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Potato Annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic Solanum tuberosum L. Plants

--Manuscript Draft--

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Abstract:	Annexins are a family of calcium- and membrane-binding proteins that are important for plant tolerance to adverse environmental conditions. Annexins function to counteract oxidative stress, maintain cell redox homeostasis, and enhance drought tolerance. In the present study, an endogenous annexin, STANN1, was overexpressed to determine whether crop yields could be improved in potato (Solanum tuberosum L.) during drought. Nine potential potato annexins were identified and their expression characterized in response to drought treatment. STANN1 mRNA was constitutively expressed at a high level and drought treatment strongly increased transcription levels. Therefore, STANN1 was selected for overexpression analysis. Under drought conditions, transgenic potato plants ectopically expressing STANN1 were more tolerant to water deficit in the root zone, preserved more water in green tissues, maintained chloroplast functions, and had higher accumulation of chlorophyll b and xanthophylls (especially zeaxanthine) than wild type (WT). Drought-induced reductions in the maximum efficiency and the electron transport rate of photosystem II (PSII), as well as the quantum yield of photosynthesis, were less pronounced in transgenic plants overexpressing STANN1 than in the WT. This conferred more efficient non-photochemical energy dissipation in the outer antennae of PSII and probably more efficient protection of reaction centers against photooxidative damage in transgenic plants under drought conditions. Consequently, these plants were able to maintain effective photosynthesis during drought, which resulted in greater productivity than WTplants despite water scarcity. Although the mechanisms underlying this stress protection are not yet clear, annexin-mediated photoprotection is probably linked to protection against light-induced oxidative stress.
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Response to Reviewers:	Dr Dorota Konopka-PostupolskaWarsaw, 06.17.2015 PLOS ONE Rewiever, Subject: revised manuscript PONE-D-14-57182 Dear Reviewer, We greatly appreciate your help and careful review of our manuscript “Potato STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic Solanum tuberosum L. Plants”. Presently, we are submitting a revised clear copy- and track - versions. Our responses to the Reviewer comments in a point-by-point manner are listed below Sincerely yours Dorota Konopka-Postupolska Responses to the Reviewer #1: 1.“They present interesting data on metabolites of the CK pathway, which could have been developed more.” The enormous diversity of cytokinins makes their measurements difficult but interpretation is even more complicated. Additionally, to our knowledge it is the very first paper showing connection between annexins and hormonal homeostasis in potato and comparative data are lacking. Hence we analyzed only this aspect of obtained data that was connected with drought tolerance and high light stress. We are planning to perform further analysis and then hopefully be able to develop this issue more. 2.“The connection between ABA biosynthesis and the xanthophyll cycle remains as cloudy as in the first submission, however. Pointing to a study in which a low ascorbate mutant was used as evidence for the importance of ascorbate recycling to the xanthophyll cycle was odd.” We apologize and did our best to make this point clear. In a new version of manuscript a new paragraph dedicated to considerations of redox poise effect on NPQ and ABA biosynthesis has been added (Discussion). 3.“There is still way too much elementary plant biochemistry in the Introduction. Why not focus the Introduction on the annexins and what is known about them, rather than explain what we know already?” According to Reviewer’s suggestion the elementary biochemical considerations has been substantially shortened and rewritten in order to clarify our idea that protective effect of annexin STANN1 on potato plants rely on increasing of antioxidant pool or efficiency. Paragraph describing plant annexin function has been deepen (Introduction).
Additional Information:	
Question	**Response**
Financial Disclosure Please describe all sources of funding that have supported your work. A complete funding statement should do the following: Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and	Transnational Access Capacities of the European Plant Phenotyping Network (EPPN, grant agreement no 284443) - FP7 Research Infrastructures Program of the European Union, http://cordis.europa.eu/fp7/ict/e-infrastructure/ (to MS). Grant N N301 567540 - Ministry of Science and Higher Education, http://www.nauka.gov.pl/en/ (to DKP) Grant PBZMNiSW-213/2006/5 - Ministry of Science and Higher Education, http://www.nauka.gov.pl/en/ (to JH) The use of CePT infrastructure financed by the European Union – the European Regional Development Fund within the Operational Programme “Innovative economy” for 2007-2013, https://cept.wum.edu.pl/ (MG and KG)

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Ethics Statement	n/a

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Additional data availability information:	Tick here if the URLs/accession numbers/DOIs will be available only after acceptance of the manuscript for publication so that we can ensure their inclusion before publication.

Dr Dorota Konopka-Postupolska Warsaw, 06.17.2015

Dr Zhulong Chan,

Ph.D Academic Editor PLOS ONE

Subject: revised manuscript PONE-D-14-57182

Dear Dr Zhulong Chan,

Thank you for reviewing the second time our manuscript entitled “Potato Annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic *Solanum tuberosum* L. Plants”. We greatly appreciate your decision to give us a chance to address the merits pointed by the Reviewer. We carefully considered the suggestions and accordingly appropriate modifications have been incorporated in the text. Hope you find that they contributed to enhancement of manuscript quality. We have shorten the consideration on elementary plant biochemistry in the Introduction and add a new paragraph for clarifying the connection between ABA biosynthesis and the xanthophyll cycle. Presently, we are submitting a revised clear copy- and track - versions. Besides, our responses to the Reviewer comments in a point-by-point manner are listed in Respond to the Reviewer paragraph.

I will be happy to answer any more questions.

Sincerely yours

On behalf of coauthors

Dorota Konopka-Postupolska

Potato annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic *Solanum tuberosum* Plants

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Abbreviations:

ABA	abscisic acid
ATANN1	annexin 1 from *Arabidopsis thaliana* L.
Car	carotenoids
Chl*a*, Chl*b*	chlorophyll *a*, chlorophyll *b*
CK	cytokinins
EAE	excess absorbed energy
Fv/Fm	the maximum quantum efficiency of photosynthesis
HSR	heat stress response
MeV	methyl viologen, paraquat
NPQ	non-photochemical quenching
pcd	programmed cell death
PETC	photodynthetic electron transport chain
PPFD	photosynthetic photon flux density [μmol m^{-2} s^{-1}]
PSI, PSII	photosystem I, photosystem II
PSIIAS	antennae system of photosystems II
PSBS	22-kD photosystem II subunit
STANN1	annexin 1 from *Solanum tuberosum* L.
SA	salicylic acid
Viol	violaxanthine
ViolDE	violaxanthin de-epoxidase
Y(II)	effective quantum yield of PSII
XCar	xanthophylls
Zea	zeaxanthine
ZEP	zeaxanthine epoxidase

Abstract

Annexins are a family of calcium- and membrane-binding proteins that are important for plant tolerance to adverse environmental conditions. Certain annexins are able to counteract oxidative stress, affect cell redox homeostasis, and enhance drought tolerance. In the present study, an endogenous annexin, STANN1, was overexpressed to determine whether yields could be improved in a crop plant, potato (*Solanum tuberosum* L.), during drought. Nine potential potato annexins were identified and their expression characterized in response to drought treatment. *STANN1* mRNA was constitutively expressed at a high level and transcription was strongly increased during drought, and this gene was therefore selected for overexpression. Under drought conditions, transgenic potato plants ectopically expressing STANN1 were more tolerant to water deficit in the root zone and preserved more water in green tissues than wild-type plants. Despite the drought stress, chloroplast functions were maintained in transgenic plants and accumulation of chlorophyll *b* and xanthophylls (especially zeaxanthine) increased compared to the wild type. Furthermore, drought-induced reductions in the maximum efficiency of PSII, the quantum yield of photosynthesis, and the electron transport rate were less pronounced in transgenic plants than in the wild type. This resulted in more efficient non-photochemical energy dissipation in the outer antennae of PSII and provided for more efficient protection of reaction centers against photooxidative damage in the transgenic plants. Consequently, these plants were able to maintain effective photosynthesis during drought, which resulted in their overall better productivity despite the period of water scarcity. Although the mechanisms underlying this stress protection are not yet clear, annexin-mediated photoprotection is probably linked to the ability of annexin to counteract light-induced oxidative stress.

Introduction

Plants have developed concurrent passive and active strategies to survive shorter or longer episodes of water deficit [1]. Several stress-response genes have already been used for bioengineering with the aim of improving plant resilience to water deficit [2]. However, ectopic expression of stress-inducible genes often results in developmental aberrations (e.g., stunted growth and irregular leaves) or impeded yield under non-stress conditions as the result of unspecific induction of programmed cell death (pcd) and/or premature senescence [3]. New approaches utilizing different mechanisms are needed to achieve sustainable improvements in crop biomass production.

Environmental stresses such as drought, salinity, and low and high temperature primarily affect chloroplast metabolism and trigger imbalances in the chloroplast redox homeostasis [4 - 6]. Thus, the ability of a plant to withstand stress and recover afterwards is strongly related to the capability to support photosynthesis and protect chloroplast structures and functions despite unfavorable conditions. In the plant cell, reactive oxygen species (ROS) are continuously formed as by-products during photosynthesis under normal conditions. The steady-state level of ROS is tightly regulated by several enzymatic and low-molecular weight antioxidant systems [7]. However, under adverse environmental conditions the capacity of antioxidant systems can be exceeded, and this leads to redox imbalance.

The two main sources of ROS in the chloroplast are the light-driven photosynthetic electron transport chains (PETC) of photosystem I (PSI) and photosystem II (PSII). PSII and PSI work sequentially to transport electrons released from a photo-excited primary electron donor (chlorophyll, Chl) on the lumenal thylakoid side of PSII to the terminal acceptor, ferrodoxin, on the stromal side in PSI. PSII catalyzes water oxidation and hence provides electrons for all further photosynthetic reactions. PSII efficiency is therefore crucial for the efficiency of photosynthesis as a whole. PSII is a highly spatially organized structure that consists of core complexes (PSIICC) associated with the peripheral antennae system (PSIIAS) [8]. PSIIAS has two main functions. First, sunlight energy is captured and transferred to PSIICC, where primary photochemical reaction occurs. Second, PSIIAS protects the whole PS against photooxidative damage. PSII is the most highly vulnerable structure of the entire photosynthetic apparatus and this mediates

plant adaptation to environmental conditions. When light intensity exceeds that which a plant can utilize in photosynthesis, over-reduction of PETC results in generation of highly reactive and low-diffusible singlet oxygen ($^1O_2$), which subsequently oxidizes PSII constituents *in situ* [9 - 11]. Although singlet oxygen cannot itself function as a signaling molecule, experimental data showed that it activates cellular signaling pathways of surprising complexity [11]. Peroxidation of different PSIIAS components gives rise to a plethora of compounds that act as secondary messengers, including products of fatty acid peroxidation, reactive electrophile species (RES), and volatile derivatives of β-carotene oxidation (e.g., β-cyclocitral and dihydroactinidiolide) [12 - 14]. In addition, the release of intrinsic chloroplast proteins after stress induction resembles similar leakage of mitochondrial proteins into the cytosol [15]. These processes collectively trigger the activation of nuclear genes involved in the molecular defense against photooxidative stress.

PSIIRC is protected against oxidation via non-photochemical quenching (NPQ), a heterologous phenomenon that allows the excess absorbed energy (EAE) from PSIIAS to dissipate before it reaches the reaction center [16 - 17]. Several NPQ components have been distinguished based on the time scale of formation and relaxation, including qE, qT, qZ, and qI [18]. qE (energy-dependent quenching) is triggered by the formation of a pH gradient across the thylakoid membrane, can be detected as heat emission within microseconds, and relaxes in minutes [19]. Two essential factors are involved in its related NPQ: zeaxanthine (Zea) and a 22-kD PSII subunit protein (PSBS) [20 - 21]. Acidification of the thylakoid lumen activates the xanthophyll cycle and produces violaxanthine de-epoxidase (ViolDe), which converts violaxanthin (Viol) into Zea. *Arabidopsis thaliana* (Arabidopsis) plants with higher levels of epoxidated Car derivatives - xanthophylls (XCar), as a result of overexpression of β-carotene hydroxylase, were more tolerant to high light and high temperature [22]. In *npq2*, another Zea-overaccumulating mutant, the kinetics of induction and relaxation of NPQ, but not its extent, were affected [17]. Zea modes of action are complex and include direct antioxidant activity [23], allosteric modulation of membrane function in PSII, and enhancement of proton binding activity [24]. In turn, PSBS affects membrane rigidity [25 - 26] and senses pH changes [27], but its precise role in energy dissipation remains unclear [28]. Arabidopsis *npq4-1* mutants that lacked PSBS and had a normal XCar cycle were deficient in qE [29 - 30]. It was

therefore suggested that PSBS functioned as a kinetic modulator of NPQ in the PSIIAS [31].

The second major source of ROS in the chloroplast is PETC of PSI. Stomatal closure and diminished CO_2 supply reduces the rate of Calvin cycle reactions and leads to inefficient regeneration of NADP+. Uncontrolled electrons flow from ferredoxin to O_2, leading to generation of the superoxide anion $O_2^{\bullet-}$ via the Mehler reaction (photochemical quenching) [4, 32]. Once formed, $O_2^{\bullet-}$ is subsequently converted into H_2O_2 or initiates chain reactions that give rise to other more reactive ROS molecules. The intensity of the Mehler reaction increases with deepening water stress. Within 0.5 s of inactivation of the chloroplast enzymatic scavenging systems, CO_2 fixation was reduced by half and 10 μM hydrogen peroxide accumulated [4]. H_2O_2 is very stable, with a half-life of ~1 ms, and can pass through biological membranes. H_2O_2 leaking induces an oxidative burst in the cytosol. The origin of this burst is poorly understood, but it likely has a complex nature consisting of both chloroplast and cytosol components. It was also showed that chloroplast-derived ROS are indispensable for intercellular ROS signaling [33].

During prolonged stress, additional sources of ROS appear in the cell. Limited CO_2 availability activates oxygenation of RUBISCO instead of carboxylation and this induces photorespiration. Neutralization of toxic products occurs in peroxisomes and is accompanied by H_2O_2 production. Photorespiratory H_2O_2 was shown to participate in the execution of pcd [34].

Plant tolerance to adverse environmental conditions is also mediated by the collective action of different hormones. Abscisic acid (ABA) and cytokinins (CK) are the most intimately involved in the plant response to decreased soil humidity. These hormones mediate mostly antagonistic effects. ABA is considered as a “stress-hormone” since it activates and coordinates different stress responses such as rapid stomatal closing and metabolic reprograming [35 - 36]. In turn, CK assists in recovery after stress by activating cell division, chloroplast development, synthesis of chloroplast proteins/photosynthetic pigments, and formation of membrane components of PETC [37]. Artificial maintenance of CK biosynthesis during conditions of stress was found to be beneficial for plant survival. Accordingly, overexpression of a CK biosynthetic enzyme, isopentynyltransferase, enhanced plant tolerance to a wide range of abiotic factors [38 - 41]. Thus, strategies to enhance

agriculture via improved protection of chloroplast structure and function under stress conditions are promising.

The role of salicylic acid (SA) in the regulation of plant interactions with the environment has been recognized in recent years and SA has emerged as an important factor involved in acclimation to certain abiotic stressors [42 - 45]. Singlet oxygen accumulation in the chloroplast was shown to induce rapid accumulation of SA [46]. At high concentrations, in cooperation with other signals, SA strongly promoted $^1O_2$-induced pcd [47 - 48]. Although their role in pcd is not clear, functional chloroplasts are necessary for the hypersensitive response [49 - 51].

Previously, we demonstrated that overexpression of endogenous annexin 1 (ATANN1), improved the tolerance of the model plant Arabidopsis Col-0 to drought [52 - 53] and that ATANN1 undergoes S-glutathionylation upon ABA induction. The objective of the present study was to verify if a similar improvement in drought tolerance could also be achieved by overexpression of annexin in the potato (*Solanum tuberosum* L.). Thus, transgenic plants with elevated levels of endogenous annexin STANN1 were created and tolerance to a transient soil water deficit was assessed. Our results showed that annexin overexpression during drought improved NPQ and alleviated photo-oxidative stress and thereby protected chloroplast function.

Materials and Methods

Generation of transgenic plants, transformation and growth conditions

S. tuberosum cultivar Sante (WT), medium-tolerant to drought, was used for transformation (http://www.europotato.org). cDNA sequence for *STANN1* without the stop codon (957 bp; Acc. No. PGSC0003DMG400017714) was fused at the 3′ end to a 6×His-tag sequence and inserted into the XbaI restriction site of pROK2 [54] between cauliflower mosaic virus 35S promoter and nopaline synthase (Nos) terminator sequences (Figure S1A in File S1Figures). The obtained clone was used for *Agrobacterium tumefaciens*-mediated transformation of WT potato plants according to [55]. Regenerated transgenic plants were transplanted into separate glass tubes filled with 10 mL of Murashige & Skoog solid medium supplemented with 50 μg mL^{-1} kanamycin. The presence of the transgene cassette was verified with genomic PCR (data not shown). Expression of recombinant STANN1_6×His protein was confirmed by purification from leaves of WT and F1 transgenic plants (lines S-2, S-3, S-7, S-83, S91, S-97, and S-123) by Ni-NTA chromatography and detection with anti-HisTag primary antibody (Sigma-Aldrich). Recombinant ATANN1_6×His protein produced by bacterial overexpression was used as a positive control (WT protein extract) (Figure S1B in File S1Figures).

Potato WT plants or WT-derived transgenic lines (S-2 and S-7) were used for further experiments. Plants were cultivated in a growth chamber (or an air-conditioned greenhouse when indicated) under standard conditions (21±2°C; 16 h/8 h day/night; light intensity 110 to 130 PPFD (photosynthetic photon flux densities); 60–80% relative humidity.

Water stress

S. tuberosum plantlets sprouted from tubers were grown in plastic pots filled with 1 kg of sterilized soil (mixture of peat and sand, pH 5.5; prepared by the Plant Breeding and Acclimatization Institute) for ~160–170 days. The field capacity (FC) was determined gravimetrically (g of water per g of soil). Pots were weighed every 2–3 days and the volume of water necessary to maintain the indicated FC was calculated individually for each plant. For well-watered control plants, FC was maintained at 65% (−0.8 MPa) for the whole experiment. Experimental drought was imposed after 8–10 weeks of growth (tuber initiation) (Figure S2A in File S1Figures). Irrigation was decreased over 10 days to gradually reduce the FC to

~25% FC (−2.0 MPa) and was then maintained at this level until the end of the water deficit period. Irrigation was subsequently resumed with full soil saturation (rewatering). To estimate the impact of drought on potato productivity, plants were cultivated for an additional 11–12 weeks after rewatering (FC 65%) until physiological maturity. An exemplary schedule of FC changes is shown in Figure S2B in File S1Figures. Samples were collected at the beginning of the water deficit period (D0), and (depending on experiment) at different days of drought, i.e. 3rd (D3), 4th (D4), 6th (D6), 10th (D10), and 14th (D14), and at the first (RW1) and third (RW3) days after rewatering.

Identification of potato annexins

Annexins were identified *in silico* by searching for the endonexin domain (PFAM definition, PF00191, 66 aa) within six translation frames of the potato genome (heterozygous diploid breeding line, *S. tuberosum* L. group Tuberosum RH89-039-16 genome) using the HMMSearch program from the HMMER3 package. According to PFAM, >93% of proteins from this family contained at least three consecutive repeats of the endonexin domain. By searching with a single repeat, the probability of missing a complete protein due to below-threshold partial hits or incorrectly defined intron-exon boundaries was minimized. Only hits with an E-value ≤0.001 were considered. To verify the presence and sequence of the predicted annexins in WT potato, genome primer sets were designed that corresponded to the 5′ (F) and 3′ (R) ends of the predicted ORFs (Table S1 in File S1Figures). Expression of putative annexin genes was verified using RT-PCR. Briefly, total RNA was isolated from WT leaves and reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific) with poly(T)$_{12\text{-}18}$ primer. Annexins were amplified from cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). PCR products were cloned with pJET Cloning Kit (Thermo Scientific) and their compliance with the predicted sequences was verified.

Semi-quantitative expression of annexins and stress-regulated genes

Gene expression was profiled over 14 days of drought in WT potatoes grown as described above. Samples were taken from the first fully-developed composite leaf at the top of the plant. For each time point, single leaf discs from four independent plants were collected, flash-frozen in liquid nitrogen, and kept at −80°C until use. Total RNA was isolated with Trizol (Invitrogen). Reverse transcription was performed as described above.

Taq DNA Polymerase (Thermo Scientific) was used to amplify specific sequences from cDNA. Genes for semi-quantitative analysis were selected from PGSC_DM_v3.4_pep_fasta, which contains a database of potato virtual translation products predicted according to similarity to annotated Arabidopsis genes. Specific primer sets for expression analysis were designed using PrimerSelect, Laser Gene10.0 DNASTAR (USA) (Table S2 in File S2Tables). The obtained sq-RT-PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and quantitated by densitometry using MultiGaugeV3.0 (Fuji) software. Expression was normalized against the expression of potato elongation factor 1 alpha mRNA (EF1a; PGSC0003DMT400050664;[56]). Each single experiment included four biological replicates that were quantitated in three technical replicates. Experiments were repeated three times for each primer set and template.

Relative water content

RWC was determined according to [57] with the exception that for full saturation (turgor weight, TW) leaves were incubated in distilled water for 4 hours instead of overnight. Experiments were performed three times on at least five biological replicates for each genotype.

Extraction and determination of plant hormones

Leaf samples of ~0.5 g (without the main vein) from 8–10-week plants subjected to drought (as described above) were collected, immediately frozen in liquid nitrogen, and kept at −80°C until use. Samples were taken from the first fully-developed composite leaf at the top of the plant at the indicated time points. Sampling was performed four hours after switching on the light. Three independent biological replicates were examined. Purification and analysis were performed according to [58, 59]. Briefly, leaf samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v) and the following labelled internal standards (10 pmol per sample) were added: $^{2}H_{6}$-ABA, $^{2}H_{5}$-*trans*Z, $^{2}H_{5}$-*trans*ZR, $^{2}H_{5}$-*trans*Z7G, $^{2}H_{5}$-*trans*Z9G, $^{2}H_{5}$-*trans*ZOG, $^{2}H_{5}$-*trans*ZROG, $^{2}H_{5}$-*trans*ZRMP, $^{2}H_{3}$-DHZ, $^{2}H_{3}$-DHZR, $^{2}H_{3}$-DHZ9G, $^{2}H_{6}$-iP, $^{2}H_{6}$-iPR, $^{2}H_{6}$-iP7G, $^{2}H_{6}$-iP9G, and $^{2}H_{6}$-iPRMP (Olchemim). Extracts were purified using a SPE-C18 column (SepPak-C18, Waters), and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction (containing ABA) was eluted with methanol and the second fraction (containing CK metabolites) was eluted with 0.35 M NH_4OH in 70% methanol. Both fractions were separated by HPLC (Ultimate 3000, Dionex) and the

hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and net photosynthesis were analyzed with a Portable Handheld Photosynthesis System CID 340 device (CID Inc., USA) according to the manufacturer's instructions. The maximum quantum efficiency of photosynthesis (Fv/Fm) and the effective quantum yield of PSII (Y(II)) were determined with CID 340 (CID Inc., USA) with a CI-510CF Chl fluorescence module and a CI-310LA light attachment providing actinic light. Measurements were performed 5 h after turning on the light, if not indicated otherwise, on the upper 4–5 fully-expanded unwrinkled leaves. Five plants were analyzed per time point. For maximal fluorescence (Fm) determination, plants were dark-adapted for 30 minutes (so all PSII reaction centers were closed) and then stimulated with saturating pulses of light (0.8 sec., 3000 PPFD). The minimal fluorescence (Fo) with all PSII reaction centers opened was measured with modulated light of 0.25 PPFD. Fv was calculated from the equation Fv= Fm-Fo. Y(II) was calculated using the equation Y(II) = (Fms-Fs)/Fms. For determination of Fms (maximal fluorescence under light), plants were adapted to light for 20 minutes and the steady-state of Chl was measured (Fs). Next, a saturating pulse (0.8 sec, 3000 PPFD) was applied and Fms was determined.

Gross NPQ was estimated with a Dual Pulse Amplitude Modulation device, PAM-100 (Walz, Germany). For a single time point, six composite leaves from 3–5 control plants were analyzed. NPQ was calculated as (Fm − Fms)/Fms, where Fm represents the fluorescence of a dark-adapted sample and Fms represents a fluorescence of the illuminated sample. Plants were dark-adapted (~20 min) and kinetics were measured after repeated light pulses of 94 PPFD for 300 sec. Leaves were subsequently relaxed in darkness for 240 seconds and fluorescence was documented throughout.

Extraction of non-polar lipids and carotenoids/chlorophyll determination

Plant material was collected from 8–10 week-old plants exposed to drought. Samples were collected 4 hours after switching on the light at D0, D6, D14, and RW3. One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded composite leaves and total six discs were combined as a single

sample. Non-polar lipids were extracted at 4°C and subjected to analysis using the ACQUITY UPLC system (Waters, USA). Shredded plant material was transferred to a 15 mL Pyrex tube. After the addition of 3 mL acetone-methanol (8:2 v/v), the sample was perfused with argon and mixed vigorously by vortexing for 2 min. For the first and third extractions, hexane (9 mL) was added and the sample was again perfused with argon before capping and shaking in a reciprocating shaker (PROMAX 2020, Heidolph, Germany) for 30 min in the dark. After shaking, the sample was incubated without agitation for 5 min to allow phase separation. The upper hexane phase was collected by aspiration and transferred to an Erlenmeyer flask, perfused with argon, capped and stored in the dark. In the second extraction stage, 2 mL of propanol was used in addition to hexane, and perfusion, shaking and phase collection were repeated as before. After removal of hexane, the polar phase was centrifuged for 15 min at 4500 rpm. The supernatant was combined with the two hexane phases, perfused with argon, and filtered through a syringe filter Milipore millex-CV13 Filter Unit (0.22 µm). The joined phases were then transferred to room temperature, evaporated to dryness under argon, and dissolved in 1 mL methanol-propanol-hexane 6:1:3 (v/v/v). Dissolved samples were transferred to 2 mL glass vials, perfused with argon, capped, and stored at −80°C. Samples (5 µL) were injected onto an ACQUITY UPLC HSS T3 1.0×150 mm 1.8 µm column and eluted with a gradient of solvents: A, water and methanol (1:9, v/v); and B, methanol:isopropanol:hexane (2:1:1, v/v/v) (210 min from A to B). Separation was monitored in the 300–750 nm range with a photodiode array detector. A single chromatogram at 436 nm was extracted, exported in ASCII format, and used for peak area integration analysis with GRAMS/AI software (Thermo Electro Corp.).

Chl*a* and Chl*b* contents were estimated by recording the absorbance of the aforementioned extract at 663, 652, and 645 nm (Cary 50 Bio UV/VIS spectrophotometer, Varian, Australia) according to [60].

Measurement of ROS levels and quantification of lipid peroxidation in high light stress

One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded leaves of potato plants and a total of six discs were combined as a single sample. Immediately after harvesting, samples were vacuum-infiltrated with methyl viologen (MeV) at the indicated concentrations and then incubated in the dark for 1

hour under normal irradiance (150 PPFD). Images were obtained after 30 hours of incubation.

A similar procedure was used for ROS quantification, with the exception that a single MeV concentration (50 μM) was used and samples were exposed to high irradiance (850 PPFD). Samples were collected at the indicated time points. Superoxide anion ($O_2^{\bullet -}$) content was determined using a colorimetric nitro blue tertrazolium (NBT) assay as described [61]. Hydrogen peroxide (H_2O_2), was detected with diaminobenzidine tetrahydrochloride (DAB) and quantified by pixel counts on scanned images using ImageJ software [62]. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA) according to [63].

Transient expression of STANN1_GFP in *Nicotiana benthamiana*

The *STANN1* sequence (without the stop codon) was introduced between NcoI and BcuI restriction sites at the 5′-end of the monomeric GFP (mGFP) coding sequence in pCAMBIA1302. Intact *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 transformed with empty pCAMBIA1302 expressing mGFP or pCAMBIA1302 expressing STANN1_mGFP as described [64]. After 3 days, 1 cm diameter leaf discs were excised and incubated with 50 μM MeV for 1 hour in darkness then 4 hours in high light (850 PPFD). Fluorescence was immediately observed using a Nikon Eclipse TE2000-E inverted C1 confocal laser scanning microscope equipped with a 40× Plan Fluore oil immersion objective lens (numerical aperture, 1.30). mGFP and chloroplast autofluorescence were excited with a solid-state Coherent Sapphire 488 nm laser and detected using 515/30 band pass and 610 long pass emission filters, respectively. All samples were analyzed in triplicate. Three independent experiments were performed.

Statistical analyses

Data were analyzed using two-way ANOVA with Duncan's Multiple Range Test (DMRT) (for yield) and MANOVA regression models (for other experiments). Multiple comparisons between means were performed with a HSD Tukey test with a confidence limit of 95%.

Results

Identification of annexin genes

Genome-wide examination of the potato sequence database for annexins revealed the presence of 11 DNA segments encoding putative proteins displaying substantial similarity to already known plant annexins. Two of these sequences were classified as pseudogenes due to a lack of continuity in any of the six open reading frames and several genetic disablements. The remaining nine genes were located on chromosomes I, IV, V, and X and each encoded 5–6 exons (Figure 1A). The positions and phases of introns in the putative potato annexin genes were consistent with those reported for rice annexins [65] (Figure 1B). The putative annexin sequences in the *S. tuberosum* genome were verified using genomic PCR (Figure 1C) and the lengths of the amplified genomic products were as expected (Table S1 in File S2Tables). The degree of nucleotide sequence identity between the putative potato annexins was in the range of 41–92%. Sequences identified by bioinformatics approaches were confirmed experimentally. Reverse transcription polymerase chain reaction indicated that all nine genes were expressed in different potato organs (data not shown).

A multiple alignment of the putative potato annexin amino acid sequences with Arabidopsis annexins revealed that all except of one the potato annexins had Arabidopsis homologs (data not shown). The newly-identified potato genes were therefore named accordingly as *STANN1*, *STANN2*, *STANN3.1*, *STANN3.2*, *STANN3.3*, *STANN4*, *STANN5*, *STANN8*, and *STANN9* (data not shown). The potato annexins constituted a functionally diverse family of proteins that was differentially expressed in different plant organs (data not shown). The most striking genomic feature of the potato annexin family was triplication of the annexin 3 gene on chromosome 1 (Figure 1D). In addition, in an arrangement resembling that in Arabidopsis, potato *STANN3.1* and *STANN4* were localized adjacent to one another and were transcribed divergently, possibly from a shared promoter. The Inparanoid database groups all annexins in the same in-paralog cluster; however, we suspect that the three variants of annexin 3 (*STANN3.1*, *STANN3.2*, and *STANN3.3*) are within-species out-paralogs. Two duplications (ancestral gene → *STANN3.1* and the ancestor of *STANN3.2*; then the ancestor of *STANN3.2* → *STANN3.2* and *STANN3.3*) seem to have occurred prior to speciation of potato and tomato, as *S. lycopersicum*

contains three orthologs of the respective annexins. In turn, *STANN4* and *STANN3.1–3.3* are out-paralogs, as *STANN4* is moderately related to all *STANN3* variants but shares high sequence similarity with other annexins from *S. lycopersicum* or Arabidopsis. Multiplication of DNA segments within this region of chromosome 1 during *Solanaceae* evolution apparently took place independently at least twice. In tomato chromosome 1, the entire dyad of *SLANN3*/S*LANN4* was duplicated [66] and gave rise to a tetrad located within a short segment of DNA (21145 bp) that was not interspersed with other genes. Collectively, this region of chromosome 1 represents a "hot-spot" in the *Solanaceae* family where duplications of a single gene or gene cluster occurred.

Characteristic of potato annexin proteins

Newly identified potato annexins had similar predicted molecular masses of 34–37 kDa and diverse isoelectric points (5.21–9.02). The overall tertiary structures (four endonexin domains containing calcium binding sites) were well preserved (Figure S3 in File S1Figures, Table S3 in File S2Tables). However, the primary amino acid sequences were quite divergent, with the lowest amino acid identity between STANN4 and STANN5 (20.9%). Conversely, groups with higher similarities (STANN3.1, STANN3.2, and STANN3.3) were also apparent. Annexins 3.2 and 3.3 were the most closely related, and had amino acid identities of 90.5% and 70.1% with STANN3.1, respectively. STANN3.2 and 3.3 differed in length (302 and 317 aa, respectively) due to lack of the 14-3-3 like domain on the C-terminus of STANN3.2. Similarly, the N-terminal end of STANN3.2 and STANN3.3, but not STANN3.1, contained a putative myristoylation motif (MG). To date, a myristoylation-mediating membrane localization has been confirmed only for mammalian AnxA13b. With respect to plant annexins, a myristoylation motif was found in poplar annexin EEE95606.1, but the functionality of this motif was not experimentally verified. In summary, despite extensive similarities, there were substantial differences between members of the STANN3 sub-family. This suggested that the family might be unique to *Solanaceae* and that evolution towards distinct cellular functions for each of the annexins has occurred.

The potato annexins contained canonical type II calcium binding sites G-X-GTD-{30-40}D/E solely in the first and occasionally fourth endonexin domains (Figure S3 in File S1Figures). STANN4 and STANN8 appear to have lost calcium

responsiveness as a result of substantial mutations (substitutions and insertions) in these regions. In STANN5, the site in the fourth repeat was probably the only one to be preserved. Tryptophan residues within the first endonexin repeat (G-W-GT) were conserved in potato annexins 1, 2, 8, and 9, but were replaced with phenylalanine (STANN5) or lysine (STANN3.1-3.3 and STANN4) in other annexins (Figure S3 in File S1Figures). This phenylalanine modification is not predicted to interfere with calcium binding as phenylalanine and tryptophan residues are both hydrophobic and possess aromatic rings. By contrast, the lysine modification may impede membrane translocation of annexin since introducing a positive charge into the calcium coordination site has the potential to disrupt calcium binding. Other amino acids or motifs important for the tertiary structure of plant annexins were preserved in the potato proteins, such as histidine 40 (except in STANN3.2 and STANN4), cysteine 111 (except in STANN4), and cysteine 239 (except in STANN1).

Expression of potato annexins during drought

Only five of the annexin genes (*STANN1*, *STANN4*, *STANN5*, *STANN9*, and *STANN2*) were expressed in the leaves of well-watered control WT plants (Figure 2). At the onset of drought (D0) the *STANN1* mRNA remained the most abundantly-expressed annexin transcript (relative to the *EF1a* mRNA). Over time, the level of *STANN1* mRNA increased whereas *STANN4*, *STANN5*, *STANN9*, and *STANN2* mRNA levels remained unchanged. The resulting difference in the accumulation of *STANN1* mRNA from D0 to D14 was statistically significant (Figure 2). Concurrently, the expression of additional annexins that were not detected in control conditions was induced. The levels of *STANN3.1* and *STANN3.2* mRNA (relative to *EF1a*) increased on D6 and remained elevated until the end of the drought period. The level of *STANN8* mRNA increased continuously during the whole period of water deficit (Figure 2). However, these induced annexins were expressed at levels at least ten-fold lower than annexin 1. This strongly suggested that STANN1 was the key annexin involved in the plant cell response to drought.

Tolerance to soil water deficit

Transgenic plants looked normal and did not display any discernible abnormalities and/or growth aberrations under well-watered conditions (both in growth chamber and in the greenhouse). Leaf turgidity was similar between transgenic and WT plants, which indicated that the leaf water status of WT and

transgenic lines was comparable (Figure 3A, upper panel; Figure S4A in File S1Figures). During soil water deficit, the overexpression of STANN1 resulted in sustained turgor maintenance. In WT plants, leaf wilting was clearly visible on D8 (Figure 3A, middle panel). By D9, the drought effect was more apparent and leaves began to shrivel, roll, and curl up. Younger leaves near to the top the of the plant were the most severely affected (Figure 3A, lower panel). Leaves of the transgenic lines S-2 and S-7 preserved stiffness and did not show signs of dehydration. Rewatering resulted in restoration of leaf turgor and normal growth resumed within 1 day for transgenic plants and 3 days for WT (Figure 3B). After 2 weeks of drought, S-2 and S-7 leaves were less damaged then those of WT. Experiments were repeated four times in succeeding years, under greenhouse and growth chamber conditions, and in all cases similar results were obtained (Figure S4 in File S1Figures). The exact number of irreversibly damaged leaves varied between experiments depending on the drought severity (intensity and length). Damage was consistently significantly lower in transgenic lines than in WT. For example, survival rates after a 3-week drought were 12% and 82% for WT and S-7, respectively.

The ability to preserve turgor in leaves is closely related to drought tolerance. Therefore, to further characterize the drought response of transgenic plants, changes to RWC under water deficit were analyzed. Under control conditions, RWC was comparable in WT and transgenic plants. Drought resulted in the continuous decline of RWC both in WT and in transgenic plants. However, differences between lines became apparent with increasing drought severity and this became statistically significant at D12 after drought onset (Figure 4A). Both in WT and in transgenic plants rewatering after 2 weeks of drought resulted in restoration of control RWC values.

Overexpression of STANN1 also improved plant yield both in terms of the total tuber mass (Figure S5A in File S1Figures) and consistency of the tuber size (Figure S5B–C in File S1Figures). The net productivity of well-irrigated WT and transgenic S-7 and S-2 lines was almost identical but tuber quality (size and uniformity) was enhanced in the transgenic lines. A 14-day drought decreased the tuber yield of the WT plants by half, whereas yield loss for S-2 and S-7 lines was statistically less significant. In addition, the tuber quality in the transgenic lines was less impaired after drought compared to WT.

Plant photosynthetic activity

Several plant-vigor-related physiological parameters were analyzed to assess the effect of STANN1 overexpression. These included stomatal conductance (a measure of water and carbon dioxide vapor through the leaf stomata) (Figure 4B), net photosynthesis (Pnet, associated with plant vitality and biomass production) (Figure 5A), maximum efficiency of PSII in the dark-adapted state (a measure of the organization and vitality of PSII) (Figure 5B), and effective quantum yield of PSII in illuminated samples (Figure 5C). Under control conditions STANN1 overexpression did not influence any of these parameters. By contrast, almost all the photosynthetic functions were disturbed during drought, and changes in the two overexpression lines were consistent. The effect of drought on gas exchange was apparent by the third day after watering reduction, but the difference between WT and transgenic lines was statistically insignificant (Figure 4B). Conductance remained low during the whole period of water deficit and returned to control levels only partially, on the third day after resumption of watering.

Pnet (Figure 5A) in WT declined virtually to zero by D3. Pnet subsequently (D6, D10) dropped to negative values, which suggested that alternative electron consumption processes were activated. In transgenic plants, Pnet remained positive until D10. After rewatering, Pnet increased in all three lines (Figure 5A).

Under control conditions, Fv/Fm values (Figure 5B) were similar in the three plant lines (~0.79). Under normal conditions, Fv/Fm in most investigated plant species is ~0.79–0.84 conditions; this value declines with plant stress. Likewise, drought negatively affected Fv/Fm in our experiments; while this was noticeable by D3 in WT, in transgenic plants the effect only became apparent on D6. In all three lines, Fv/Fm recovered to baseline within 3 days of rewatering. Measurements were performed on upper non-wrinkled leaves, indicating that apical shoot meristems were not irreversibly damaged by dehydration.Y(II) (Figure 5C) in both WT and transgenic plants declined steadily from the onset of drought but the reduction appeared only on D6 in transgenic plants and the effect was significantly reduced compared to WT. Moreover, Y(II) fully recovered in S-2 and S-7 but, even on the third day after soil resaturation, remained reduced in WT. This showed that impairment of PSII in transgenic plants was fully reversible.

Photosynthetic pigments content in transgenic plants

Photosynthetic pigment contents were determined to better understand the effect of STANN1 on the cellular photosynthetic machinery.

Chla and Chlb accumulation

In WT and transgenic lines under well-watered conditions both the total Chl content (11.2±0.01 and 10.6±2.29 mg mL^{-1}, respectively) and the ratio of Chl*a* to Chl*b* were similar. Accumulation of Chl*a* (Figure 6A) and Chl*b* (Figure 6B) did not change during drought in WT. After rewatering, the level of Chl*a* increased to 180% of the control value at D0. During water deficit in S-7 the level of Chl*a* was stable; however, accumulation of Chl*b* increased and reached 168% at D14 compared to D0. As a consequence, the Chl*a*/*b* ratio rose to 2.0. After rewatering, the level of Chl*a* doubled and Chl*b* remained stable.

XCar accumulation

In non-stress conditions the elevated expression of STANN1 did not significantly change the total Car level; however, the XCar content was increased (Zea - 188%, Viol - 144%) in comparison to levels in WT plants (Figs. 6C–D). Since samples were collected at the same time, these differences were not related to the possible diurnal fluctuation induced by light. During drought in WT, Zea content increased progressively and reached a similar level as in S-7 plants only after rewatering (0.35±0.01 pmol g^{-1} FW). In S-7 plants, the Zea level remained largely stable and fluctuated in the range 0.31–0.34 g^{-1} FW. Viol declined significantly during the drought in both plant lines. The most significant reduction was observed during the first 6 days of drought and was more noticeable in S-7 than in WT (57% and 10.5% reduction, respectively). At subsequent time points the difference between lines disappeared and Viol remained at a stable level after rewatering (0.45±0.01 g^{-1} FW in WT and 0.44±0.06 g^{-1} FW in S-7).

Annexin overexpression affects hormonal homeostasis in plants subjected to drought

The drought phenotype of transgenic potato plants resembled that of plants overproducing CK. We therefore assessed stress-hormone levels (ABA, CK, and SA) in leaves of WT and S-7 plants subjected to drought. In well-watered conditions the level of biologically active ABA in transgenic plants was significantly lower than in WT (Figure 7A). However, this difference was lost by D6 after initiation of drought. This suggested that biosynthesis of ABA in transgenic plants during the first week of

drought was more intense than in WT. This is in accordance with a more pronounced decrease in Viol (ABA precursor) levels in transgenic plants compared to WT (Figure 6D). In the second week of water deficit, only a slight increase in ABA level was observed and maximum levels on D14 were similar between WT and S-7 (3.21±264.01 and 3.02±101.59 nmol g^{-1} FW, respectively) (Figure 7A). As expected, ABA levels decreased to control values on resumption of watering.

Under control conditions, annexin overexpression had no significant effect on CK levels (Figure 7B; Table S4 in File S1Figures). The contents of active and total CK were similar and amounted to 6.35 and 6.90 pmol g^{-1} FW, and 506.34 and 542.08 pmol g^{-1} FW, in WT and S-7 plants, respectively. Drought stress was associated with down-regulation of *trans*-zeatin (tZ), the most physiologically active CK involved in the stimulation of cell division. At the early stage of drought progression (RWC ~85%, only minor difference from control conditions), the level of active CK in WT increased, especially compared to the less active isopentenyladenosine (iPR). Active CK decreased under severe drought conditions, with the exception of *cis*-zeatin (cZ) and its riboside (cZR), both of which were CK species associated with stress responses. After rewatering, active CK content strongly increased, especially of *trans*-zeatin (tZ). Levels of cZ and cZR substantially decreased. High levels of active CK (including high levels of cZ) were maintained in S-7 even under severe drought conditions. These levels were substantially higher than in parental plants. After rewatering, active CK elevation was much more pronounced in S-7 than in WT. The level of storage compounds (CK O-glucosides) was generally low. By contrast, levels of deactivation products (CK N-glucosides) substantially increased during drought, probably as a result of the enhanced deactivation of CK (data not shown).

Overexpression of STANN1 had no effect on SA levels under well-watered conditions. SA accumulation in WT and S-7 did not change significantly under moderate drought (D6). During the second week of water limitation, the SA level increased in both lines and the level of accumulation in S-7 was about twice that in WT (Figure 7C). SA accumulated further after rewatering in WT but declined in S-7, albeit to a higher level than at D0. These data indicated that ROS modulating systems were activated more rapidly and to a higher extent in transgenic plants than in WT.

In summary, genetic modification did not influence ABA synthesis (and, therefore, ABA-dependent responses). The changes in CK metabolism (significant elevation upon rewatering) were in line with phenotypic observations. SA levels

increased rapidly but the elevation was transient. This suggested that SA-mediated activation of antioxidant systems was faster in transgenic than WT plants but that longer-lasting effects (like pcd) might be alleviated.

PSII antennae complexes functions in transgenic plants

Overexpression of annexin resulted in an increase of Zea content, which is essential for the harmless dissipation of excess excitation energy as heat (NPQ). In addition, expression of another key NPQ factor (*PSBS*) was reduced during drought (Figure S6A in File S1Figures). These data strongly suggested the XCar cycle might be more efficient in transgenic than in WT plants. Gross NPQ performance in transgenic plants was therefore analyzed directly in attached leaves of control, well-watered WT and transgenic plants (Figure 8). As expected, NPQ of S-7 and WT differed. Maximal NPQ occurred later after initiation of light stimulation in S-7 than in WT plants, and NPQ amplitude in S-7 was ~25% higher than that of WT plants. Similarly, the steady-state level of NPQ was elevated and saturation was delayed in S-7 compared with WT. Overall, these results showed that protection of PSII against photooxidative damage was more effective in S-7 than in WT as a result of improved NPQ.

Experience of stress in transgenic plants

Activation of the heat stress response (HSR) is a consequence of drought stress in nature. However, experimental plants are usually cultivated in controlled constant temperatures. Despite this limitation, activation of HSR was observed in both WT and transgenic plants. Stomatal closing due to water deficiency in the root zone was sufficient to induce expression of heat shock proteins (HSP). Thus, to characterize the effect of overexpression of annexin on potato plants, the accumulation of chloroplast-specific (*HSP100*) and cytosol-targeted (*HSP40*) mRNAs were analyzed during drought. In WT, water deficit resulted in an increased accumulation of both HSP mRNAs (compared to the EF1a normalization control) that peaked during the second week of drought. In transgenic plants, *HSP100* expression was induced but *HSP40* expression was not stimulated (Figs. S6C–D).

Annexin-mediated protection of chloroplast

Leaf disc senescence *assay*

To assess whether STANN1 overexpression directly alleviated photooxidative stress, leaf discs from WT and transgenic S-2 and S-7 plants were exposed to normal

light (150 PPFD) in the presence of two concentrations (10 and 50 μM) of a photosensitizer, methyl viologen (MeV). MeV induces oxidative burst by accepting electrons from PSI and transferring them to molecular oxygen, resulting in chloroplast damage. The damage caused by MeV was visualized by the degree of bleaching of leaf tissues. In the absence of MeV, exposure to light for up to 30 hours had almost no effect on leaf discs. By contrast, exposure to both light and MeV induced bleaching that increased according to MeV concentration (Figure S7 in File S1Figures). Transgenic plants S-2 and S-7 showed higher tolerance to MeV, as demonstrated by a reduction in leaf disc bleached margins.

Quantification of ROS and lipid peroxidation

To further analyze STANN1-mediated protection against light stress, leaf discs from WT and S-7 plants were subjected to the combined action of high light (850 PPDF) and MeV (50 μM) as described above. The levels of superoxide anion, hydrogen peroxide, and malonyldialdehyde (MDA) were quantified at the indicated time points (Figure 9).

Exposure of WT induced biphasic accumulation of superoxide anions, with an initial peak seen 30 min after induction and a second, more substantial and long-lasting, peak beginning 9 hours after induction. In S-7, an initial increase in superoxide level was noticeable but was not statistically significantly different to WT. The maximum level of O_2^- was the same in WT and S-7, but the kinetics of the second peak differed (Figure 9A). In WT, the level of superoxide increased steadily from 6 to12 h. By contrast, accumulation occurred in S-7 between 6 and 9 h, reaching a similar maximal level as in WT, and the level of superoxide then remained unchanged until 12 h after induction.

In WT, light-induced changes in H_2O_2 level followed the biphasic pattern of accumulation with a second higher and sustained peak (Figure 9B). The first peak occurred at 30 min and the second occurred at 12h after induction. In S-7, the first peak was observed and this had a similar magnitude as in WT. After several hours, no further accumulation of H_2O_2 was observed in S-7 and overall levels were significantly lower than in WT.

Lipid peroxidation, measured as an MDA equivalent, was apparent in WT only after 30 min and 12 hours. No statistically significant changes in the lipid peroxidation state were observed under high light stress in S-7 (Figure 9C).

Annexin 1 attenuates cell death when exposed to oxidative stress

A transient mGFP expression assay was performed to confirm that the aforementioned tolerance to photooxidative stress was indeed due to elevated STANN1 levels. In this experiment, STANN1 was produced as an in-frame C-terminal fusion with mGFP. *N. benthamiana* leaf discs were transformed with STANN1_mGFP or mGFP-alone constructs. The leaf discs were then subjected to high light or to the combined action of high light and MeV, as described above. For analysis, leaves with similar fluorescent protein expression levels were used. Exposure to high light alone had no effect on cell structure, regardless of the construct used (mGFP-alone, Figure 10A–D; STANN1_mGFP, Figure 10E–H). High light plus MeV induced cytosol condensation and chloroplast damage (as determined by a decrease in chloroplast autofluorescence) in mGFP-expressing cells (Figure 10I–L). Overexpression of annexin 1 attenuated both these effects and the cells resembled those from control samples (Figure 10M–P). The intensity of chloroplast fluorescence was quantified and, while there was no significant difference in mGFP fluorescence between plants transiently expressing STANN1_mGFP and mGFP-only, the difference in chloroplast red autofluorescence between the mGFP-only and STANN1_mGFP expressing leaves was statistically significant. This strongly suggested that chloroplast structure was maintained in the presence of STANN1 protein.

Discussion

The experiments presented here clearly demonstrate that overexpression of endogenous annexin 1 can be successfully employed to improve crop tolerance to water deficit. In potato, a reduction in the photosynthetic rate in response to water deficit is usually attributed to damage of the lipids, pigments, and proteins in the photosynthetic apparatus. Elevation of STANN1 produced an increased tolerance to the photooxidative damage that accompanied drought. When plants were grown under optimal conditions, genetic modification had no negative effects on plant phenotype, growth, or productivity. We therefore propose that annexin overexpression is a valuable new approach for crop improvement that focuses not on intensification and/or more rapid induction of ABA-mediated stress responses, but rather on delay and/or attenuation of leaf senescence and maintenance of physiological processes when plants are exposed to challenging environmental conditions.

The selection of annexin

As has been observed in other plant species, annexins in potato form a multigene family that encodes proteins with similar overall tertiary structure but different primary amino acid similarities. Despite some extensive similarities, the individual annexins displayed unique expression patterns in the different plant organs (data not shown) and in response to drought. This suggests the specialization of individual family members towards unique roles in growth/development and adaptation to environmental conditions. For example, although no specialized function could be inferred from primary amino acid sequence, functional knock-out of annexin 5 (At1g68090) was lethal in Arabidopsis. Recently [67, 68] showed that expression of this protein took place only during microsporogenesis and that lethality was due to impairment in pollen development.

Expression of several potato annexins was induced during drought. However, only *STANN1* was constitutively expressed and the level of its mRNA during water deficit was several-fold higher than other annexins. This strongly suggested that STANN1 was the primary annexin involved in the response to stress. The constitutive overexpression of *STANN1* minimized the risk of developmental aberrations resulting from ectopic expression of protein during development. We therefore selected STANN1 as a drought tolerance candidate in potato.

Hormonal stress response of transgenic plants

Our results showed that steady-state ABA levels were reduced in transgenic compared to WT. Despite this, the drought-induced accumulation of ABA had similar kinetics in transgenic and WT plants, and levels similar to those in WT were eventually achieved. This suggested that, despite some modifications, ABA synthesis was fully functional in transgenic plants. ABA is of key importance in the abiotic stress response and it was therefore of utmost importance that ABA content, and hence the ABA-dependent stress signaling pathways, were activated equally in WT and transgenic plants. Other signaling pathways were modified. Concurrent sustained CK biosynthesis and higher accumulation of SA might delay senescence and improve plant photosynthetic performance. Elevated CK content resulted in maintenance of substantial photosynthetic activity under drought in tobacco [69]. Exogenous salicylate (SA, aspirin, benzoic acid) affected gas exchange rates and processes related to the PETC due to enhancement of NPQ (Janda *et al*. 2014). SA is also able to exert a protective effect in plants against a wide range of different abiotic factors via its ability to modulate antioxidant systems and affect ASA-GSH homeostasis [70, 71]. Elevated SA in transgenic plants may therefore alleviate photo oxidative stress. In this study, we found that an elevated level of STANN1 protein alleviated oxidative stress and affected the biphasic response of H_2O_2, $O_2^{\bullet-}$, and lipid peroxidation induced by high light. Moreover, stress levels in transgenic plants was reduced. Accumulation of cytosolic *HSP40* mRNA during drought in the transgenic plants was substantially reduced, suggesting that the HSR developed more slowly and was less pronounced in transgenic lines than in WT. This is in accordance with data presented by [57] for *Solanum andigeanum*. In *S. andigeanum*, the degree of stress (estimated by the level of respective *HSP* mRNAs) experienced during drought by a less tolerant landrace was higher than for a more resistant race. One of the consequences of delayed stress detection could be more efficient protection of the chloroplast, resulting in sustained efficient functioning of photosynthetic apparatus.

Improved NPQ in transgenic plants

In control conditions, NPQ in the leaves of transgenic plants was improved. During water scarcity the relative content of Zea in the total Car pool steadily increased relative to WT. In addition, the drought-induced decrease of *PSBS* mRNA was delayed. Collectively, these data suggested that transgenic plants possessed an

improved ability to dissipate the EAE (produced by high light and stomatal closure) that could not be used for photosynthetic purposes. In several plant species, Viol to Zea reconversion is essential for pH-dependent qE, a major and quickly reversible component of NPQ [10, 17, 72]. PSBS was shown to be essential for qE [73]. Zea accumulation in turn correlates with the second, sustained, and slowly reversible component of NPQ: photoinhibition (qI) [74]. Under prolonged light stress, qI replaces qE [75]. The efficiency of Viol pool reconstitution is therefore also essential for effective NPQ.

The PSIIAS dissipates excess energy more efficiently in transgenic plants

Chlorophyll content

In higher plants PSIIAS contains chlorophyll *a* (Chl*a*) and *b* (Chl*b*) and accessory pigments (oxygenated Car derivatives, XCar). Transgenic potato plants with elevated STANN1 preserved a higher Chl*b* content and increased their Chl *a*/*b* ratio during water stress. This indicated that fine-tuning of photosynthetic complexes in response to light stress was altered in the transgenic plants. A similar protective effect of annexin overexpression on the total Chl level was noted in different plant species. However, in contrast to other papers [76 - 78] we analyzed physiological changes in attached leaves of intact plants that were experiencing gradually progressing drought and quantitated different chlorophylls separately. Upon drought, biosynthesis of Chl*b* was induced in transgenic plants, while in WT plants it did not change, even after rewatering. Chl*a* and Chl*b* are differently distributed in the thylakoid membranes, with Chl*b* being restricted to the peripheral PSIIAS. An Arabidopsis mutant that did not synthesize Chl*b* (*chlorine 1, ch1*) due to disruption of chlorophyllide-*a* oxygenase (CAO) gene lacked AS and lost the ability to dissipate the excess energy in NPQ [79]. This loss resulted in increased plant photosensitivity due to an augmented release of $^1O_2$ from PSII [80]. Correspondingly, overexpression of CAO resulted in a decrease in the Chl *a*/*b* ratio and led to transcriptional reprogramming and retardation of senescence [81]. The ability to support Chl*b* synthesis during drought probably helps to improve NPQ and preserves PSII integrity during stress in transgenic plants.

XCar content

During drought stress, transgenic plants suffered less from depression of photosynthetic efficiency (photoinhibition) resulting at least partially from overaccumulation of Zea and, to a lesser extent, Viol. However, the mechanisms underlying the annexin-mediated changes to the XCar pool are unclear. Zea and Viol are produced in chloroplasts as intermediates in the Car biosynthetic pathway that ultimately leads to ABA synthesis. The relative contents of both XCars changes daily and is strongly dependent on environmental conditions (primarily light). In darkness or under sub-saturating irradiance, Zea is epoxidated into Viol in a two-step reaction by zeaxanthin epoxidase (ZEP), resulting in a Viol level approximately ten-fold higher than that of the Zea precursor [82]. However, under specific conditions, this epoxidation step is reversible. When light absorption exceeds the ability of a plant to assimilate CO_2, due to excessive sun radiation or to other environmental factors, preexisting Viol can be re-converted into Zea by ViolDE [83]. In our experiments, samples were collected at the same time to avoid fluctuations in the level of epoxidated and non-epoxidated XCars. The persistent overaccumulation of Zea affected the kinetics of induction and relaxation but not the extent of NPQ. This overaccumulation also led to permanent down-regulation of PSII activity both under experimental conditions and in the field [84, 85]. In turn, overaccumulation of Viol and inability to reconvert to Zea resulted in increased photosensitivity and bleaching as a consequence of the reduction in NPQ [17]. Thus, ZEP/ViolDE activities must be strictly regulated to optimize the efficiency of photosynthesis in a varying environment without increasing the overall XCar pool size. ViolDE is activated when the lumen pH falls below 6.2. ZEP activity requires higher pH (~7.4), molecular oxygen as a second substrate, and NADPH as a cofactor. Optimal activity of ViolDE also relies on lipid bilayer membrane fluidity and requires the presence of phospholipids favoring the formation of a reversed hexagonal structure, monogalactosyldiacylglycerol [86]. Independent of stress durability and severity, 20–50% of Viol remains unconverted. Suggested mechanisms include feed-back inhibition of ViolDE by Zea [87] or curvature membrane stress elicited on the lipid bilayer by Zea [88]. Alternatively, since Viol is also a precursor of the side-branch synthetic pathway leading eventually to ABA synthesis [89] it cannot be excluded that some enantiomer of Viol is inaccessible for ViolDE [87].

At constant light intensity, moderate water deficit leads to an increase in Zea level. Increasing amounts of Viol are needed as water deficit progresses as it is both

re-converted into Zea and used to synthesize ABA. The presence of spatially (shoot *versus* root) or metabolically (e.g., stereoisomers, 9-*cis*, and 9-*trans*) separate pools of Viol were postulated, as these would support the continued functions of xanthophyll cycle or ABA synthesis, respectively (reviewed in [90]). However, the factors determining the functional partitioning of Viol into different pools remain largely unknown.

Finally, the lower steady-state level of ABA and concomitant increased Zea level in transgenic plants may be simply due to the lower levels of oxidative stress in the chloroplasts of those plants. Light-stress-dependent ABA biosynthesis results from inefficient reduction of ascorbic acid (AA) under these conditions. Protonated AA is an essential cofactor for ViolDE and synthesis of Zea in the thylakoid lumen [91]. The XCar cycle cannot function properly when ascorbate recycling is inefficient and Viol accumulates. This can result in ABA synthesis. The ABA increased by 60% in leaves of ascorbate-deficient *vte1* Arabidopsis plants [92]. Hence, improved NPQ may accelerate ascorbate recycling and therefore support Zea synthesis.

How does annexin affect chloroplast-localized processes?

Although annexins contain no specific signaling sequences that target them to the chloroplast, annexins were found in the chloroplast proteome of some plant species (reviewed in [93]). For example, a mustard (*Sinapsis alba L.*) annexin was found to be a component of a multi-subunit chloroplast RNA polymerase A complex [94]; however, these results were not confirmed in a subsequent study [95]). Recently, ATANN1 was detected in the hydrogen peroxide-sensitive chloroplast proteome [96]. These chloroplast localizations remain exceptions. The question then remains as to how annexins can affect the processes within the chloroplast?

It is probable that annexin-mediated protection of the chloroplast and photosynthesis could be an indirect effect of the ability of annexin to counteract photooxidative stress. Chloroplasts are one of the major sources of ROS during stress in plant cells [11]. ROS derived from electron leakage from overloaded PETC can be released to the cytosol where they pose a threat to cellular macromolecules and structures and also participate in retrograde chloroplast-to-nucleus signaling [97] and pcd induction [33], [98]. Annexin STANN1 was able to attenuate both phases of chloroplast-derived oxidative stress. In transgenic plants, the expression of nucleus-encoded PSII proteins (Figs. S6A–B) and HSPs was modified correspondingly (Figs.

S6C–D), which strongly suggested that annexins were able to modulate retrograde oxidative signaling. The mechanisms underlying this activity are unclear. Annexins were proposed to undergo thiol-disulphide cycles in a similar manner to the low-molecular-weight antioxidant glutathione or thioredoxins [99]. The presence of redox-sensitive cysteines has been shown for mammalian AnxA2 [100] and the ability of AnxA2 to regulate cellular redox homeostasis by participation in reversible cycles of oxidation and reduction of their cysteines was recently confirmed *in vivo* [100, 101]. Since annexins are abundant (it is estimated that in the plant cell they can constitute up to 2% of the total soluble proteins) they could participate significantly in the cellular protein thiol pool. In our previous study [52], we showed that Arabidopsis annexin 1 (ATANN1) contains two cysteines (111 and 239) that are not involved in formation of intramolecular disulfide bridge but can undergo S-gluthathionylation upon ABA treatment. [96] recently showed that Arabidopsis ATANN1 participated in the cellular thiol pool. ATANN1 was one of 24 proteins that were oxidized *in vivo* in Arabidopsis plants after MeV-induced photooxidative stress. However, only the first cysteine is preserved in potato annexin STANN1 (Figure S3 in File S1Figures). STANN1 nevertheless exerts a protective effect, which suggests that a different mechanism is involved.

Conclusions

The results obtained in this study clearly indicate that annexin overexpression is of potential utility in developing drought-tolerant crop plants. Enhanced drought tolerance is connected to the ability of transgenic potato plants to better deal with light stress induced by stomatal closure and diminished CO_2 supply. The attenuated ROS accumulation improved chloroplast function and genetically modified plants were able to maintain efficient PSII under stress conditions. Maintenance of a high photosynthetic yield even under sub-optimal conditions had a beneficial effect on final plant yields. Thus, annexins are a promising target for manipulation of plant tolerance to environmental conditions.

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Figure legends.Figure 1. Annexin genes in potato genome.

(A) Localization of annexin genes on potato chromosomes. The Roman numerals at the top denote the chromosome, digits in brackets indicate chromosome size.

(B) Intron-exon organization of potato annexin genes.

(C) Genomic PCR confirming the presence of predicted annexin genes in WT potato. Specific primers anneal to the 5'- and 3'- ends of coding sequence of certain annexin gene, hence the length of the resulting PCR product is a sum of the respective coding sequence with introns.

(D) Schematic arrangement of *STANN3.1*, *STANN3.2*, *STANN3.3* and *STANN4* on chromosome I.

Figure 2. Profiling of annexin expression in WT potato leaves during drought.

Potato WT plants grew in the walk-in growth chamber under controlled conditions. After 8-10 weeks irrigation was gradually reduced to decrease the field capacity (FC) to 25% (which took approximately 10 days) and then maintained at this level till 14^{th} day. Samples were collected from the first fully developed composite leaf from the top at indicated time points (D0 – beginning of drought, D6 – sixth day of drought, and D14 – fourteenth day of drought). RNA was isolated with Trizol and sq-RT-PCR was performed with primer sets specific for certain annexins. The level of expression was normalized against *EF1a* mRNA. Results are means ±SE ($n \leq 4$). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 3. Drought tolerant phenotype of transgenic plants.

Potato WT plants and transgenic lines (S-2, S-7) was subjected to drought as described above in Fig. 2. (A) Drought stress phenotype of WT (left column), S-2 (middle column) and S-7 (right column) plants. Photographs were taken on the beginning (D0), on eighth (D-8) and ninth (D-9) day of drought. Experiments were repeated twice in greenhouse and twice in growth chamber and gave similar results.

(B) Regeneration of potato plants after prolonged drought. The procedure of drought imposition was the same as described above but the FC was maintained at 25% until the

twenty first day of drought (D21). On D22 plants were rewatered and after draining of gravitationally bound water FC was kept up at 65%. Photograph was taken on the third day after rewatering. Left side - two WT plants; middle – two S-2 plants, and right – two S-7 plants. Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.

Figure 4. Examination of leaf water status.

Potato WT plants (white bars) and transgenic lines: S-2 (gray bars) and S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. (A) Relative water content (RWC) analysis. Samples from the first fully developed undamaged leaf from the top of plant were collected at D0, D4, D7, D12 and 3 days after rewatering (RW3) and relative water content (RWC) was determined. Results are means ±SE (n=3). (B) Stomatal conductance were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results.
Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated 3 times and gave comparable results.

Figure 5. Netto photosynthesis and photosynthetic performance of PSII in potato plants during drought.

Potato WT (white bars) and transgenic lines: S-2 (grey bars) and S-7 (black bars) were subjected to drought as described in Fig. 2. (A) Netto photosynthesis, (B) maximum quantum yield of photosystem II (Fv/Fm) and (C) effective quantum yield of photosystem II,Y(II) were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters

designate values which are not significantly different at $p<0.05$ and belong to the same homogenic group.

Figure 6. Photosynthetic pigment content during drought.

WT (white bars) and transgenic line S-7 (black bars) were exposed to drought as described in Fig. 2. Samples were collected at the same time during the day at D0, D6, D14 and RW3 from third, fourth and fifth fully expanded leaves from top at 4 hours after turning the light. The level (A) chlorophyll *a*; (B) chlorophyll *b*; (C) zeaxanthine; and (D) violaxanthine were determined Non-polar lipids were separated on an ACQUITY UPLC system (Waters) and peaks were integrated at 436 nm. The level of xanthophylls is expressed as percent of the total carotenoids. The level of chlorophyll is expressed as mg mL^{-1}. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 7. Accumulation of stress-related hormones during drought.

WT (white bars) and transgenic line S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. The level of (A) absicisic acid ABA; (B) sum of active cytokinins, CK; (C) salicylic acid, SA were determined at D0, D6, D14 and RW1. Samples (0.5g of fresh leaf tissue without the midrib) were collected from the first fully developed, undamaged leaf from the top of plant at 4 hours after turning the light. Labeled internal standards were added to the leaf samples before homogenization. Hormones were then extracted, purified using a SPE-C18 column and separated on a reverse phase-cation exchange SPE column. Hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer. The level of ABA and SA is expressed as nmol g^{-1} of fresh weight; the levels of cytokinins – as pmol g^{-1} of fresh weight. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 8. NPQ assayed in leaf of well-watered potato plants.

Potato WT (dashed line) and transgenic S-7 (solid line) grew in the walk-in growth chamber under controlled conditions and were watered to maintained FC at 65%. Performance of gross non-photochemical quenching (NPQ) were assayed on the first fully developed composite leaf from the top of plant at 4 hours after turning the light with Dual PAM-100. For measurement plants were adapted to dark for 20 minutes and then stimulated with repeated light pulses of actinic light (94 PPFD) for 5 minutes and once again subjected to dark for 6 minutes. Each point represents the mean ±SD (*n*=3-4). Experiment was repeated three times and gave comparable results.

Figure 9. Accumulation of ROS (hydrogen peroxide and superoxide anion) and lipid peroxidation.

Potato WT (white bars) and transgenic line S-7 (black bars) grew in walk-in growth chamber under controlled conditions. Leaf discs were expunged from the third, fourth and fifth upper fully expanded leaves and immediately vacuum infiltrated with methyl viologen (50 μM). After 1 hour incubation in dark discs were exposed to high light irradiance (850 PPFD) for indicated times (0.5 – 24 hours). Superoxide anion was determined colorimetrically with nitro blue tetrazolium chloride 9NBT). Hydrogen peroxide was stained in tissue with diaminobenzidine tetrahydrochloride (DAB) and quantified using the ImageJ. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA). Results are means ±SE (n=5). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 10. STANN1 attenuated MeV-induced photooxidative stress.

Confocal laser scanning image of the leaf epidermis of tobacco plant transiently expressing GFP (A-D and I-L) or STANN1_GFP (E-H and M-P). 3 days after infiltration leaf discs were excised and subjected to high light (850 PPFD) (A-H) or the combine treatment of high light (850 PPFD) and 50 μM MeV (G-L). The fluorescence was monitored with Nikon TE-2000E EZ-C1 exc. 488 nm and emission 515/30 and 605/75 for GFP and chloroplast, respectively. First column represent single focal plane, second – chloroplast autofluorescence acquired with the same excitation parameter for each construction to visualized the difference between

responses to the same treatment, third – overlay of green and red fluorescence channels with GFP enhanced to visualized cells; right column – stack obtained with Volume Render program EZ-C1 combined with chloroplasts. Scale bar is 20 μm. Experiment was performed 3 times.

Supporting information legends:

File S1.

Figure S1. Construction of transgenic plants.

A) Structure of the T-DNA region from pROK2 carrying STANN1_His6x that was used for *Agrobacterium* -mediated transformation. LB – left border; RB – right border; NPTII – neomycin phosphotransferase II, CaMV – cauliflower mosaic virus 35S promoter; NOS – nopaline synthase terminator;
(B) Expression of STANN1_His6x protein in F1 transgenic potato lines. Proteins were isolated from leaves of WT and F1 transgenic lines S-2, S-3, S-7, S-83, S-91, S-97 and S-123 grown *in vitro*. His-tagged proteins were purified with Ni-NTA agarose, subjected to SDS_PAGE and blotting followed by detection with anti-His primary Ab. The band detected in WT represents *Arabidopsis* annexin ATANN1_His6x (molecular weight *ca* 36 kD) produced in *Escherichia coli* that was added before purification to the ground protein to STANN1_His6x easily dimerized hence the two bands were detected, the lower with molecular weight corresponding to monomer and the upper corresponding to dimer.

Figure S2. Characteristics of experimental drought.

(A) Potato WT and S-7 plants after 8 week of growth at the phase of experimental drought implementation. Transgenesis has no impact on tuber development. Formation of stolon hooks and stolon swelling as well as first tubers are visible.
(B) Field capacity (FC) was normalized at the beginning of experiment and maintained at constant level (app. 65%); for control (well-irrigated plants) FC was maintained at this level throughout the whole experiment. For experimental drought FC was gradually lowered to 20% and kept at this level un till the end of drought. Rewatering was applied by full water saturation of the soil and after gravity draining of excess water FC was kept at the 65% untill the end of experiment.

Figure S3. Multiple alignment of amino acid sequences of putative annexins from potato and selected annexins from human, Arabidopsis and cotton.

The alignment was done with Cobalt (Constrain-based Multiple Alignment Tool). Gene Bank Acc Nos of employed sequences are as follows: human AnxA5 (NP_001145.1), Gossypium hirsutum GHANN1 (1N00), Arabidopsis thaliana ATANN1 (2Q4C) and for potato annexins: STANN1 PGSC0003DMG4000177114, STANN2, STANN3.1 PGSC0003DMG4000221817, STANN3.2 PGSC0003DMG401019427, STANN3.3 PGSC0003DMG402019427, STANN4 PGSC0003DMG400019446, STANN5 PGSC0003DMG400007966, STANN8 PGSC0003DMG400007482 and STANN9 PGSC0003DMG40001879.

The boundaries of endonexin repeats were marked on the basis of crystal structures obtained for GHANN1 (Hofmann et al., 2003) and ATANN1 (Levin et al., 2007) and are, respectively:

- 1st endonexin domain: 14-80 and 13-81;
- 2nd endonexin domain: 83-153 and 84-154;
- 3rd endonexin domain: 164-239 and 165-241
- 4th endonexin domain: 241-309 and 244-3111 respectively for cotton and Arabidopsis annexin.

Conserved histidine 40 is in red; methionine and cysteines from C3 cluster are in blue and underlined.

Calcium binding motifs G-X-GTD-{38-40}-D/E are marked by black boxes; potential N-terminal acylation motif is in bold; potential actin-binding domains IRI are in bold and italic;

C-terminal peptide similar to 14-3-3 proteins is marked by pale-green rectangle.

Amino acid residues of high conservation are shown in red, medium - in blue.

Figure S4. Drought tolerant phenotype of transgenic S-7 potato plants.

Each image depicts two WT plants (left side) and two transgenic S-7 plants (right side) subjected to experimental drought. Drought was started on D0 and lasted 21 days. During that time watering was gradually reduced so as to lower the FC to 20%. After reaching that level it was maintained until 21 days after onset of experiment. The soil was than fully saturated with water (rewatering) and FC was maintained at 65% until the end of experiment.

D10 - irrigation withheld for 10 days, D14 - irrigation withheld for 14 days, D21 - irrigation withheld for 21 days, RW5 – rewatered for 5 days.
Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.
In WT symptoms of wilting clearly appeared after 10 days of drought; in S-7 they were apparent only after 2 weeks. On the 21st day WT were severely affected with damaged stems and dry leaves. At the same time in S-7 plants the upper leaves still maintained turgor. After rewatering only a few leaves in WT regenerated; instead, new shoots developed from below-ground parts after at least a week of regular irrigation. In contrast, the S-7 plants preserved their upper leaves and after rewatering returned to a normal healthy look within hours. The exact number of irreversibly damaged leaves varied between experiments, but it was always significantly lower than in WT.

Figure S5. Potato yield during drought.

(A) Irrigated Water Use Efficiency (IWUE) is a quotient of crop produced per unit per amount of water supplied (IWUE = Y / W [g/pot/mL of water])
(B) An exemplary tuber yield per plant. Potato plants WT, S-2, and S-7 were grown in a greenhouse. After 8-10 weeks of growth plants were subjected to drought stress by restricting irrigation to achieve 20% FC and kept at this level until 14th day. After that time plants were rewatered and cultivated in optimal conditions for additional 10 weeks until physiological maturity. Tubers were lifted immediately after withering of haulms. The weight of all fresh tubers from single plant was determined immediately after harvesting. Experiments were repeated twice and gave similar results.
(C) Quantification of tuber yield experiments. Results are shown as mean ±SD (n=10)

Figure S6. Expression of genes coding for PSII proteins and HSPs.

Relative quantification of *PSBS* (A), *LHCB4* (B) *HSP100* (C) and *HSP40* (D) mRNAs in leaves of WT (white bars) and transgenic S-7 (black bars) potato plants during three-week drought and after rewatering. The data represents the mean ±SE from at least four measurements. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test, the same letters designate

days which are not significantly different at $P < 0.05$ and belong to the same homogenic group.

Figure S7. The effect of photooxidative stress on potato leaves.

Leaf discs (F ~ 1 cm) were excised from leaves of WT or transgenic plants S-2 and S-7 and immediately infiltrated with (A) 50 mM Tris-Cl, pH 7.5 (B)10 mm MeV or (C) 50 mM MeV in 50 mM Tris-Cl, pH 7.5. Subsequently, leaf discs were exposed to light of 150 PPFD for 30 h.

File S2.

Table S1. Primer pairs used for identification of potato annexins.

Primer pairs corresponding to the predicted 5' (F) and 3' (R) ends of the particular annexin genes were designed on the basis of published potato genome sequence. Gene length refers to the total length of exons and introns. Individual primer pairs (F - forward, R - reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA).

Table S2. Primer pairs used for sq-RT-PCR.

Primers for semi-quantitative analysis of expression of annexins and other genes in potato. Individual primer pairs (F- forward, R- reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA) to span intron–exon boundaries to exclude interference from genomic DNA contamination. Amplified fragments were between 300 and 500 base pairs. The genes were selected from PGSC_DM_v3.4_pep_fasta containing database of potato virtual translation products on the basis of their homology to annotated Arabidopsis genes. Analyzed genes were as follows: annexins: STANN1-9; HSP100 (heat shock protein 100 kDa); HSP40 (heat shock protein 40kDa, DNAJ); PSBS (chlorophyll a/b- binding photosystem II 22kD subunit S); LHCB4 (light-harvesting complex binding protein 4). As a reference the housekeeping gene for Elongation Factor a1 (EF1a) was used.

Table S3. Characterization of putative potato annexin proteins.

chlo – chloroplast; cyto – cytoplasm; cyto_ER – cytoplasm/membrane of endoplasmatic reticulum; cysk – cytoskeleton; ER – endoplasmatic reticulum; extr – extracellular; mito – mitochondria; nucl – nucleus; plas – plastids; vacu – vacuole

Table S4. Cytokinins in leaves of WT and S-7 potato plants under drought.

S. tuberosum WT and transgenic S-7 plants were subjected to 2-week drought or well-watered. At time points indicated 0.5 g of tissue (without the main vein) was collected 4 hours after beginning of the day from fully expanded leaves. Hormone levels were analyzed by LC-MS as described in Materials and Methods (n=3). Data are shown as pmol g-1 FW.

Abbreviations: tZR, trans-zeatine riboside; tZ, trans-zeatin; iPR, isopentenyl adenosine riboside; iP, isopentenyl adenine; cZR, cis-zeatin riboside; cZ, cis-zeatin.

Potato ~~annexin~~ Annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic *Solanum tuberosum* L. Plants

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Abbreviations:

ABA	abscisic acid
ATANN1	annexin 1 from *Arabidopsis thaliana* L.
Car	carotenoids
Chl*a*, Chl*b*	chlorophyll *a*, chlorophyll *b*
CK	cytokinins
~~EAE~~	~~excess absorbed energy~~
Fv/Fm	the maximum quantum efficiency of photosynthesis
~~HSR~~	~~heat stress response~~
MeV	methyl viologen, paraquat
NPQ	non-photochemical quenching
~~pcd~~PCD	programmed cell death
PETC	photodynthetic electron transport chain
PPFD	photosynthetic photon flux density [μmol m^{-2} s^{-1}]
PSI, PSII	photosystem I, photosystem II
~~PSIIAS~~	~~antennae system of photosystems II~~
PSBS	22-kD photosystem II subunit
STANN1	annexin 1 from *Solanum tuberosum* L.
SA	salicylic acid
Viol	violaxanthine
~~ViolDE~~	~~violaxanthin de-epoxidase~~
Y(II)	effective quantum yield of PSII
XCar	xanthophylls
Zea	zeaxanthine
~~ZEP~~	~~zeaxanthine epoxidase~~

Abstract

Annexins are a family of calcium- and membrane-binding proteins that are important for plant tolerance to adverse environmental conditions. ~~Certain a~~Annexins ~~are able~~function to counteract oxidative stress, ~~affect~~ maintain cell redox homeostasis, and enhance drought tolerance. In the present study, an endogenous annexin, STANN1, was overexpressed to determine whether crop yields could be improved in ~~a crop plant,~~ potato (*Solanum tuberosum* L.)~~,~~ during drought. Nine potential potato annexins were identified and their expression characterized in response to drought treatment. *STANN1* mRNA was ~~constitutively~~ expressed at a high level and drought treatment ~~transcription was~~ strongly increased ~~during drought~~transcription levels~~., and this gene was t~~Therefore, *STANN1* was selected for overexpression analysis. Under drought conditions, transgenic potato plants ectopically expressing STANN1 were more tolerant to water deficit in the root zone~~,~~ ~~and~~ preserved more water in green tissues~~, than wild-type plants. Despite the drought stress, chloroplast functions were~~ maintained chloroplast functions, and had higher ~~in transgenic plants and~~ accumulation of chlorophyll *b* and xanthophylls (especially zeaxanthine) ~~increased compared to the~~ than wild type. ~~Furthermore, d~~Drought-induced reductions in the maximum efficiency of PSII (PSII), the quantum yield of photosynthesis, and the electron transport rate were less pronounced in transgenic plantsoverexpressing STANN1 than in the wild type. This ~~resulted in~~conferred more efficient non-photochemical energy dissipation in the outer antennae of PSII and ~~provided for~~ probably more efficient protection of reaction centers against photooxidative damage in ~~the~~ transgenic plants under drought conditions. Consequently, these plants were able to maintain effective photosynthesis during drought, which resulted in ~~their overall better~~greater productivity than wild-type

plants despite ~~the period of~~ water scarcity. Although the mechanisms underlying this stress protection are not yet clear, annexin-mediated photoprotection is probably linked to ~~the ability of annexin to counteract~~ protection against light-induced oxidative stress.

Introduction

Plants have developed ~~concurrent~~ passive and active strategies to survive environmental stresses such as drought, salinity, chilling, heat shock, heavy metals, UV radiation, ozone, mechanical stress, nutrient deficiency, hypoxia, and biotic stress ~~shorter or longer episodes of water deficit~~ [1]. Several stress-response genes have already been ~~used~~ targets for bioengineering ~~with the aim of improving plant resilience to water deficit~~ studies to improve plant stress tolerance [2]. However, ectopic expression of stress-inducible genes often results in developmental aberrations (e.g., stunted growth and irregular leaves) or ~~impeded~~ reduced crop yields under non-stress conditions ~~as the result of uns~~due to non-specific induction of programmed cell death (~~pcd~~PCD) and/or premature senescence [3]. Current knowledge of stress-responsive pathways is based primarily on results obtained by imposing each stress individually, whereas plants in natural settings are generally challenged with multiple concurrent stresses, and the resultant signaling pathways may be superimposed and/or induce/antagonize each another [4-6]. New approaches ~~utilizing different mechanisms~~ to bioengineering stress tolerance in crop plants are needed to achieve sustainable improvements in crop biomass production [2].

~~Environmental stresses such as drought, salinity, and low and high temperature primarily affect chloroplast metabolism and trigger imbalances in the chloroplast redox homeostasis [4 - 6]. Thus, the ability of a plant to withstand stress and recover afterwards is strongly related to the capability to support photosynthesis and protect chloroplast structures and functions despite unfavorable conditions. In the plant cell, reactive oxygen species (ROS) are continuously formed as by-products during photosynthesis under normal conditions. The steady-state level of ROS is tightly regulated by several enzymatic and low-molecular-weight antioxidant systems [7].~~

However, under adverse environmental conditions the capacity of antioxidant systems can be exceeded, and this leads to redox imbalance.

The two main sources of ROS in the chloroplast are the light-driven photosynthetic electron transport chains (PETC) of photosystem I (PSI) and photosystem II (PSII). PSII and PSI work sequentially to transport electrons released from a photo-excited primary electron donor (chlorophyll, Chl) on the lumenal thylakoid side of PSII to the terminal acceptor, ferrodoxin, on the stromal side in PSI. PSII catalyzes water oxidation and hence provides electrons for all further photosynthetic reactions. PSII efficiency is therefore crucial for the efficiency of photosynthesis as a whole. PSII is a highly spatially organized structure that consists of core complexes (PSIICC) associated with the peripheral antennae system (PSIIAS) [8]. PSIIAS has two main functions. First, sunlight energy is captured and transferred to PSIICC, where primary photochemical reaction occurs. Second, PSIIAS protects the whole PS against photooxidative damage. PSII is the most highly vulnerable structure of the entire photosynthetic apparatus and this mediates plant adaptation to environmental conditions. When light intensity exceeds that which a plant can utilize in photosynthesis, over-reduction of PETC results in generation of highly reactive and low-diffusible singlet oxygen ($^1O_2$), which subsequently oxidizes PSII constituents *in situ* [9 - 11]. Although singlet oxygen cannot itself function as a signaling molecule, experimental data showed that it activates cellular signaling pathways of surprising complexity [11]. Peroxidation of different PSIIAS components gives rise to a plethora of compounds that act as secondary messengers, including products of fatty acid peroxidation, reactive electrophile species (RES), and volatile derivatives of β-carotene oxidation (e.g., β-cyclocitral and dihydroactinidiolide) [12 - 14]. In addition, the release of intrinsic chloroplast proteins after stress induction resembles

similar leakage of mitochondrial proteins into the cytosol [15]. These processes collectively trigger the activation of nuclear genes involved in the molecular defense against photooxidative stress.

PSIIRC is protected against oxidation via non-photochemical quenching (NPQ), a heterologous phenomenon that allows the excess absorbed energy (EAE) from PSIIAS to dissipate before it reaches the reaction center [16 - 17]. Several NPQ components have been distinguished based on the time scale of formation and relaxation, including qE, qT, qZ, and qI [18]. qE (energy-dependent quenching) is triggered by the formation of a pH gradient across the thylakoid membrane, can be detected as heat emission within microseconds, and relaxes in minutes [19]. Two essential factors are involved in its related NPQ: zeaxanthine (Zea) and a 22-kD PSII subunit protein (PSBS) [20 - 21]. Acidification of the thylakoid lumen activates the xanthophyll cycle and produces violaxanthine de-epoxidase (ViolDe), which converts violaxanthin (Viol) into Zea. *Arabidopsis thaliana* (Arabidopsis) plants with higher levels of epoxidated Car derivatives - xanthophylls (XCar), as a result of overexpression of β-carotene hydroxylase, were more tolerant to high light and high temperature [22]. In *npq2*, another Zea-overaccumulating mutant, the kinetics of induction and relaxation of NPQ, but not its extent, were affected [17]. Zea modes of action are complex and include direct antioxidant activity [23], allosteric modulation of membrane function in PSII, and enhancement of proton binding activity [24]. In turn, PSBS affects membrane rigidity [25 - 26] and senses pH changes [27], but its precise role in energy dissipation remains unclear [28]. Arabidopsis *npq4-1* mutants that lacked PSBS and had a normal XCar cycle were deficient in qE [29 - 30]. It was therefore suggested that PSBS functioned as a kinetic modulator of NPQ in the PSIIAS [31].

~~The second major source of ROS in the chloroplast is PETC of PSI. Stomatal closure and diminished CO_2 supply reduces the rate of Calvin cycle reactions and leads to inefficient regeneration of NADP+. Uncontrolled electrons flow from ferredoxin to O_2, leading to generation of the superoxide anion $O_2^{\bullet -}$ via the Mehler reaction (photochemical quenching) [4, 32]. Once formed, $O_2^{\bullet -}$ is subsequently converted into H_2O_2 or initiates chain reactions that give rise to other more reactive ROS molecules. The intensity of the Mehler reaction increases with deepening water stress. Within 0.5 s of inactivation of the chloroplast enzymatic scavenging systems, CO_2 fixation was reduced by half and 10 μM hydrogen peroxide accumulated [4]. H_2O_2 is very stable, with a half-life of ~1 ms, and can pass through biological membranes. H_2O_2 leaking induces an oxidative burst in the cytosol. The origin of this burst is poorly understood, but it likely has a complex nature consisting of both chloroplast and cytosol components. It was also showed that chloroplast-derived ROS are indispensable for intercellular ROS signaling [33].~~

~~During prolonged stress, additional sources of ROS appear in the cell. Limited CO_2 availability activates oxygenation of RUBISCO instead of carboxylation and this induces photorespiration. Neutralization of toxic products occurs in peroxisomes and is accompanied by H_2O_2 production. Photorespiratory H_2O_2 was shown to participate in the execution of pcd [34].~~

~~Plant tolerance to adverse environmental conditions is also mediated by the collective action of different hormones. Abscisic acid (ABA) and cytokinins (CK) are the most intimately involved in the plant response to decreased soil humidity. These hormones mediate mostly antagonistic effects. ABA is considered as a "stress-hormone" since it activates and coordinates different stress responses such as rapid stomatal closing and metabolic reprograming [35 – 36]. In turn, CK assists in~~

recovery after stress by activating cell division, chloroplast development, synthesis of chloroplast proteins/photosynthetic pigments, and formation of membrane components of PETC [37]. Artificial maintenance of CK biosynthesis during conditions of stress was found to be beneficial for plant survival. Accordingly, overexpression of a CK biosynthetic enzyme, isopentynyltransferase, enhanced plant tolerance to a wide range of abiotic factors [38 - 41]. Thus, strategies to enhance agriculture via improved protection of chloroplast structure and function under stress conditions are promising.

The role of salicylic acid (SA) in the regulation of plant interactions with the environment has been recognized in recent years and SA has emerged as an important factor involved in acclimation to certain abiotic stressors [42 - 45]. Singlet oxygen accumulation in the chloroplast was shown to induce rapid accumulation of SA [46]. At high concentrations, in cooperation with other signals, SA strongly promoted $^1O_2$-induced pcd [47 - 48]. Although their role in pcd is not clear, functional chloroplasts are necessary for the hypersensitive response [49 - 51]. Previously, we demonstrated that overexpression of endogenous annexin 1 (ATANN1), improved the tolerance of the model plant Arabidopsis Col-0 to drought [52 - 53] and that ATANN1 undergoes S-glutathionylation upon ABA induction. The objective of the present study was to verify if a similar improvement in drought tolerance could also be achieved by overexpression of annexin in the potato (*Solanum tuberosum* L.). Thus, transgenic plants with elevated levels of endogenous annexin STANN1 were created and tolerance to a transient soil water deficit was assessed. Our results showed that annexin overexpression during drought improved NPQ and alleviated photo-oxidative stress and thereby protected chloroplast function.

Recent work shows that changes in redox poise can regulate plant cell function [7-10] by acting as cellular signals [11]. Numerous studies have investigated redox-mediated stress responses [12-16]. Our understanding of the mechanisms that control production and metabolism of reactive oxygen species (ROS) in plants is incomplete. ROS are generated in several cellular compartments. In light, the predominant location of ROS production is in chloroplasts [17, 18], where ROS are continuously formed as photosynthetic by-products under non-stress conditions. Environmental stress can trigger an imbalance in redox homeostasis and affect chloroplast metabolism [17, 18]. The chloroplast antioxidant system has been described as "loosely tailored" to maintain an endogenous ROS pool [20], which enables plants to quickly respond to fluctuating light levels. Consequently, significantly improving ROS scavenging enhances protection against sustained stress, but also desensitizes plant light responses and impairs environmental fitness. This result suggests that a delicate balance has to be maintained between ROS production and the activity of antioxidant systems. Chloroplasts also are involved in biosynthesis of hormones, carotenoids (Car), amino acids, and lipids. Therefore, the protection of chloroplast structure and function from environmental stresses is crucial for plant stress resistance and recovery.

The two main ROS sources in chloroplasts are the light-driven photosynthetic electron transport chains (PETCs) of photosystem I (PSI) and photosystem II (PSII). Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the PETCs [21]. Under these conditions, oxygen can be utilized instead of $NADP^+$ as an alternative acceptor for excess electrons [22]. Several mechanisms have evolved to dissipate excess excitation energy, including nonphotochemical quenching (NPQ), which dissipates excess electrons as heat in PSII [23] and via the water-water cycle

in PSI [21]. The water-water cycle produces hydrogen peroxide (H_2O_2), which is the most stable small ROS that can passively and actively cross membranes [24, 25]. In isolated intact spinach chloroplasts and *Arabidopsis thaliana* (Arabidopsis) protoplasts, H_2O_2 produced under illumination can avoid neutralization by chloroplast antioxidant systems, and escape the organelle in a light-intensity-dependent manner [26-28].

Changes in chloroplast redox poise activate secondary ROS-producing sources in other cellular compartments, such as membrane NADPH oxidases [respiratory burst oxidase homologs (RBOHs)]. Abscisic acid (ABA) induces *RBOH* genes expression in Arabidopsis leaves and guard cells [29-30], *Hordeum vulgare* aleurone layer [31], and *Zea mays* seedlings [32]. NADPH oxidase-mediated ROS accumulation has been reported in ozone-treated Arabidopsis leaf [33, 34] and salt-treated Arabidopsis root tips [35].

Prolonged drought stress results in CO_2 deficiency due to stomatal closing and activates photorespiration. This rescue reaction dissipates excess reducing equivalents and energy, but generates H_2O_2 during salvage of the toxic byproduct 2-phosphoglycolate in peroxisomes. Under drought conditions, photorespiration is estimated to generate 70% of the H_2O_2 produced [36]. One of the major roles of photorespiration was proposed to be readjustment of redox homeostasis under abiotic stress conditions [37]. Cytokinin (CK)-mediated stimulation of photorespiration in transgenic tobacco plants resulted in substantially enhanced drought resistance [38].

Different buffering systems in discrete subcellular compartments are involved in maintaining cellular redox homeostasis and redox signaling. The primary pathway of H_2O_2 scavenging is the glutathione-ascorbate cycle (GSH-As cycle), which operates in chloroplasts, cytosol, mitochondria, and peroxisomes [21, 39]. Each cellular

compartment contains a specific set of H_2O_2 scavenging enzymes, such as peroxidases, catalases (CAT), dismutases, glutaredoxins (GRX), and thioredoxins (TRX) [40, 41]. In Arabidopsis and tobacco plants enhancing the antioxidant capacity of chloroplast and cytosol had a beneficial effect on photosynthesis and stress tolerance [42], whereas removal of any single antioxidant component reduced photosynthesis and stress tolerance [43-47]. There is evidence for cross-talk between antioxidant systems from different compartments [40, 48].

Annexins are a multigene, evolutionarily conserved family of calcium- and phospholipid-binding proteins [49]. They are present in all eukaryotes and are characterized by a highly conserved tertiary structure. The annexin superfamily is defined by the presence of approximately 70 amino acid endonexin motifs, which contain five α-helices that are repeated four (or eight) times in the protein. The contribution of annexins to plant cell adaptation to adverse environmental conditions is well documented [49-53]. However, an understanding of the primary physiological functions of plant annexins remains elusive.

Annexin 1 was identified in a genome-wide search of Arabidopsis sequences capable of rescuing *Escherichia coli* ΔoxyR growth on high H_2O_2 concentrations [54]. Subsequent analyses confirmed that Arabidopsis annexin 1 (ATANN1), *Brassica juncea* annexins BJANN1 and BJANN3, and *Nelumbo nucifera* annexin NNANN1 ameliorated oxidative stress in homologous or heterologous cells and improved stress tolerance [55-61]. Transgenic tobacco plants expressing BJANN1 were more tolerant of different abiotic stresses and exhibited enhanced resistance to *Phytophthora parasitica* var. *nicotianae*, possibly due to constitutively increased expression of several pathogenesis-related proteins [55]. NNANN1 overexpression in Arabidopsis conferred enhanced tolerance to heat and oxidative stress [58]. Deletion

of functional ATANN1 reduced expression of glutathione-*S*-transferase Tau 1 (GSTU1) in Arabidopsis seedlings after H_2O_2 treatment [62]. Under salinity conditions, ATANN1 mediates ROS-dependent Ca^{2+} signaling in roots at low (1.5 mM) external Ca^{2+} concentrations [63]. These studies used seedlings or leaf discs subjected to short-term stress treatments (in hours). There is a lack of information regarding annexin function in cell physiology and metabolism during long-term exposure to environmental stress.

Potato is one the most important vegetable crops. Its global annual production in 2010 exceeded 300 million tons (FAOStat). Potato plants are highly efficient in terms of water usage (http://www.fao.org/potato-2008/en/potato/water.html), and produce more food per water unit than any other crop [64]. Therefore, potato could be a promising alternative to cereal crops. Modern potato cultivars are susceptible to drought, which is defined as a shortage of water in the root zone [65]. Water deficit affects nearly all stages of potato development, and negatively impacts tuber numbers and quality (crop yield) [66, 67]. Only a few attempts to engineer potato drought tolerance have been reported (reviewed in [68]). These studies had limited success because most transgenic plants did not exhibit good performance and productivity under non-stress and stress conditions. Potato annexin has not been considered for bioengineering applications; however, new proteomics research showed that STANN1 could be a candidate gene to improve stress tolerance. STANN1 was differentially expressed in potato tubers in response to wounding [69, 70], bruising (personal observation; [71]), osmotic stress and salinity [72], and was differentially expressed in potato aerial parts in response to osmotic stress and salinity [73]. In this study, we overexpress potato annexin STANN1 and observe the effects on drought tolerance.

First, we investigated if increased expression of ATANN1 affected potato drought tolerance. We used the *S. tuberosum* genome to identify all potato annexins, and analyzed potential involvement in drought responses using semi-quantitative RT-PCT. Then, we characterized photosynthetic performance in transgenic plants overexpressing ATANN1 during prolonged water deficit around the root zone. We also investigated possible annexin functions in modulating redox signaling, and assessed changes in drought stress responses. Our working hypothesis was that annexin modulated plant stress responses by increasing the cytosolic antioxidant buffering capacity in transgenic plants. Studies on Arabidopsis ecotypes indicate that *ATANN1* mRNA levels differ in ecotypes adapted to very different local climatic condition (TAIR and our non-published data). In potato tubers, STANN1 levels did not differ in proteomes from different genetic backgrounds [74]. Further experiments are necessary to elucidate if drought-tolerant potato landraces and cultivars could be generated by enhancing the level of annexin expression.

Materials and Methods

Generation of transgenic plants, transformation and growth conditions

S. tuberosum cultivar Sante (WT), medium-tolerant to drought, was used for transformation experiment (~~http://www.europotato.org~~http://www.europotato.org).The *STANN1* cDNA sequence ~~for *STANN1*~~ without the stop codon (957 bp; Acc. No. PGSC0003DMG400017714) was fused at the 3′ end to a 6×His-tag sequence and inserted into the XbaI restriction site of pROK2 [75~~54~~] between cauliflower mosaic virus 35S promoter and nopaline synthase (Nos) terminator sequences (Fig. S1A in Supporting Information file S1 Figures~~Figure S1A in File S1Figures~~). ~~The obtained clone~~This construct was used for *Agrobacterium tumefaciens*-mediated transformation of WT potato plants according to a previously published method [76~~55~~]. Regenerated transgenic plants were transplanted into separate glass tubes filled with 10 mL of Murashige & Skoog solid medium supplemented with 50 μg/~~-~~mL^{-1} kanamycin. The presence of the transgene cassette was verified with genomic PCR (data not shown). Expression of recombinant STANN1_6×His protein was confirmed by purification from leaves of WT and F1 transgenic plants (lines S-2, S-3, S-7, S-83, S91, S-97, and S-123) by Ni-NTA chromatography and detection with anti-HisTag primary antibody (Sigma-Aldrich). Recombinant ATANN1_6×His protein produced by bacterial overexpression was used as a positive control (WT protein extract) (Fig. S1B in Supporting Information file S1Figures~~Figure S1B in File S1Figures~~).

Potato WT plants (*S. tuberosum* cv Sante) or ~~WT-derived~~ transgenic lines in the “Sante” background (S-2 and S-7) were used for further experiments. Plants were cultivated in a growth chamber (or an air-conditioned greenhouse when indicated) under standard

conditions (21±2°C; 16 h/8 h day/night; light intensity 110 to 130 PPFD (photosynthetic photon flux densities); 60–80% relative humidity.

Water stress

S. tuberosum plantlets sprouted from tubers were grown in plastic pots filled with 1 kg of sterilized soil (mixture of peat and sand, pH 5.5; prepared by the Plant Breeding and Acclimatization Institute) for ~~≃~~160–170 days. The field capacity (FC) was determined gravimetrically (g of water per g of soil). Pots were weighed every 2–3 days and the volume of water necessary to maintain the indicated FC was calculated individually for each plant. For well-watered control plants, FC was maintained at 65% (−0.8 MPa) for the whole experiment. Experimental drought was imposed after 8–10 weeks of growth (tuber initiation) (Fig. S2A in Supporting Information file S1Figures~~Figure S2A in File S1Figures~~). Irrigation was decreased over 10 days to gradually reduce the FC to ~25% FC (−2.0 MPa) and was then maintained at this level until the end of the water deficit period. Irrigation was subsequently resumed with full soil saturation (rewatering). To estimate the impact of drought on potato productivity, plants were cultivated for an additional 11–12 weeks after rewatering (FC 65%) until physiological maturity. An exemplary schedule of FC changes is shown in~~-~~ Fig. S2B (Supporting Information file S1Figures)~~Figure S2B in File S1Figures~~. Samples were collected at the beginning of the water deficit period (D0), and (depending on experiment) at different days of drought, i.e. 3rd (D3), 4th (D4), 6 th (D6), 10 th (D10), and 14 th (D14), and at the first (RW1) and third (RW3) days after rewatering.

Identification of potato annexins

Annexins were identified in silico by searching for the endonexin domain (PFAM definition, PF00191, 66 aa) ~~with~~in six translation frames of the heterozygous ~~potato genome (heterozygous~~ diploid potato breeding line, *S. tuberosum* L. group Tuberosum RH89-039-16 genome~~)~~ using the HMMSearch program from the HMMER3 package. According to PFAM, >93% of proteins from this family contained at least three consecutive repeats of the endonexin domain. By searching with a single repeat, the probability of missing a complete protein due to below-threshold partial hits or incorrectly defined intron-exon boundaries was minimized. Only hits with an E-value ≤0.001 were considered. To verify the presence and sequence of the predicted annexins in WT potato, genome primer sets were designed that corresponded to the 5′ (F) and 3′ (R) ends of the predicted open reading frames ~~ORFs~~ (ORFs , Table S1 in Supporting Information file S2Tables~~Table S1 in File S1Figures~~). Expression of putative annexin genes was verified using RT-PCR. Briefly, total RNA was isolated from WT leaves and reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania) with poly(T)$_{12-18}$ primer. Annexins were amplified from cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). PCR products were cloned with pJET Cloning Kit (Thermo Scientific) and their compliance with the predicted sequences was verified.

Semi-quantitative expression of annexins and stress-regulated genes

Gene expression was profiled over 14 days of drought in WT potatoes grown as described above. Samples were taken from the first fully-developed composite leaf at the top of the plant. For each time point, single leaf discs from four independent plants were collected, flash-frozen in liquid nitrogen, and ~~kept~~ stored at −80°C until use. Total RNA was isolated

with Trizol (Invitrogen, Scottland). Reverse transcription was performed as described above. Taq DNA Polymerase (Thermo Scientific) was used to amplify specific sequences from cDNA. Genes for semi-quantitative analysis were selected from PGSC_DM_v3.4_pep_fasta, which contains a database of potato virtual translation products predicted according to similarity to annotated Arabidopsis genes. Specific primer sets for expression analysis were designed using PrimerSelect, Laser Gene10.0 DNASTAR (USA) (Table S2 in Supporting Information file S2Tables ~~Table S2 in File S2Tables~~). The obtained sq-RT-PCR products were subjected~~separated~~ by agarose gel electrophoresis, stained with ethidium bromide, and quantitated by densitometry using MultiGaugeV3.0 (Fuji) software. Expression was normalized ~~against~~ with respect to the expression of potato elongation factor 1 alpha mRNA (EF1a; PGSC0003DMT400050664;[77~~56~~]). Each single experiment included four biological replicates, ~~that~~ which were quantitated in three technical replicates. Experiments were repeated three times for each primer set and template.

Relative water content

Relative water content (RWC) was determined ~~according to~~as described previously [~~5~~778] with the ~~exception that~~ slight modification. F~~f~~or full saturation ~~(~~[equivalent to turgor weight~~,~~ (TW)] leaves were incubated in distilled water for 4 hours instead of overnight. Experiments were performed three times on at least five biological replicates for each genotype.

Extraction and determination of plant hormones

Leaf samples of ~0.5 g (without the main vein) from 8–10-week plants subjected to drought (as described above) were collected, immediately frozen in liquid nitrogen, and kept at −80°C until use. Samples were taken from the first fully-developed composite leaf at the top of the plant at the indicated time points. Sampling was performed four hours after ~~switching on the light~~the start of daily illumination. Three independent biological replicates were examined. Purification and analysis were performed ~~according to~~ as described previously [~~58, 59~~79, 80]. Briefly, leaf samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v) and the following labelled internal standards (10 pmol per sample) were added: $^{2}H_{6}$-ABA, $^{2}H_{5}$-*trans*Z, $^{2}H_{5}$-*trans*ZR, $^{2}H_{5}$-*trans*Z7G, $^{2}H_{5}$-*trans*Z9G, $^{2}H_{5}$-*trans*ZOG, $^{2}H_{5}$-*trans*ZROG, $^{2}H_{5}$-*trans*ZRMP, $^{2}H_{3}$-DHZ, $^{2}H_{3}$-DHZR, $^{2}H_{3}$-DHZ9G, $^{2}H_{6}$-iP, $^{2}H_{6}$-iPR, $^{2}H_{6}$-iP7G, $^{2}H_{6}$-iP9G, and $^{2}H_{6}$-iPRMP (Olchemim, Czech Republic). Extracts were purified using a SPE-C18 column (SepPak-C18, Waters), and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction (containing ABA) was eluted with methanol and the second fraction (containing CK metabolites) was eluted with 0.35 M NH_4OH in 70% methanol. Both fractions were separated by HPLC (Ultimate 3000, Dionex) and the hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and net photosynthesis were analyzed with a Portable Handheld Photosynthesis System CID 340 device (CID Bio-Science, Camas, WA, USA~~CID Inc., USA~~) according to the manufacturer's instructions. The maximum quantum efficiency of photosynthesis (Fv/Fm) and the effective quantum yield of PSII [~~(~~Y(II)]~~)~~ were determined with CID 340 (CID Inc., USA) with a CI-510CF Chl

fluorescence module and a CI-310LA light attachment (CID Bio-Science) providing actinic light. Measurements were performed 5 h after turning on the light, if not indicated otherwise, on the upper ~~4–5~~five fully-expanded unwrinkled leaves. Five plants were analyzed per time point. For maximal fluorescence (Fm) determination, plants were dark-adapted for 30 minutes (so all PSII reaction centers were closed) and then stimulated with saturating pulses of light (0.8 seconds~~.,~~, 3,000 PPFD). The minimal fluorescence (Fo) with all PSII reaction centers opened was measured with modulated light of 0.25 PPFD. Fv was calculated from the equation Fv= Fm-Fo. Y(II) was calculated using the equation Y(II) = (Fms-Fs)/Fms. ~~For determination of Fms (~~The maximal fluorescence under light (Fms)~~, plants were~~ was determined by allowing plants to adapt~~ed~~ to light for 20 minutes and measuring the steady-state of chlorophyll (Chl) fluorescence ~~was measured~~ (Fs). Next, a saturating pulse (0.8 seconds, 3,000 PPFD) was applied and Fms was determined.

Gross NPQ was estimated with a Dual Pulse Amplitude Modulation device, PAM-100 (Walz, Germany). For a single time point, six composite leaves from three to five ~~3–5~~ control plants were analyzed. NPQ was calculated as (Fm − Fms)/Fms, where Fm represents the fluorescence of a dark-adapted sample and Fms represents a fluorescence of the illuminated sample. Plants were dark-adapted ~~(~~for ~20 minutes~~)~~ and kinetics were measured after repeated light pulses of 94 PPFD for 300 seconds. Leaves were subsequently relaxed in darkness for 240 seconds and fluorescence ~~was documented throughout~~while continuously measuring and recording fluorescence.

~~Extraction of~~ N~~n~~on-polar lipids extraction and carotenoids/chlorophyll determination

Plant material was collected from 8–10-week-old plants exposed to drought. Samples were collected 4 hours after switching on the light at D0, D6, D14, and RW3. One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded composite leaves, and total six discs harvested from two plants were combined as a single sample. Non-polar lipids were extracted at 4°C and subjected to analysis using the ACQUITY UPLC system (Waters, USA). Shredded pPlant material after shredding in cooled mortar was transferred to a 15 mL Pyrex tube. After the addition of 3 mL acetone-methanol (8:2 v/v), the sample was perfused with argon and mixed vigorously by vortexing for 2 min. For the first second and third extractions, hexane (9 mL) was added and the sample was again perfused with argon before capping and shaking in a reciprocating shaker (PROMAX 2020, Heidolph, Germany) for 30 min in the dark. After shaking, the sample was incubated without agitation for 5 min to allow phase separation. The upper hexane phase was collected by aspiration and transferred to an a 100 mL Erlenmeyer flask, perfused with argon, capped and stored in the dark in 4°C. In the second extraction stage, 2 mL of propanol was used in addition to hexane, and perfusion, shaking and phase collection were repeated as before. After removal of hexane, the polar phase was centrifuged for 15 min at 4500 rpm. The supernatant was combined with the two hexane phases, perfused with argon, and filtered through a Milipore syringe filter unit Milipore millexMillex-CV13 Filter Unit (0.22 µm). The joined combined hexan phases were then transferred to room temperature, evaporated to dryness under argon, and dissolved in 1 mL methanol-propanol-hexane 6:1:3 (v/v/v). Dissolved samples were transferred to 2 mL glass vials, perfused with argon, capped, and stored at −80°C.

Non-polar lipids were analyzed by injecting 5 µL of sample extract Samples (5 µL) were injected onto an ACQUITY UPLC HSS T3 1.0×150 mm 1.8 µm column

and eluted with a gradient of solvents: A, [water and methanol (1:9, v/v)]; and solvent B, [methanol:isopropanol:hexane (2:1:1, v/v/v)], with a total of (210 minutes to transition from solvent A to B from A to B). Separation was monitored in the 300–750 nm range with a photodiode array detector. A single chromatogram at 436 nm was extracted, exported in ASCII format, and used for peak area integration analysis with GRAMS/AI software (Thermo Electro Corp, Finland.).

Chl*a* and Chl*b* contents were estimated by recording the absorbance of the aforementioned extract at 663, 652, and 645 nm (Cary 50 Bio UV/VIS spectrophotometer, Varian, Australia) according toas described previously [6081].

Measurement of ROS levels and quantification of lipid peroxidation during high light stress

One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded leaves of potato plants, and for a total of six discs three discs were harvested from each of two plants were combined as a single sampleand a total of six discs were combined as a single sample. Immediately after harvesting, samples were vacuum-infiltrated with methyl viologen (MeV) at the indicated concentrations and then incubated in the dark for 1 hour under normal irradiance (150 PPFD). Images were obtained after 30 hours of incubation.

A similar procedure was used for ROS quantification, with the exception that a single MeV concentration (50 μM) was used and samples were exposed to high irradiance (850 PPFD). Samples were collected at the indicated time points. Superoxide anion ($O_2^{\bullet-}$) content was determined using a colorimetric nitro blue tertrazolium (NBT) assay as described previously [6182]. Hydrogen peroxide

(H_2O_2), was detected with diaminobenzidine tetrahydrochloride (DAB) and quantified by counting pixels ~~counts~~ on scanned images using ImageJ software [~~62~~83]. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA) ~~according to~~as described previously [~~63~~84].

Transient expression of STANN1_GFP in *Nicotiana benthamiana*

The *STANN1* sequence (without the stop codon) was introduced between NcoI and BcuI restriction sites at the 5′-end of the monomeric GFP (mGFP) coding sequence in pCAMBIA1302. Intact *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 transformed with empty pCAMBIA1302 expressing mGFP or pCAMBIA1302 expressing STANN1_mGFP as described previously [~~64~~85]. After 3 days, 1 cm diameter leaf discs were excised and incubated with 50 μM MeV for 1 hour in darkness, and then incubated for 4 hours in high light (850 PPFD). Fluorescence was immediately observed using a Nikon Eclipse TE2000-E inverted C1 confocal laser scanning microscope equipped with a 40× Plan Fluore oil immersion objective ~~lens~~ (numerical aperture, 1.30). mGFP and chloroplast autofluorescence were excited with a solid-state Coherent Sapphire 488 nm laser and detected using 515/30 band pass and 610 long pass emission filters, respectively. All samples were analyzed in triplicate. Three independent experiments were performed.

Statistical analyses

Data were analyzed using two-way ANOVA with Duncan's Multiple Range Test (DMRT) (for yield) and MANOVA regression models (for other experiments).

Multiple comparisons between means were performed with a HSD Tukey test with a confidence limit of 95%.

Results

Identification of potato annexin genes

Genome-wide examination of the potato sequence database for annexins revealed the presence of 11 DNA segments encoding putative proteins displaying substantial similarity to ~~already known~~ previously characterized plant annexins. Two of these sequences were classified as pseudogenes due to several defects and a lack of continuity in any of the six ~~open reading frames and several genetic disablements~~ORFs. The remaining nine genes were located on chromosomes I, IV, V, and X and each encoded 5–6 exons (Fig.~~ure~~ 1A). The positions and phases of introns in the putative potato annexin genes were consistent with those reported for rice annexins [~~65~~86] (Fig.~~ure~~ 1B). The putative annexin sequences in the *S. tuberosum* genome were verified using genomic PCR (Fig.~~ure~~ 1C), and the lengths of the amplified genomic products were as expected (Table S1 in Supporting Information file S2Tables~~Table S1 in File S2Tables~~). The degree of nucleotide sequence identity between the putative potato annexins was 41–92%.~~The degree of nucleotide sequence identity between the putative potato annexins was in the range of 41–92%.~~ Sequences identified by bioinformatics approaches were confirmed experimentally. Reverse transcription polymerase chain reaction indicated that all nine genes were expressed in different potato organs (data not shown).

~~A m~~Multiple alignment of the putative potato annexin amino acid sequences with Arabidopsis annexins revealed that all ~~except of~~ but one of the potato annexins had Arabidopsis homologs (data not shown). The newly-identified potato genes were ~~therefore~~ named accordingly as *STANN1*, *STANN2*, *STANN3.1*, *STANN3.2*, *STANN3.3*, *STANN4*, *STANN5*, *STANN8*, and *STANN9* (data not shown). The potato

annexins ~~constituted~~ formed a functionally diverse protein family ~~of proteins~~ that was differentially expressed in different plant organs (data not shown). The most striking genomic feature of the potato annexin family was triplication of the annexin 3 gene on chromosome 1 (Fig~~ure~~ 1D). In addition, in an arrangement resembling that in Arabidopsis, potato *STANN3.1* and *STANN4* were adjacently localized ~~adjacent to one another~~ and ~~were~~ divergently transcribed ~~divergently~~, possibly from a shared promoter. The Inparanoid database groups all annexins in the same in-paralog cluster; however, we suspect that the ~~three variants of~~ annexin 3 variants (*STANN3.1*, *STANN3.2*, and *STANN3.3*) are within-species out-paralogs. Two duplications (ancestral gene → *STANN3.1* and the ancestor of *STANN3.2*; then the ancestor of *STANN3.2* → *STANN3.2* and *STANN3.3*) ~~seem~~ appear to have occurred prior to ~~speciation of~~ potato and tomato speciation, as *S. lycopersicum* contains ~~three~~ two orthologs of the ~~respective~~ annexin~~s~~ STANN4 and STANN3.1~~s~~. In turn, *STANN4* and *STANN3.1*–*3.3* are out-paralogs, as *STANN4* is moderately related to all *STANN3* variants but shares high sequence similarity with other annexins from *S. lycopersicum* or Arabidopsis. Multiplication of DNA segments within this region of chromosome 1 during *Solanaceae* evolution apparently took place independently at least twice. In tomato chromosome 1, the entire dyad of *SLANN3*/S*LANN4* was duplicated [~~66~~87] and gave rise to a tetrad located within a short segment of DNA (21,145 bp) that was not interspersed with other genes. ~~Collectively, t~~This region of chromosome 1 represents a “hot-spot” in the *Solanaceae* family where duplications of a single gene or gene cluster occurred.

Characteristic of potato annexin proteins

Newly identified potato annexins had similar predicted molecular masses of 34–37 kDa and diverse isoelectric points (5.21–9.02). The overall tertiary structures ~~(~~, which were defined by four endonexin domains containing calcium binding sites~~,)~~ were well preserved (Fig. S3 in Supporting Information file S1Figures, Table S3 in Supporting Information file S2Tables~~Figure S3 in File S1Figures, Table S3 in File S2Tables~~). However, the primary amino acid sequences ~~were quite divergent~~diverged significantly, with the lowest amino acid identity of 20.9% between STANN4 and STANN5 ~~(20.9%)~~. ~~Conversely, g~~Groups with higher similarities were identified ~~(~~, such as STANN3.1, STANN3.2, and STANN3.3~~)~~ ~~were also apparent~~. Annexins 3.2 and 3.3 were the most closely related~~, and had~~ with amino acid identities of 90.5% and 70.1% with STANN3.1, respectively. STANN3.2 and 3.3 differed in length (302 and 317 aa, respectively) due to lack of the 14-3-3 like domain on the C-terminus of STANN3.2. Similarly, the N-terminal end of STANN3.2 and STANN3.3, but not STANN3.1, contained a putative myristoylation motif (MG). To date, a myristoylation-mediating membrane localization has been confirmed only for mammalian AnxA13b. With respect to plant annexins, a myristoylation motif was found in poplar annexin EEE95606.1, but the functionality of this motif was not experimentally verified. In summary, despite extensive similarities, there were substantial differences between members of the STANN3 sub~~-~~family. This suggested that this ~~the~~ subfamily might be unique to *Solanaceae*, and that ~~evolution towards~~ distinct cellular functions evolved for each of the annexins ~~has occurred~~.

The potato annexins contained canonical type II calcium binding sites G-X-GTD-{30-40}D/E solely in the first and occasionally fourth endonexin domains (Fig. S3 in Supporting Information file S1Figures~~Figure S3 in File S1Figures~~). STANN4 and

STANN8 appear to have lost calcium responsiveness as a result of substantial mutations (substitutions and insertions) in these regions. The calcium binding site in the fourth endonexin repeat was probably the only one preserved in STANN5.~~In STANN5, the site in the fourth repeat was probably the only one to be preserved.~~ Tryptophan residues within the first endonexin repeat (G-W-GT) were conserved in potato annexins 1, 2, 8, and 9, but were replaced with phenylalanine (STANN5) or lysine (STANN3.1-3.3 and STANN4) in other annexins (Fig. S3 in Supporting Information file S1Figures~~Figure S3 in File S1Figures~~). This phenylalanine modification is not predicted to interfere with calcium binding ~~as~~ because phenylalanine and tryptophan residues are both hydrophobic and possess aromatic rings. By contrast, the lysine modification may impede membrane translocation of annexin ~~since~~ because introducing a positive charge into the calcium coordination site has the potential to disrupt calcium binding. Other amino acids or motifs important for the plant annexin tertiary structure ~~of plant annexins~~ were preserved in the potato proteins, such as histidine 40 (except in STANN3.2 and STANN4), cysteine 111 (except in STANN4), and cysteine 239 (except in STANN1).

Potato annexin gene ~~E~~expression ~~of potato annexins~~ during drought

To generate drought-tolerant potato, the genes whose products confer drought tolerance have to be identified. Potato annexins are a multigene family; therefore, we characterized the expression of all annexins during drought. Only five ~~of the~~ annexin genes (*STANN1*, *STANN4*, *STANN5*, *STANN9*, and *STANN2*) were expressed in the leaves of well-watered control WT plants (Fig~~ure~~ 2). At the onset of drought (D0),

~~the~~ *STANN1* mRNA ~~remained~~ was the most abundant~~ly expressed annexin~~ transcript (relative to the *EF1a* mRNA). Over time, the level of *STANN1* mRNA increased whereas *STANN4*, *STANN5*, *STANN9*, and *STANN2* mRNA levels remained unchanged. The ~~resulting~~ difference in the accumulation of *STANN1* mRNA from D0 to D14 was statistically significant (Fig~~ure~~ 2). Concurrently, ~~the expression of~~ additional annexins were expressed that were not detected ~~in~~ under control conditions ~~was induced~~. The levels of *STANN3.1* and *STANN3.2* mRNA (relative to *EF1a*) increased on D6 and remained elevated until the end of the drought period. The level of *STANN8* mRNA increased continuously during the whole period of water deficit (Fig~~ure~~ 2). However, these induced annexins were expressed at levels at least ten-fold lower than that of annexin 1. This strongly suggested that STANN1 was the key annexin involved in the plant cell response to drought.

Tolerance to soil water deficit

Drought is one of the most devastating environmental stresses in modern agriculture; it reduces global crop yields in developed and developing countries [88]. Continued efforts are required to obtain new crop varieties that assure food security. Annexins were shown to be a promising target in model plants; thus we wanted to verify if they can be used to improve stress tolerance in crop plants. To investigate the effect of STANN1 on drought tolerance in potato transgenic plants overexpressing STANN1 were generated by introducing the STANN1coding sequence under control of the 35S promoter. Transgenic plants ~~looked normal~~displayed normal morphology without ~~and did not display~~ any discernible abnormalities and/or growth aberrations under well-watered conditions ~~(both~~ in growth chamber and in the greenhouse~~)~~. Leaf turgidity was similar between transgenic and WT plants, which indicated that the leaf

water status of WT and transgenic lines was comparable (Fig. 3A, upper panel; Fig. S4A in Supporting Information file S1Figures~~Figure 3A, upper panel; Figure S4A in File S1Figures~~). During soil water deficit, ~~the overexpression of~~ STANN1 overexpression conferred ~~resulted in~~ sustained turgor maintenance, whereas~~. In WT plants,~~ leaf wilting was clearly visible on D8 in WT plants (Fig.~~ure~~ 3A, middle panel). ~~By D9,~~In WT the effect of drought ~~effect~~ was more apparent by D9, and leaves began to shrivel, roll, and curl up. Younger leaves near ~~to~~ the top the of the plant were ~~the~~ most severely affected (Fig.~~ure~~ 3A, lower panel). Leaves of the transgenic lines S-2 and S-7 ~~preserved stiffness~~maintained turgor and did not show signs of dehydration. Rewatering ~~resulted in restoration of~~restored leaf turgor and normal growth resumed within 1 day for transgenic plants and 3 days for WT (Fig.~~ure~~ 3B). After 2 weeks of drought, S-2 and S-7 leaves were less damaged then those of WT. Experiments were repeated four times in succeeding years, under greenhouse and growth chamber conditions, and in all cases similar results were obtained (Fig. S4 in Supporting Information file S1Figures~~Figure S4 in File S1Figures~~). The exact number of irreversibly damaged leaves varied between experiments depending on the drought severity (intensity and length). Damage was consistently significantly lower in transgenic lines than in WT. For example, survival rates after a 3-week drought were 12% and 82% for WT and S-7, respectively.

The ability to preserve turgor in leaves is closely related to drought tolerance. ~~Therefore, t~~To further characterize ~~the~~ drought responses ~~of~~in transgenic plants overexpressing STANN1, RWC changes ~~to RWC~~ under water deficit were analyzed. ~~Under control conditions,~~ RWC was comparable in WT and transgenic plants. Drought ~~resulted in the continuous decline of~~reduced RWC ~~both~~ in WT and ~~in~~ transgenic plants. However, differences between lines became apparent with

increasing drought severity and this became statistically significant at D12 ~~after drought onset~~ (Fig.~~ure~~ 4A). ~~Both in WT and in transgenic plants rewatering~~ Rewatering after 2 weeks of drought treatment ~~resulted in restoration of~~ restored control RWC values in WT and transgenic plants. The effect of drought on stomatal conductance (a measure of water and carbon dioxide vapor through the leaf stomata) was apparent by the third day after reducing watering, but the difference between WT and transgenic lines was statistically insignificant (Fig. 4B). Conductance remained low during the whole period of water deficit and only partially returned to control levels on the third day after the resumption of watering.

The real goal for any genetic engineering efforts in crop plants is to improve crop yield. We examined the productivity of transgenic plants under control conditions and under drought. ~~Overexpression of~~ STANN1 overexpression ~~also~~ improved plant yield both in terms of the total tuber mass (Fig. S5A in Supporting Information file S1Figures~~Figure S5A in File S1Figures~~) and consistency of the tuber size (Fig. S5B–C in Supporting Information file S1Figures~~Figure S5B–C in File S1Figures~~). The net productivity of well-irrigated WT and transgenic S-7 and S-2 lines was almost identical but tuber quality (size and uniformity) was enhanced in the transgenic lines. A 14-day drought decreased the tuber yield of the WT plants by half, whereas yield loss for S-2 and S-7 lines under comparable conditions was statistically less significant. ~~In addition, the t~~Tuber quality in the transgenic lines was less impaired after drought compared with~~to~~ WT. On the basis of these results, we concluded that increasing STANN1 levels is a promising strategy to improve drought tolerance in potato.

Plant photosynthetic activity during drought

We showed that elevation of ATANN1 levels enhanced drought tolerance in potato, but the mechanism of this process was unknown. There is some indication that this could be due to annexin-mediated modulation of redox homeostasis. During drought, ROS accumulation in chloroplasts leads to oxidative damage of photosystems [89, 90]. PSII catalyzes water oxidation and provides electrons for all further photosynthetic reactions; thus, its efficiency is crucial for the entire pathway. The PSII complex is a highly vulnerable structure that is constantly photodamaged; to maintain activity, constant repair and reassembly of reaction centers with newly synthesized proteins is necessary [91]. PSII activity is involved in mediating plant adaptation to environmental conditions. Drought impairs photosynthetic capacity and reduces leaf netto carbon uptake due to increased photorespiration activity (another sink for the absorbed energy) [92]. To directly estimate the effect of STANN1 on drought-induced PSII damage, the photosynthetic performance of PSII in transgenic plants overexpressing STANN1 was characterized under drought conditions. Several physiological parameters related to plant ~~-~~vigor~~-related physiological parameters~~ were analyzed to assess the effect of STANN1 overexpression. These included ~~stomatal conductance (a measure of water and carbon dioxide vapor through the leaf stomata) (Figure 4B)~~, net photosynthesis (Pnet, associated with plant vitality and biomass production) (Fig.~~ure~~ 5A), maximum efficiency of PSII in the dark-adapted state (a measure of the organization and vitality of PSII) (Fig.~~ure~~ 5B), and effective quantum yield of PSII in illuminated samples (Fig.~~ure~~ 5C). Under control conditions, STANN1 overexpression did not influence any of these parameters. By contrast, ~~almost~~ essentially all ~~the~~ photosynthetic functions were disturbed during drought, and changes in the two overexpression lines were consistent.

~~The effect of drought on gas exchange was apparent by the third day after watering reduction, but the difference between WT and transgenic lines was statistically insignificant (Figure 4B). Conductance remained low during the whole period of water deficit and returned to control levels only partially, on the third day after resumption of watering.~~

Wild-type Pnet ~~(Figure 5A) in WT~~ declined ~~virtually~~ to zero by D3 (Fig. 5A). ~~Pnet s~~Subsequently, ~~(D6, D10)~~ it dropped to negative values by D3 and D10.~~, which suggested that alternative electron consumption processes were activated.~~ In the two transgenic plant lines, Pnet remained positive until D10. After rewatering, Pnet increased in all three lines (Fig.~~ure~~ 5A).

Under control conditions, Fv/Fm values (Fig.~~ure~~ 5B) were similar in the all three plant lines (~0.79), and~~. Under normal conditions, Fv/Fm in~~was in the same range as in most investigated plant species ~~is ~0.79–0.84 conditions;. this value declines with plant stress. Likewise, d~~Drought negatively affected Fv/Fm in our experiments; ~~while~~ this was ~~noticeable~~ observed by D3 in WT, but become apparent in transgenic plants ~~the effect only became app~~arent on D6. In all three lines, Fv/Fm recovered to baseline within 3 days of rewatering. Measurements were performed on upper non-wrinkled leaves, indicating that apical shoot meristems were not irreversibly damaged by dehydration. In WT and transgenic plants Y(II) (Fig.~~ure~~ 5C) ~~in both WT and transgenic plants~~ declined steadily from the onset of drought, but the reduction appeared ~~only~~ on D6 in transgenic plants, and the effect was significantly reduced compared to WT. ~~Moreover,~~ Y(II) fully recovered in S-2 and S-7~~; but~~however, even on the third day after soil resaturation, ~~remained reduced in WT~~the physiological efficiency of PSII was not restored in WT. This ~~showed~~ suggested that ~~impairment of PSII in transgenic plants was fully reversible~~photorespiration was activated later in

STANN1 overexpressing plants then in WT. Thus, PETC was protected for a longer time against irreversible damage and diminished photorespiration-induced H_2O_2 accumulation in cytosol. These results show that PSII impairment in transgenic plants was fully reversible

Photosynthetic pigments content in transgenic plants

Drought activates premature senescence in plants [93] and stimulates catabolism of photosynthetic pigments [94], particularly chlorophyll (Chl) and carotenoids (Car). We determined the photosynthetic pigment contents under drought conditions to better understand the effect of STANN1 on photosynthetic machinery.~~Photosynthetic pigment contents were determined to better understand the effect of STANN1 on the cellular photosynthetic machinery.~~

Chla and Chlb accumulation

In WT and transgenic lines under well-watered conditions, ~~both~~ the total Chl content (11.2±0.01 and 10.6±2.29 mg mL^{-1}, respectively) and the ratio of Chl*a* to Chl*b* were similar. ~~Accumulation~~ In WT accumulation of Chl*a* (Fig.~~ure~~ 6A) and Chl*b* (Fig.~~ure~~ 6B) did not change during drought i~~n WT. A~~, and after rewatering, the level of Chl*a* increased to 180% of the control value at D0. During water deficit in S-7 line, the Chl*a* level ~~of Chl*a*~~ was stable; however, ~~accumulation of~~ Chl*b* levels increased and reached 168% at D14 compared to D0. ~~As a c~~Consequently~~ce~~, the Chl*a*/*b* ratio rose to 2.0. After rewatering, ~~the level of~~ Chl*a* levels doubled and Chl*b* levels remained stable.

~~XCar~~ Xanthophyll accumulation

The xanthophyll (XCar) cycle is essential for harmless dissipation of excess excitation energy in PSII as heat (NPQ). The relative XCar abundance in the total Car pool changes during the day depending on the incident light [95]. To exclude diurnal fluctuations, samples were collected at the same time (approximately 4 hours after the start of daily illumination). Under non-stress conditions, STANN1 overexpression did not significantly affect the total Car level, but the XCar content increased [zeaxanthin (Zea), 188%; violaxanthin (Viol), 144%] compared with that in WT plants (Fig. 6C–D). This result indicates that the xanthophyll cycle activity was higher in transgenic plants than in WT plants under the same light conditions. In WT plants, Zea content increased progressively during drought and reached a similar level to that in transgenic S-7 plants only after rewatering [0.35±0.01 pmol/g fresh weight (FW)]. In transgenic S-7 plants, the Zea level remained largely stable and fluctuated in the range of 0.31–0.34 pmol/g FW. Viol declined significantly during drought in both plant lines. The most significant reduction was observed during the first 6 days of drought, and was more pronounced in S-7 than in WT (57% and 10.5% reduction, respectively). At subsequent time points, the differences between lines disappeared and Viol remained at a stable level after rewatering (0.45±0.01 pmol/g FW in WT and 0.44±0.06 g^{-1} FW in S-7).

~~In non-stress conditions the elevated expression of STANN1 did not significantly change the total Car level; however, the XCar content was increased (Zea - 188%, Viol - 144%) in comparison to levels in WT plants (Figs. 6C–D). Since samples were collected at the same time, these differences were not related to the possible diurnal fluctuation induced by light. During drought in WT, Zea content increased progressively and reached a similar level as in S-7 plants only after rewatering (0.35±0.01 pmol g^{-1} FW). In S-7 plants, the Zea level remained largely stable and fluctuated in the range 0.31–0.34 g^{-1} FW. Viol declined significantly during the drought in both plant lines. The most significant reduction was observed during the first 6 days of drought and was more noticeable in S-7 than in WT (57% and 10.5%~~

~~reduction, respectively). At subsequent time points the difference between lines disappeared and Viol remained at a stable level after rewatering (0.45±0.01 g^{-1} FW in WT and 0.44±0.06 g^{-1} FW in S-7).~~

Nonphotochemical quenching activity

The observed differences in XCar accumulation prompted us to analyze gross NPQ performance in attached leaves of control, well-watered WT, and transgenic plants (Fig. 7). As expected, NPQ of S-7 and WT differed. Maximal NPQ occurred in S-7 plants after the start of daily illumination, and NPQ amplitude was ~25% higher in S-7 than in WT. The steady-state NPQ level was elevated and saturation was delayed in S-7 compared with those of WT. The *PSBS* (another key NPQ factor) mRNA level during drought was higher in S-7 than in WT plants (Fig. S6A in Supporting Information file S1Figures). These results indicate that the NPQ capacity in transgenic line S-7 was greater than that of WT, which likely conferred better protection of PSII against photooxidative damage. Electrons were redirected to H_2O_2 in transgenic plants and subsequent ROS accumulation was lower than in WT.

Annexin overexpression affects hormonal homeostasis in plants subjected to drought

The drought phenotype of transgenic potato plants overexpressing STANN1resembled that of plants overproducing CK. Compelling evidence indicates that the redox signaling network integrates with phytohormone-activated pathways [96]. ROS are positioned upstream and downstream of at least some hormone-signaling pathways [14].We therefore ~~assessed~~ stress-hormone levels [~~(ABA, CK, and SA)~~ (pro-senescing: ABA and salicylic acid (SA); anti-senescing: CK] in leaves

of WT and S-7 plants subjected to drought. UnderIn well-watered conditions, the level of biologically active ABA in transgenic plants was significantly lower than in WT (Fig.ure 87A). However, this difference was insignificant by D6 after the initiation of drought lost by D6 after initiation of drought. This suggested that biosynthesis of ABA in transgenic plants during the first week of drought was more intense active than that in WT. This, which is in accordanceconsistent with a more pronounced decrease inreduction of Viol (ABA precursor) levels in transgenic plants compared to WT (Fig.ure 6D). In During the second week of water deficit, only a slight increase in ABA level was observed and maximum levels on D14 were similar between in WT and S-7 (3.21±264.01 and 3.02±101.59 nmol g^{-1} FW, respectively) (Fig.ure 87A). As expected, ABA levels decreased declined to control values on resumption of watering.

Under control conditions, annexin overexpression had no significant effect on CK levels (Fig. 8B; Table S4 in Supporting Information file S1FiguresFigure 7B; Table S4 in File S1Figures). The contents of active and total CK were similar and amounted to 6.35 and 6.90 pmol g^{-1} FW, and 506.34 and 542.08 pmol g^{-1} FW, in WT and S-7 plants, respectively. Drought stress was associated with down-regulation of *trans*-zeatin (tZ), the most physiologically active CK involved in the stimulation of cell division. At theDuring early drought stages of drought progression (RWC ~85%, only minor difference from control conditions), the level of active CK in WT increased, especially compared to the less active isopentenyladenosine (iPR) levels. Active CK decreased declined under severe drought conditions, with the exception of *cis*-zeatin (cZ) and its riboside (cZR), both of which were CK species associated with stress responses. After rewatering, active CK content strongly increased, especially that of *trans*-zeatin (tZ),. Levels of whereas cZ and cZR levels substantially

~~decreased~~declined. High levels of active CK (including high levels of cZ) were maintained in S-7 even under severe drought conditions. These levels were substantially higher than in parental plants. After rewatering, active CK elevation was much more pronounced in S-7 than in WT. The level of storage compounds (CK O-glucosides) was generally low. By contrast, levels of deactivation products (CK N-glucosides) substantially increased during drought, probably as a result of the enhanced deactivation of CK (data not shown).

SA accumulation was reported in response to different abiotic stresses [97]. ~~Overexpression of~~ STANN1 overexpression had no effect on SA levels under well-watered conditions. SA accumulation in WT and S-7 did not change significantly under moderate drought (D6). During the second week of water limitation, the SA level increased in both lines, and ~~the level of~~SA accumulation in S-7 was ~~about~~ approximately twice that in WT (Fig.~~ure~~ 8~~7~~C). ~~SA accumulated further after rewatering in WT but declined in S-7, albeit to a higher level than at D0. These data indicated that ROS-modulating systems were activated more rapidly and to a higher extent in transgenic plants than in WT.~~ The SA level declined in S-7 during recovery, but remained slightly higher than that observed at D0. By contrast, SA continued to increase in WT and exceeded the level observed in S-7. These data indicate that ROS-modulating systems are activated more rapidly and to a higher extent in transgenic plants overexpressing STANN1 than in WT plants.

In summary, genetic modification did not influence ABA synthesis ~~(~~and~~,~~ ~~therefore,~~ ABA-dependent responses~~)~~. The ~~changes in~~elevation in CK metabolism ~~(significant elevation~~ upon rewatering~~)~~ ~~were in line~~ was consistent with phenotypic observations. SA levels in S-7 increased rapidly during drought and peaked by D14 but declined rapidly after rewatering~~but the elevation was transient~~. This suggested

that SA-mediated activation of antioxidant systems during drought was faster in STANN1 overexpressing plants. ~~transgenic than WT plants but that longer-lasting effects (like pcd) might be alleviated.~~In WT plants, delayed SA-mediated effects such as induction of PCD might be induced.

STANN1 mitigates drought-mediated oxidative stress in cytosol and chloroplasts

Although the experimental plants were grown under constant temperature conditions, heat stress response (HSR) was induced in WT and transgenic plants during drought. In WT plants, water deficit increased the accumulation of chloroplast-specific *HSP100* and cytosol-targeted *HSP40* mRNAs (compared to the EF1a normalization control), which peaked during the second week of drought. In transgenic plants, only *HSP100* expression was induced under water deficit (Fig. S6C–D). This result suggests that STANN1 overexpression mitigates cytosolic oxidative stress.

~~PSII antennae complexes functions in transgenic plants~~

~~Overexpression of annexin resulted in an increase of Zea content, which is essential for the harmless dissipation of excess excitation energy as heat (NPQ). In addition, expression of another key NPQ factor (*PSBS*) was reduced during drought (Figure S6A in File S1Figures). These data strongly suggested the XCar cycle might be more efficient in transgenic than in WT plants. Gross NPQ performance in transgenic plants was therefore analyzed directly in attached leaves of control, well-watered WT and transgenic plants (Figure 8). As expected, NPQ of S-7 and WT differed. Maximal NPQ occurred later after initiation of light stimulation in S-7 than~~

~~in WT plants, and NPQ amplitude in S-7 was ~25% higher than that of WT plants. Similarly, the steady-state level of NPQ was elevated and saturation was delayed in S-7 compared with WT. Overall, these results showed that protection of PSII against photooxidative damage was more effective in S-7 than in WT as a result of improved NPQ.~~

~~Experience of stress in transgenic plants~~

~~Activation of the heat stress response (HSR) is a consequence of drought stress in nature. However, experimental plants are usually cultivated in controlled constant temperatures. Despite this limitation, activation of HSR was observed in both WT and transgenic plants. Stomatal closing due to water deficiency in the root zone was sufficient to induce expression of heat shock proteins (HSP). Thus, to characterize the effect of overexpression of annexin on potato plants, the accumulation of chloroplast-specific (*HSP100*) and cytosol-targeted (*HSP40*) mRNAs were analyzed during drought. In WT, water deficit resulted in an increased accumulation of both HSP mRNAs (compared to the EF1a normalization control) that peaked during the second week of drought. In transgenic plants, *HSP100* expression was induced but *HSP40* expression was not stimulated (Figs. S6C–D).~~

~~Annexin-mediated protection of chloroplast~~

STANN1 mitigates photooxidative stress induced by MeV

Enhanced stress tolerance frequently reduces plant responsiveness to light [98]. To verify if annexin-mediated drought tolerance influenced light stress responses, we analyzed the effect of the photosensitizer MeV on transgenic plants overexpressing STANN1. MeV induces oxidative stress, which enables studies of oxidative tolerance and stress cross-tolerance in plants [99]. MeV induces an oxidative burst by accepting electrons from PSI and transferring them to molecular oxygen, which

results in massive H_2O_2 accumulation in light and generates oxidative stress in chloroplasts.

Leaf disc senescence assay

~~To assess whether STANN1 overexpression directly alleviated photooxidative stress, l~~Leaf discs from WT~~, and transgenic~~ S-2 and S-7 plants were exposed to normal light (150 PPFD) in the presence of ~~two concentrations (10 and 50 μM) of a photosensitizer,~~ 10 and 50 μM ~~methyl viologen (~~MeV~~)~~. ~~MeV induces oxidative burst by accepting electrons from PSI and transferring them to molecular oxygen, resulting in chloroplast damage.~~ The damage caused by MeV was visualized ~~by~~ as the degree ~~of bleaching~~ of leaf tissue~~s~~ bleaching. In the absence of MeV, exposure to light for up to 30 hours had ~~almost~~ no significant effect on leaf discs. By contrast, exposure to ~~both~~ light during~~and~~ MeV treatment induced leaf tissue bleaching, which ~~that~~ increased according to MeV concentration (Fig. S7 in Supporting Information file S1Figures~~Figure S7 in File S1Figures~~). Transgenic plants S-2 and S-7 had~~showed~~ higher tolerance to MeV, ~~as demonstrated by a reduction in~~and exhibited lower levels of leaf disc bleaching in light~~ed margins~~.

Quantification of ROS and lipid peroxidation

To further analyze STANN1-mediated protection against light stress, leaf discs from WT and S-7 plants were subjected to the ~~combined~~ combinationaction of relative excess ~~high~~ light (850 PPDF) and 50 μM MeV ~~(50 μM) as described above~~. The levels of superoxide anion, hydrogen peroxide, and malonyldialdehyde (MDA) were quantified at the indicated time points (Fig~~ure~~ 9).

Exposure of WT to excess light and high MeV concentration induced biphasic accumulation of superoxide anions, with an initial peak ~~seen~~ at 30 minutes after

induction and a second, more substantial and long-lasting, peak beginning at 9 hours after induction. In S-7, an initial increase in superoxide anion level was ~~noticeable~~ observed, which ~~but~~ was ~~not statistically~~ significantly lower than that in WT.~~different to WT~~. The maximum level of O_2^- was the same in WT and S-7, but the kinetics of the second peak differed (Fig.~~ure~~ 9A). In WT, the level of superoxide increased steadily from 6 to12 h after induction. ~~By contrast, accumulation occurred I~~In S-7 superoxide anion accumulation occurred ~~between~~ during 6~~ and~~ -9 hours after induction, reaching a similar maximal level as in WT at this time point, and the ~~level of~~ superoxide level then~~then~~ remained unchanged until 12 h after induction.

In WT, light-induced changes in H_2O_2 level ~~followed the~~were biphasic, ~~pattern of accumulation~~ with a second higher and sustained peak (Fig.~~ure~~ 9B). The first peak occurred ~~at~~ within 30 minutes and the second peak occurred ~~at~~ by 12 hours after induction. In S-7, the first peak ~~was observed and this~~ had a similar magnitude ~~as in~~to that in WT. After several hours, no further accumulation of H_2O_2 was observed in S-7, and overall levels were significantly lower than in WT.

Lipid peroxidation, measured as an MDA equivalent, was apparent in WT only after 30 minutes and 12 hours. No statistically significant changes in the lipid peroxidation state were observed under high light stress in S-7 (Fig.~~ure~~ 9C).

Annexin 1 attenuates cell death and protects chloroplast structure against oxidative stress~~when exposed to oxidative stress~~

In our experiments, the annexin STANN1 attenuated both phases of chloroplast-derived oxidative stress. In transgenic plants overexpressing STANN1, the expression of nucleus-encoded PSII proteins (Fig. S6A–B) and HSPs was modified correspondingly (Fig. S6C–D). A transient mGFP expression assay was performed to

confirm that ~~the aforementioned~~ tolerance to photooxidative stress was ~~indeed~~ due to elevated STANN1 levels. In this experiment, STANN1 was produced as an in-frame C-terminal fusion with mGFP. *N. benthamiana* leaf discs were transformed with STANN1_mGFP (experiment) or mGFP~~-alone~~ (control) constructs. ~~The l~~Leaf discs were then subjected to high light or to the ~~combined~~ combination~~action~~ of high light and MeV~~,~~ as described above. ~~For analysis, l~~Leaves with similar fluorescent protein expression levels were used for analysis. Exposure to high light alone had no effect on cell structure, regardless of the construct used (mGFP-alone, Fig.~~ure~~ 10A–D; STANN1_mGFP, Fig.~~ure~~ 10E–H). High light plus MeV induced cytosol condensation and chloroplast damage (as determined by a ~~decrease~~ decline in chloroplast autofluorescence) in mGFP-expressing cells (Fig.~~ure~~ 10I–L). ~~Overexpression of a~~Annexin 1 overexpression attenuated both of these effects, and the cell~~s~~ morphology resembled ~~those from~~that of control samples (Fig.~~ure~~ 10M–P). ~~The intensity of c~~Chloroplast fluorescence intensity was quantified and, ~~while~~ there was no significant difference in mGFP fluorescence between plants transiently expressing mGFP and STANN1_mGFP~~ and mGFP only,~~. ~~the~~ The difference in chloroplast ~~red~~ autofluorescence (red) between the mGFP~~ only~~ and STANN1_mGFP expressing leaves was statistically significant. This strongly ~~suggested~~ suggests that the chloroplast structure was maintained in the presence of STANN1 protein.

Discussion

~~The experiments presented here~~This study clearly demonstrate that ~~overexpression~~ elevation of endogenous ~~annexin~~ STANN1 expression can be successfully employed to improve ~~crop~~ potato tolerance to water deficit. Under optimal conditions, genetic modification had no negative effects on plant phenotype, growth, or productivity. Reduction of the photosynthetic rate in response to water deficit is usually attributed to ROS-induced damage of lipids, pigments, and proteins in the photosynthetic apparatus. Overexpressed STANN1 relieved the negative effects of drought stress, such as degradation of photosynthetic pigments, reduction of photosynthetic activity, and loss of productivity. In transgenic plants NPQ was induced more rapidly and had higher capacity in STANN1-overexpressing plants, which contributed to increased tolerance to photooxidative stress. Exposure to MeV reduced ROS accumulation and membrane lipid damage, so STANN1-overexpressing plants were not desensitized to light. Consequently, we assume that maintenance of photosynthesis during water deficit was due to protection against drought-induced oxidative stress and/or modification of redox/hormonal signaling in STANN1-overexpressing plants. ~~In potato, a reduction in the photosynthetic rate in response to water deficit is usually attributed to damage of the lipids, pigments, and proteins in the photosynthetic apparatus. Elevation of STANN1 produced an increased tolerance to the photooxidative damage that accompanied drought. When plants were grown under optimal conditions, genetic modification had no negative effects on plant phenotype, growth, or productivity.~~ We ~~therefore~~ propose that manipulation of annexin ~~over~~expression is a valuable new approach for crop improvement that focuses ~~not on intensification and/or more rapid induction of ABA-mediated stress responses, but rather~~ on delay and/or attenuation of leaf

senescence and maintenance of physiological processes when plants are exposed to challenging environmental conditions.

~~The selection of a~~Annexin selection for transgenic experiment

~~As has been observed in other plant species,~~ Potato annexins ~~in potato~~ form a multigene family that encodes proteins with similar ~~overall~~ tertiary structures but different primary amino acid similarities. Despite some extensive similarities, the individual annexins displayed unique expression patterns in the different plant organs (data not shown) and in response to drought. This suggests the specialization of individual family members towards unique roles in growth/development and adaptation to environmental conditions. Recently it was shown that~~For example, although no specialized function could be inferred from primary amino acid sequence,~~ functional knock-out of annexin 5 (At1g68090) in Arabidopsis was ~~lethal~~ male-sterile due to the abortion of pollen grains before mature pollen stage;~~in Arabidopsis~~ however, on the basis of primary amino acid sequence, no specialized functions could be predicted for annexin 5. ~~Recently~~Detailed investigations ~~[67, 68] showed~~ revealed that ATANN5 was ~~expression~~ expressed primarily ~~of this protein took place only~~ during microsporogenesis. ~~and that~~ The observed pollen lethality was due to impairment in pollen development [100, 101]. This suggests that the specialization of individual annexin family members for unique roles in growth/development may be reflected in specific expression patterns. This also may be observed for adaptation to environmental conditions. It will be necessary to test if ectopic expression of any other Arabidopsis annexin under the ATANN5 promoter restores pollen development.

This suggests that the specialization of individual annexin family members for unique roles in growth/development may be reflected in specific expression patterns. This also may be observed for adaptation to environmental conditions. It will be necessary to test if ectopic expression of any other Arabidopsis annexin under the ATANN5 promoter restores pollen development.

Expression of ~~several~~ four potato annexins was induced during drought. However, ~~only~~ STANN1 ~~was constitutively expressed and the level of its mRNA during water deficit~~mRNA level was several-fold higher than other annexins. This strongly suggested that STANN1 was ~~the primary annexin~~ involved primarily in ~~the~~ stress responses ~~to stress~~ rather than complementation within the annexin family, at least at the transcriptional level. *STANN1* is expressed in all plant organs (data not shown); therefore~~The constitutive overexpression of *STANN1* minimized~~ the risk of inducing developmental aberrations ~~resulting from~~due to ectopic *STANN1* expression ~~of protein~~ during development is minimal. ~~We therefore~~Hence, we considered ~~selected~~ STANN1 as a good candidate to improve drought tolerance ~~candidate~~ in potato and possibly other crops.

~~Hormonal stress response of transgenic plants~~

~~Our results showed that steady-state ABA levels were reduced in transgenic compared to WT. Despite this, the drought-induced accumulation of ABA had similar kinetics in transgenic and WT plants, and levels similar to those in WT were eventually achieved. This suggested that, despite some modifications, ABA synthesis was fully functional in transgenic plants. ABA is of key importance in the abiotic stress response and it was therefore of utmost importance that ABA content, and hence the ABA-dependent stress signaling pathways, were activated equally in WT and transgenic plants. Other signaling pathways were modified. Concurrent sustained CK biosynthesis and higher accumulation of SA might delay senescence and improve plant photosynthetic performance. Elevated CK content resulted in maintenance of substantial photosynthetic activity under drought in tobacco [69].~~

~~Exogenous salicylate (SA, aspirin, benzoic acid) affected gas exchange rates and processes related to the PETC due to enhancement of NPQ (Janda *et al*. 2014). SA is also able to exert a protective effect in plants against a wide range of different abiotic factors via its ability to modulate antioxidant systems and affect ASA-GSH homeostasis [70, 71]. Elevated SA in transgenic plants may therefore alleviate photo oxidative stress. In this study, we found that an elevated level of STANN1 protein alleviated oxidative stress and affected the biphasic response of H_2O_2, $O_2^{\bullet-}$, and lipid peroxidation induced by high light. Moreover, stress levels in transgenic plants was reduced. Accumulation of cytosolic *HSP40* mRNA during drought in the transgenic plants was substantially reduced, suggesting that the HSR developed more slowly and was less pronounced in transgenic lines than in WT. This is in accordance with data presented by [57] for *Solanum andigeanum*. In *S. andigeanum*, the degree of stress (estimated by the level of respective *HSP* mRNAs) experienced during drought by a less tolerant landrace was higher than for a more resistant race. One of the consequences of delayed stress detection could be more efficient protection of the chloroplast, resulting in sustained efficient functioning of photosynthetic apparatus.~~

~~Improved~~ STANN1 affects NPQ ~~in transgenic plants~~and mitigates chloroplast-induced oxidative stress in cytosol

Chloroplasts are one of the major stress-induced ROS sources in plant cells. Disruption of chloroplast redox poise permeates throughout the cell and activates secondary ROS sources in other compartments. In mesophyll cells of *Eupatorium adenophorum*, tenuazonic acid (TeA) inhibits electron flow along PSI and PSII and induces H_2O_2 accumulation in chloroplasts within 1 hour. By 4 hours, H_2O_2 spread to the cell walls facing intercellular spaces [102]. We provided evidence that photooxidative stress in potato leaves induced a biphasic oxidative burst, with the first transient peak after 1 hour and the second more significant peak occurring by 12 hours. In STANN1-overexpressing plants, both phases of ROS accumulation were reduced. Biphasic ROS accumulation with a similar kinetics was reported in

response to ozone and salt stress treatments [33-35]. In Arabidopsis and tobacco, the first transient ROS peak occurring after O_3 treatment originated in the chloroplast, whereas the second required NADPH oxidase activity and undisturbed functioning of PETC [34].

We assume that the first peak of ROS accumulation during photooxidative stress in potato is due primarily to chloroplast-generated ROS, whereas the second resulted from activation of secondary ROS sources. The question arises how proteins such as annexins that contain no specific signal sequences that target them to chloroplasts can modulate processes inside chloroplasts. Annexins are found occasionally in chloroplast proteomes of some plants (reviewed in [49]). For example, a mustard (*Sinapis alba* L.) annexin was identified as a component of a multisubunit chloroplast RNA polymerase A complex [103]; however, these results were not confirmed in a subsequent study [104]. Overall, these chloroplast localizations remain exceptions. We believe that annexin-mediated protection of chloroplasts and photosynthesis could be an indirect effect of increased redox homeostasis buffering in the cytosol. In Arabidopsis, the cross-talk between different ROS-scavenging systems in distinct cellular compartments was reported. During light stress, cytosolic antioxidant capacity had an essential role in protecting chloroplasts. Deletion of cytosolic ascorbate peroxidase APX1 induced degradation of thylakoid and stromal/mitochondrial APXs, a cytochrome b6f complex subunit protein, and the small subunit protein of Rubisco [43]. This result is in agreement with observations from other studies [26, 105], which reported that ROS accumulation rapidly reduced chloroplast antioxidant capacity due to APX inhibition.

Manipulation of chloroplast/cytosol antioxidant capacity was successfully used to modulate potato tolerance to adverse conditions. Transgenic potato lines engineered

to express cytosolic or chloroplast Cu/Zn-superoxide dismutase (SOD) from tomato displayed enhanced tolerance to MeV [106]. Overexpression of cytosolic Cu/Zn-SOD from *Potentilla atrosanguinea* improved drought stress tolerance and enhanced net photosynthetic rates [107]. Co-expression of Cu/Zn-SOD and APX in chloroplasts enhanced potato tolerance to multiple abiotic stresses, including chilling, high temperature, photooxidative stress, and drought [108]. Accordingly, the lack of chloroplast thioredoxin CDSP32 resulted in greater susceptibility of potato plants to oxidative stress [109]. Taken together, these data show that elevating cytosolic antioxidant capacity is a promising way to enhance stress tolerance in potato. STANN1 overexpression improved drought tolerance and mitigated photooxidative stress, similarly to that observed for plants with overexpression of ROS-scavenging enzymes. The accumulation of mRNAs coding for cytosolic HSP40 and chloroplast HSP100 was entirely or partially reduced during drought, suggesting that the HSR in transgenic lines developed more slowly and to a lesser extent. This is in agreement with a previous report [78] for *Solanum andigeanum*, in which the expression level of respective *HSP* mRNAs during drought was higher in less tolerant lines than in more resistant landraces.

In summary, chloroplasts are the site of cross-talk between basic metabolic pathways and stress responses, which places them in a key position with respect to coordination of defense responses [110]. This suggests that protection of chloroplasts from ROS-induced damage is of utmost importance.

Cross-talk between redox signaling and phytohormone-mediated pathways in transgenic plants overexpressing STANN1

Cross-talk between ABA, SA, CK, JA and other phytohormone pathways modulates plant development and stress adaptation. Our results showed that increased STANN1 expression modified drought-induced hormone accumulation. We assume that this is an indirect consequence of STANN1-mediated modulation of cellular redox homeostasis. Accumulating data indicate that multi-faceted and multi-level feedback interactions orchestrate hormone- and ROS-mediated signaling networks. Alterations in the cellular redox state were sufficient to modify hormone accumulation and downstream effects [96]. ROS signaling is positioned upstream and downstream of hormone-signaling pathways [13, 14]. Redox cues integrate with the action of different phytohormones such as ABA and SA in the coordination of plant growth and stress tolerance [111, 112].

Annexin elevation reduced the ABA steady-state level, but drought-induced ABA accumulation had similar kinetics in transgenic and WT plants, and levels similar to those in WT were eventually achieved in STANN1-overexpressing plants. Stress-dependent ABA accumulation typically results from elevated ABA synthesis [113]. ABA biosynthesis is induced by protonation of the thylakoid lumen and accumulation of oxidized ascorbic acid as a result of a reduction in PETC efficiency. Oxidized AA cannot function as a cofactor for violaxanthin de-epoxidase, which deactivates the XCar cycle in favor of ABA biosynthesis [114]. In leaves of ascorbate-deficient *vte1* Arabidopsis plants, the ABA level increased by 60% [115]. In transgenic potato plants overexpressing STANN1, the ABA biosynthetic pathway

was fully functional. ABA is a key factor in abiotic stress responses (induces stomatal closing and transcriptional reprograming); therefore, it is of utmost importance that ABA content and ABA-dependent stress signaling pathways are similarly activated in WT and transgenic plants. However, the reduced steady-state ABA level indicated that biosynthetic pathways in STANN1-overexpressing plants under control conditions were directed toward XCar activity at the expense of ABA synthesis.

The control SA levels in transgenic plants were not significantly different than those in WT plants, and were similar to previously reported values for potato cv Desiree [116]. *S. tuberosum* has higher basal SA levels than Arabidopsis, maize, tobacco, or tomato [117]. Increases in SA levels in potato are relatively moderate (e.g., two-fold) after infection with *Phytophthora infestans*, compared with a 20-fold increase in Arabidopsis [118]. There is a lack of data on SA accumulation in potato leaves during drought, although it has been shown that SA functions as a regulatory signal mediating drought stress responses in several plant species [119, 120]. In our experiments, SA increases during water deficit in both plant lines were similar (6-fold in WT and 5-fold in S-7 over basal level), which was in perfect agreement with observations in *Phillyrea angustifolia* [121]. However, the SA peak was observed in WT plants only after rewatering, whereas in transgenic potato it occurred earlier, even during drought.

Recently, it became clear that SA is an important regulator of photosynthesis. In Arabidopsis, SA influences plant photosynthetic performance, and properly balanced SA levels are necessary for acclimation to changing light [122, 123]. The SA-mediated signaling pathway in Arabidopsis is involved in optimal photosynthetic activity under stress conditions by modulating redox homeostasis [124]. SA enhances

the cell antioxidant capacity during drought, although the mechanism of this process is unclear. Endogenous SA deficiency in potato results in ineffective induction of stress defense system and enhances stress sensitivity [116, 125, 126]. During plant response to pathogen infection, SA inhibits the ROS-scavenging enzymes CAT and APX [127-129] and stabilizes H_2O_2 levels. SA and H_2O_2 function as a positive-feedback amplifying loop; if not properly balanced, this loop exerts detrimental effects for cell survival. In rice, reduced endogenous SA levels enhanced H_2O_2 accumulation and the appearance of spontaneous necrotic lesions during senescence and development of oxidative damage and in response to high light intensities [130]. SA accumulation induces different responses depending on the timing and accumulation level; it induces stress-responsive defense systems such as antioxidant enzymes, or induces PCD in response to long-term elevations of SA levels. In WT plants, slow and prolonged SA accumulation despite the resumption watering may ultimately lead to PCD. Rapid SA accumulation in transgenic plants appears to indicate more efficient mobilization of SA-induced stress responses, and accounts for improved photosynthetic performance.

Recent work shows that CK has an important role in plant adaptation to environmental stresses such as drought, cold, osmotic stress, and light stress [131-134]. In our experiments, CK species and their levels under control conditions were similar to those previously reported for potato cv Desiree [135]. STANN1 overexpression did not influence CK profiles or steady-state levels, but CK levels were maintained during water deficit and rapidly increased after rewatering.

CK antagonizes many ABA-induced physiological responses to drought such as stomatal closure or leaf senescence [136]. Maintaining CK biosynthesis during drought improves stress tolerance, confers protection against photooxidative stress,

and mitigates reductions in photosynthesis [38, 83, 137-141]. CK activity is anti-senescent and associated with maintenance of greater antioxidant activity. In creeping bentgrass, elevated CK levels due to senescence-driven expression of isoprenyl transferase (IPT), a key enzyme in CK biosynthesis pathways, conferred drought resistance, increased the levels and activity of scavenging enzymes such as APX and CAT1, and reduced MDA accumulation [142]. Similarly, elevated CK levels in tobacco plant leaves and chloroplasts conferred higher physiological parameters than those in controls [143], and increased APX and dehydroascorbate reductase (DHAR) activity, which prevented over-oxidation of the chloroplastic ascorbate pool. CK regulates stress responses on several levels, such as inducing stress-inducible gene expression [144-145], including peroxidases, GRX, and glutathione S-transferases. Plants with reduced CK levels had lower ROS-scavenging capacity, exhibited more severe photodamage after high-light treatment, and had reduced neoxanthin and Zea levels under control conditions, which declined further during photooxidative stress. Similar effects were observed in scavenging enzyme activities, and a strong reduction in APX and SOD activities were observed under control conditions and in response to light stress [146]. We assume that sustained biosynthesis of CK during drought in transgenic potato plants overexpressing STANN1 remediates oxidative stress and improves photosynthetic performance.

STANN1 affects redox homeostasis

Different hypotheses have been proposed to explain the molecular basis of annexin-mediated alleviation of oxidative stress, including innate peroxidase activity [54, 147, 148], calcium-induced stabilization of peroxidases activity [149], and modulation of calcium influx [62, 150]. Based on the results of our experiments, we

assume that annexin-mediated reduction in oxidative stress in transgenic potato overexpressing STANN1 results from the annexin effect on thiol-disulfide homeostasis.

Downstream transmission of several environmental cues for H_2O_2 accumulation is sensed and mediated by several ROS-neutralizing systems, which are low-molecular-weight antioxidant buffers such as ascorbate (ASC) and glutathione (GSH), and oxidoreductases such as GRX, TRX, and scavenging enzymes [39]. The most prominent ROS-scavenging and redox-signal perception system is GSH accumulation and GSH oxidation to disulfide (GSSG) during ascorbate regeneration in the glutathione-ascorbate cycle [151, 152]. This type of redox imbalance is transduced downstream by reversible formation of a mixed disulfide between GSH and a target protein (*S*-glutathionylation). An increased GSH:GSSG ratio was observed in plants exposed to chilling, heat stress, heavy metals, xenobiotics, drought, ozone, pathogen [153-159], and during oxidative stress resulting from deficiency in the H_2O_2-scavenging photorespiratory enzymes CAT or APX [36, 47, 160-164]. In maize and rice, the ability to maintain higher GSH:GSSG ratios was associated with greater stress tolerance [164]. GSSG accumulation is a key determinant of cell death and growth arrest [165, 166].

Immunolocalization studies revealed that stress-triggered GSSG accumulation occurred in discrete subcellular compartments. Localization studies in Arabidopsis detected little or no accumulation in mitochondria, slight but significant accumulation in the cytosol, and prominent accumulation in vacuole and chloroplasts [162]. GSSG sequestration in metabolically inert vacuole is thought to initiate catabolism, whereas accumulation in chloroplasts could have functional consequences for photosynthetic efficiency. Increased GSSG level is sufficient to

trigger protein *S*-glutathionylation [167], which is thought to regulate enzymatic protein activity [168]. A large number of unidentified targets of this posttranslational modification represent chloroplast proteins (e.g., RuBisCO or glucose-6-phosphate dehydrogenase) [167]. However, it is not clear if GSSG accumulation in chloroplast results from import or *in situ* synthesis. Isolated wheat chloroplasts can take up GSSG from the medium [36]. Re-engineering of compartment-specific glutathione synthesis pathways suggested that cytosol-to-chloroplast GSSG transport also occurs *in vivo* [169]. Specific GSSG transporters have been identified in tonoplast but not in the chloroplast envelope [170].

The presence of redox-sensitive cysteines has been shown for mammalian ANXA2 [171] and ANXA1 [172]. They are located in the extended C- or N - terminal end (Cys324 aa for ANXA1, and Cys8 and Cys334 for ANXA2), which confer structural diversity to proteins from this family. It cannot be easily generalized if other annexins contain cysteines susceptible to oxidation. ANXA2 was proposed to directly neutralize H_2O_2 with accompanying oxidation of only Cys8 [173-175]; subsequently, it would be reduced via the NADPH-dependent thioredoxin system (NTS) being thus an ultimate acceptor of electrons from NADPH. Oxidative damage in annexin A2-depleted cells enhanced oxidation of the ANXA2-binding proteins actin and transcription factor JunD. This suggests that ANXA2 can function directly as a protein reductase [174].

The presence of reactive cysteines was confirmed in Arabidopsis annexin ATANN1. Implicated amino acids are localized within the endonexin domains and are highly evolutionarily conserved [53] (Fig. S3 in Supporting Information file S1Figures). ATANN1 Cys underwent *in planta S*-nitrosylation within 20 minutes after NO treatment [176] and *S*-glutathionylation within 30 minutes after ABA induction [56]. MeV treatment resulted in oxidation of both cysteines

residues of ATANN1 (Cys111 and Cys 239), although the exact type of modification (mixed disulfide bonds with GSH, or intramolecular disulfide bond) has not been defined [177]. The closest ATANN1 homolog in *Brassica rapa*, BRANN1, appears to form a complex with peroxidase in floral buds [178].

STANN1 contains two cysteine residues, Cys17 and Cys111. Among potato annexins, the former is unique for STANN1 and STANN3.2, whereas the latter is homologous to Arabidopsis Cys111. This arrangement resembles that of ANXA1, with reactive Cys8 in the N-terminal amino acid. Elevated STANN1 levels mitigated photooxidative stress and diminished ROS accumulation, which suggested that STANN1 enhanced the capacity of cytosolic antioxidant buffer. Therefore, it appears that the evolutionarily conserved cysteine homologous to ATANN1 Cys239 is not necessary for such activity. Plant annexins can prevent ROS over-accumulation in a similar way to that previously shown for ANXA2: by direct ROS neutralization and further regeneration by NADPH-dependent thioredoxin/glutaredoxin system. Therefore, annexin would function as an ultimate acceptor of excess electrons leaking from over-reduced PETC. Alternatively, STANN1 may be used as an acceptor for ROS diminishing thus GSSG formation (Fig. 11). Annexins are abundant cytosolic proteins (accounting for up to 2% of the total soluble proteins). They possess redox-sensitive cysteines and could participate significantly in the cellular protein thiol pool. In transgenic plants overexpressing STANN1, annexin levels are higher and the antioxidant protective effect is increased. Reduced GSSG accumulation prevents a decline in the GSH:GSSG ratio and over-oxidation of the cellular environment. The latter mechanism could explain the broad-specificity of annexin-mediated protection, which is functional in bacteria and photosynthetic and non-photosynthetic eukaryotic cells. Glutathione is one of the most abundant non-protein thiols; it is present in cyanobacteria and proteobacteria, and in all mitochondria- or chloroplast-containing eukaryotes [179, 180]. The mechanism of GSH-mediated regulation and maintenance of cellular redox status is similar in all living

organisms. Reversible oxidative thiol modifications modulate the function of proteins involved in many different pathways, including gene transcription, translation, protein folding, metabolism, signal transduction, and apoptosis.

In control conditions, NPQ in the leaves of transgenic plants was improved. During water scarcity the relative content of Zea in the total Car pool steadily increased relative to WT. In addition, the drought-induced decrease of *PSBS* mRNA was delayed. Collectively, these data suggested that transgenic plants possessed an improved ability to dissipate the EAE (produced by high light and stomatal closure) that could not be used for photosynthetic purposes. In several plant species, Viol to Zea reconversion is essential for pH-dependent qE, a major and quickly reversible component of NPQ [10, 17, 72]. PSBS was shown to be essential for qE [73]. Zea accumulation in turn correlates with the second, sustained, and slowly reversible component of NPQ: photoinhibition (qI) [74]. Under prolonged light stress, qI replaces qE [75]. The efficiency of Viol pool reconstitution is therefore also essential for effective NPQ.

The PSIIAS dissipates excess energy more efficiently in transgenic plants

Chlorophyll content

In higher plants PSIIAS contains chlorophyll *a* (Chl*a*) and *b* (Chl*b*) and accessory pigments (oxygenated Car derivatives, XCar). Transgenic potato plants with elevated STANN1 preserved a higher Chl*b* content and increased their Chl *a*/*b* ratio during water stress. This indicated that fine-tuning of photosynthetic complexes in response to light stress was altered in the transgenic plants. A similar protective effect of annexin overexpression on the total Chl level was noted in different plant species. However, in contrast to other papers [76 – 78] we analyzed physiological changes in attached leaves of intact plants that were experiencing gradually progressing drought and quantitated different chlorophylls separately. Upon drought, biosynthesis of Chl*b* was induced in transgenic plants, while in WT plants it did not change, even after rewatering. Chl*a* and Chl*b* are differently distributed in the thylakoid membranes, with Chl*b* being restricted to the peripheral PSIIAS. An Arabidopsis mutant that did not synthesize Chl*b* (*chlorine 1*, *ch1*) due to disruption of chlorophyllide *a*

oxygenase (CAO) gene lacked AS and lost the ability to dissipate the excess energy in NPQ [79]. This loss resulted in increased plant photosensitivity due to an augmented release of $^1O_2$ from PSII [80]. Correspondingly, overexpression of CAO resulted in a decrease in the Chl *a*/*b* ratio and led to transcriptional reprogramming and retardation of senescence [81]. The ability to support Chl*b* synthesis during drought probably helps to improve NPQ and preserves PSII integrity during stress in transgenic plants.

XCar content

During drought stress, transgenic plants suffered less from depression of photosynthetic efficiency (photoinhibition) resulting at least partially from overaccumulation of Zea and, to a lesser extent, Viol. However, the mechanisms underlying the annexin-mediated changes to the XCar pool are unclear. Zea and Viol are produced in chloroplasts as intermediates in the Car biosynthetic pathway that ultimately leads to ABA synthesis. The relative contents of both XCars changes daily and is strongly dependent on environmental conditions (primarily light). In darkness or under sub-saturating irradiance, Zea is epoxidated into Viol in a two-step reaction by zeaxanthin epoxidase (ZEP), resulting in a Viol level approximately ten-fold higher than that of the Zea precursor [82]. However, under specific conditions, this epoxidation step is reversible. When light absorption exceeds the ability of a plant to assimilate CO_2, due to excessive sun radiation or to other environmental factors, preexisting Viol can be re-converted into Zea by VioIDE [83]. In our experiments, samples were collected at the same time to avoid fluctuations in the level of epoxidated and non-epoxidated XCars. The persistent overaccumulation of Zea affected the kinetics of induction and relaxation but not the extent of NPQ. This overaccumulation also led to permanent down-regulation of PSII activity both under experimental conditions and in the field [84, 85]. In turn, overaccumulation of Viol and inability to reconvert to Zea resulted in increased photosensitivity and bleaching as a consequence of the reduction in NPQ [17]. Thus, ZEP/VioIDE activities must be strictly regulated to optimize the efficiency of photosynthesis in a varying environment without increasing the overall XCar pool size. VioIDE is activated when the lumen pH falls below 6.2. ZEP activity requires higher pH (~7.4), molecular oxygen as a second substrate, and NADPH as a cofactor. Optimal activity of VioIDE also relies on lipid bilayer membrane fluidity and requires the presence of phospholipids favoring the formation of a reversed hexagonal structure,

monogalactosyldiacylglycerol [86]. Independent of stress durability and severity, 20–50% of Viol remains unconverted. Suggested mechanisms include feed-back inhibition of ViolDE by Zea [87] or curvature membrane stress elicited on the lipid bilayer by Zea [88]. Alternatively, since Viol is also a precursor of the side-branch synthetic pathway leading eventually to ABA synthesis [89] it cannot be excluded that some enantiomer of Viol is inaccessible for ViolDE [87].

At constant light intensity, moderate water deficit leads to an increase in Zea level. Increasing amounts of Viol are needed as water deficit progresses as it is both re-converted into Zea and used to synthesize ABA. The presence of spatially (shoot *versus* root) or metabolically (e.g., stereoisomers, 9-*cis*, and 9-*trans*) separate pools of Viol were postulated, as these would support the continued functions of xanthophyll cycle or ABA synthesis, respectively (reviewed in [90]). However, the factors determining the functional partitioning of Viol into different pools remain largely unknown.

Finally, the lower steady-state level of ABA and concomitant increased Zea level in transgenic plants may be simply due to the lower levels of oxidative stress in the chloroplasts of those plants. Light-stress-dependent ABA biosynthesis results from inefficient reduction of ascorbic acid (AA) under these conditions. Protonated AA is an essential cofactor for ViolDE and synthesis of Zea in the thylakoid lumen [91]. The XCar cycle cannot function properly when ascorbate recycling is inefficient and Viol accumulates. This can result in ABA synthesis. The ABA increased by 60% in leaves of ascorbate-deficient *vte1* Arabidopsis plants [92]. Hence, improved NPQ may accelerate ascorbate recycling and therefore support Zea synthesis.

How does annexin affect chloroplast-localized processes?

Although annexins contain no specific signaling sequences that target them to the chloroplast, annexins were found in the chloroplast proteome of some plant species (reviewed in [93]). For example, a mustard (*Sinapsis alba L.*) annexin was found to be a component of a multi-subunit chloroplast RNA polymerase A complex [94]; however, these results were not confirmed in a subsequent study [95]). Recently, ATANN1 was detected in the hydrogen peroxide-sensitive chloroplast proteome [96]. These chloroplast localizations remain exceptions. The question then remains as to how annexins can affect the processes within the chloroplast?

It is probable that annexin-mediated protection of the chloroplast and photosynthesis could be an indirect effect of the ability of annexin to counteract photooxidative stress. Chloroplasts are one of the major sources of ROS during stress in plant cells [11]. ROS derived from electron leakage from overloaded PETC can be released to the cytosol where they pose a threat to cellular macromolecules and structures and also participate in retrograde chloroplast-to-nucleus signaling [97] and pcd induction [33], [98]. Annexin STANN1 was able to attenuate both phases of chloroplast-derived oxidative stress. In transgenic plants, the expression of nucleus-encoded PSII proteins (Figs. S6A–B) and HSPs was modified correspondingly (Figs. S6C–D), which strongly suggested that annexins were able to modulate retrograde oxidative signaling. The mechanisms underlying this activity are unclear. Annexins were proposed to undergo thiol-disulphide cycles in a similar manner to the low-molecular-weight antioxidant glutathione or thioredoxins [99]. The presence of redox-sensitive cysteines has been shown for mammalian AnxA2 [100] and the ability of AnxA2 to regulate cellular redox homeostasis by participation in reversible cycles of oxidation and reduction of their cysteines was recently confirmed *in vivo* [100, 101]. Since annexins are abundant (it is estimated that in the plant cell they can constitute up to 2% of the total soluble proteins) they could participate significantly in the cellular protein thiol pool. In our previous study [52], we showed that Arabidopsis annexin 1 (ATANN1) contains two cysteines (111 and 239) that are not involved in formation of intramolecular disulfide bridge but can undergo S-gluthathionylation upon ABA treatment. [96] recently showed that Arabidopsis ATANN1 participated in the cellular thiol pool. ATANN1 was one of 24 proteins that were oxidized *in vivo* in Arabidopsis plants after MeV-induced photooxidative stress. However, only the first cysteine is preserved in potato annexin STANN1 (Figure S3 in File S1Figures). STANN1 nevertheless exerts a protective effect, which suggests that a different mechanism is involved.

Conclusions

The results obtained in this study clearly indicate that annexin overexpression has~~is of~~ potential ~~utility in~~application for developing drought-tolerant crop~~ plant~~s. Enhanced drought tolerance in transgenic potato plants overexpressing STANN1 confers greater tolerance ~~is connected to the ability of transgenic potato plants to better deal with~~ to high-light stress induced by stomatal closure, and diminished CO_2 supply. ~~The attenuated~~ ROS accumulation was attenuated, ehich improved chloroplast function ;~~and~~ genetically modified plants ~~were able to~~ maintained efficient PSII under stress conditions. Maintenance of a high photosynthetic yield even under sub-optimal conditions had a beneficial effect on final crop ~~plant~~ yields and biomass production. ~~Thus, a~~Annexins are a promising target for manipulation of plant tolerance to environmental conditions.

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Figure legends.Figure 1. Annexin genes in potato genome.

(A) Localization of annexin genes on potato chromosomes. The Roman numerals at the top denote the chromosome, digits in brackets indicate chromosome size.

(B) Intron-exon organization of potato annexin genes.

(C) Genomic PCR confirming the presence of predicted annexin genes in WT potato. Specific primers anneal to the 5'- and 3'- ends of coding sequence of certain annexin gene, hence the length of the resulting PCR product is a sum of the respective coding sequence with introns.

(D) Schematic arrangement of *STANN3.1*, *STANN3.2*, *STANN3.3* and *STANN4* on chromosome I.

Figure 2. Profiling of annexin expression in WT potato leaves during drought.

Potato WT plants grew in the walk-in growth chamber under controlled conditions. After 8-10 weeks irrigation was gradually reduced to decrease the field capacity (FC) to 25% (which took approximately 10 days) and then maintained at this level till 14th day. Samples were collected from the first fully developed composite leaf from the top at indicated time points (D0 – beginning of drought, D6 – sixth day of drought, and D14 – fourteenth day of drought). RNA was isolated with Trizol and sq-RT-PCR was performed with primer sets specific for certain annexins. The level of expression was normalized against *EF1a* mRNA. Results are means ±SE ($n \leq 4$). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 3. Drought tolerant phenotype of transgenic plants.

Potato WT plants and transgenic lines (S-2, S-7) was subjected to drought as described above in Fig. 2. (A) Drought stress phenotype of WT (left column), S-2 (middle column) and S-7 (right column) plants. Photographs were taken on the beginning (D0), on eighth (D-8) and ninth (D-9) day of drought. Experiments were repeated twice in greenhouse and twice in growth chamber and gave similar results.

(B) Regeneration of potato plants after prolonged drought. The procedure of drought imposition was the same as described above but the FC was maintained at 25% until the twenty first day of drought (D21). On D22 plants were rewatered and after draining of gravitationally bound water FC was kept up at 65%. Photograph was taken on the third day after rewatering. Left side - two WT plants; middle – two S-2 plants, and right – two S-7 plants. Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.

Figure 4. Examination of leaf water status.

Potato WT plants (white bars) and transgenic lines: S-2 (gray bars) and S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. (A) Relative water content (RWC) analysis. Samples from the first fully developed undamaged leaf from the top of plant were collected at D0, D4, D7, D12 and 3 days after rewatering (RW3) and relative water content (RWC) was determined. Results are means ±SE (n=3). (B) Stomatal conductance were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results.

Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated 3 times and gave comparable results.

Figure 5. Netto photosynthesis and photosynthetic performance of PSII in potato plants during drought.

Potato WT (white bars) and transgenic lines: S-2 (grey bars) and S-7 (black bars) were subjected to drought as described in Fig. 2. (A) Netto photosynthesis, (B) maximum quantum yield of photosystem II (Fv/Fm) and (C) effective quantum yield of photosystem II,Y(II) were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values which are not significantly different at $p<0.05$ and belong to the same homogenic group.

Figure 6. Photosynthetic pigment content during drought.

WT (white bars) and transgenic line S-7 (black bars) were exposed to drought as described in Fig. 2. Samples were collected at the same time during the day at D0, D6, D14 and RW3 from third, fourth and fifth fully expanded leaves from top at 4 hours after turning the light. The level (A) chlorophyll *a*; (B) chlorophyll *b*; (C) zeaxanthine; and (D) violaxanthine were determined Non-polar lipids were separated on an ACQUITY UPLC system (Waters) and peaks were integrated at 436 nm. The level of xanthophylls is expressed as percent of

the total carotenoids. The level of chlorophyll is expressed as mg mL^{-1}. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

~~Figure 7. Accumulation of stress-related hormones during drought.~~

~~WT (white bars) and transgenic line S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. The level of (A) absicisic acid ABA; (B) sum of active cytokinins, CK; (C) salicylic acid, SA were determined at D0, D6, D14 and RW1. Samples (0.5g of fresh leaf tissue without the midrib) were collected from the first fully developed, undamaged leaf from the top of plant at 4 hours after turning the light. Labeled internal standards were added to the leaf samples before homogenization. Hormones were then extracted, purified using a SPE-C18 column and separated on a reverse phase-cation exchange SPE column. Hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer. The level of ABA and SA is expressed as nmol g^{-1} of fresh weight; the levels of cytokinins – as pmol g^{-1} of fresh weight. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).~~

Figure 7~~8~~. NPQ assayed in leaf of well-watered potato plants.

Potato WT (dashed line) and transgenic S-7 (solid line) grew in the walk-in growth chamber under controlled conditions and were watered to maintained FC at 65%. Performance of gross non-photochemical quenching (NPQ) were assayed on the first fully developed composite leaf from the top of plant at 4 hours after turning the light with Dual PAM-100. For measurement plants were adapted to dark for 20 minutes and then stimulated with repeated light pulses of actinic light (94 PPFD) for 5 minutes and once again subjected to dark for 6 minutes. Each point represents the mean ±SD (*n*=3-4). Experiment was repeated three times and gave comparable results.

Figure 8. Accumulation of stress-related hormones during drought.

WT (white bars) and transgenic line S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. The level of (A) absicisic acid ABA; (B) sum of active cytokinins, CK; (C) salicylic acid, SA were determined at D0, D6, D14 and RW1. Samples (0.5g of fresh leaf tissue without the midrib) were collected from the first fully developed, undamaged leaf from the top of plant at 4 hours after turning the light. Labeled internal standards were added to the leaf samples before homogenization. Hormones were then extracted, purified using a SPE-C18 column and separated on a reverse phase-cation exchange SPE column. Hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer. The level of ABA and SA is expressed as nmol g^{-1} of fresh weight; the levels of cytokinins – as pmol g^{-1} of fresh weight. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 9. Accumulation of ROS (hydrogen peroxide and superoxide anion) and lipid peroxidation.

Potato WT (white bars) and transgenic line S-7 (black bars) grew in walk-in growth chamber under controlled conditions. Leaf discs were expunged from the third, fourth and fifth upper fully expanded leaves and immediately vacuum infiltrated with methyl viologen (50 μM). After 1 hour incubation in dark discs were exposed to high light irradiance (850 PPFD) for indicated times (0.5 – 24 hours). Superoxide anion was determined colorimetrically with nitro blue tetrazolium chloride 9NBT). Hydrogen peroxide was stained in tissue with diaminobenzidine tetrahydrochloride (DAB) and quantified using the ImageJ. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid

(TBA). Results are means ±SE (n=5). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 10. STANN1 attenuated MeV-induced photooxidative stress.

Confocal laser scanning image of the leaf epidermis of tobacco plant transiently expressing GFP (A-D and I-L) or STANN1_GFP (E-H and M-P). 3 days after infiltration leaf discs were excised and subjected to high light (850 PPFD) (A-H) or the combine treatment of high light (850 PPFD) and 50 μM MeV (G-L). The fluorescence was monitored with Nikon TE-2000E EZ-C1 exc. 488 nm and emission 515/30 and 605/75 for GFP and chloroplast, respectively. First column represent single focal plane, second – chloroplast autofluorescence acquired with the same excitation parameter for each construction to visualized the difference between responses to the same treatment, third – overlay of green and red fluorescence channels with GFP enhanced to visualized cells; right column – stack obtained with Volume Render program EZ-C1 combined with chloroplasts. Scale bar is 20 μm. Experiment was performed 3 times.

Figure 11. A simplified scheme depicting the interactions between cellular redox state and participation in ROS scavenging mechanisms.

Oxidative stress is an unavoidable consequence of environmental stresses. ROS accumulation begins in chloroplasts and then it spreads throughout the whole cell. Activation of a secondary ROS sources e.g. NADPH oxidase complex or photorespiration resulted in substantial H_2O_2 accumulation in cytosol. To avoid deleterious effects of ROS several compartment-specific mechanisms evolved,

including accumulation of low-molecular-weight antioxidants (glutathione, ascorbate), scavenging enzymes (CAT, APX, SOD) and protein thiols (PRX, GRX and TRX) that undergoes a reversible cycles the thiol-disulphide exchange.

The redox-sensitive proteins sense, transduce, and translate ROS signals into appropriate cellular responses. Thus, precise regulation of size and redox status of the thiol pool is of essential importance for induction of appropriate responses. In plant cells glutathione is present in different compartments in milimolar concentrations and in quiescents it maintained largely in reduced state due to activity of glutathione reductases (GR) at expense of NADPH. Stress-induced ROS accumulation stimulates oxidation of glutathione (GSSG) and in the same time *de novo* synthesis of GSH. Disturbances in GSH/GSSG ratio might non-specifically influence several downstream pathways, e.g. by induction of thiol-disulfide exchange on target proteins. Cellular redox potential depends primarily on the total concentration of the total glutathione and the extend of its oxidation. GSSG accumulation did not disturb the redox potential if it is compensated by increasing the total glutathione concentration. However, if size of total pool remains unchanged when the GSH:GSSG ratio increased the cell redox potential in the cytosol become more positive.

We propose that the improved stress tolerance of annexin STANN1-overexpressing potato plants results from amelioration of oxidative shift of the cytosolic glutathione redox potential. Elevation of STANN1 level had a pleiotropic effect on plant metabolism and physiology what suggested that not one specific but several downstream signaling pathways were touched. Disruption of the glutathione redox potential is sufficient to induce such effect; e.g., in transgenic tobacco with constitutive upregulation of glutathione content MAPK and SA signaling pathways

were modified. Annexin posses oxidation-sensitive cysteines and can act as a reductant influencing thus the redox potential. During stress in transgenic plants the capacity of cytosol redox buffer was more reducing compared to WT what prevents oxidation of downstream targets and modulate timing as well as magnitude of stress response. It had a beneficial effect on cell survival, photosynthesis and delay senescence. Similar effects were observed in tobacco and Arabidopsis plants and over-expressing particular elements of antioxidant systems.

Supporting information legends:

File S1Figures.

Figure S1. Construction of transgenic plants.

A) Structure of the T-DNA region from pROK2 carrying STANN1_His6x that was used for *Agrobacterium* -mediated transformation. LB – left border; RB – right border; NPTII – neomycin phosphotransferase II, CaMV – cauliflower mosaic virus 35S promoter; NOS – nopaline synthase terminator;
(B) Expression of STANN1_His6x protein in F1 transgenic potato lines. Proteins were isolated from leaves of WT and F1 transgenic lines S-2, S-3, S-7, S-83, S-91, S-97 and S-123 grown *in vitro*. His-tagged proteins were purified with Ni-NTA agarose, subjected to SDS_PAGE and blotting followed by detection with anti-His primary Ab. The band detected in WT represents *Arabidopsis* annexin ATANN1_His6x (molecular weight *ca* 36 kD) produced in *Escherichia coli* that was added before purification to the ground protein to STANN1_His6x easily dimerized hence the two bands were detected, the lower with molecular weight corresponding to monomer and the upper corresponding to dimer.

Figure S2. Characteristics of experimental drought.

(A) Potato WT and S-7 plants after 8 week of growth at the phase of experimental drought implementation. Transgenesis has no impact on tuber development. Formation of stolon hooks and stolon swelling as well as first tubers are visible.
(B) Field capacity (FC) was normalized at the beginning of experiment and maintained at constant level (app. 65%); for control (well-irrigated plants) FC was maintained at this level throughout the whole experiment. For experimental drought FC was gradually lowered to 20% and kept at this level un till the end of drought. Rewatering was applied by full water saturation of the soil and after gravity draining of excess water FC was kept at the 65% untill the end of experiment.

Figure S3. Multiple alignment of amino acid sequences of putative annexins from potato and selected annexins from human, Arabidopsis and cotton.

The alignment was done with Cobalt (Constrain-based Multiple Alignment Tool). Gene Bank Acc Nos of employed sequences are as follows: human AnxA5

(NP_001145.1), Gossypium hirsutum GHANN1 (1N00), Arabidopsis thaliana ATANN1 (2Q4C) and for potato annexins: STANN1 PGSC0003DMG4000177114, STANN2, STANN3.1 PGSC0003DMG4000221817, STANN3.2 PGSC0003DMG401019427, STANN3.3 PGSC0003DMG402019427, STANN4 PGSC0003DMG400019446, STANN5 PGSC0003DMG400007966, STANN8 PGSC0003DMG400007482 and STANN9 PGSC0003DMG40001879.

The boundaries of endonexin repeats were marked on the basis of crystal structures obtained for GHANN1 (Hofmann et al., 2003) and ATANN1 (Levin et al., 2007) and are, respectively:

- 1st endonexin domain: 14-80 and 13-81;
- 2nd endonexin domain: 83-153 and 84-154;
- 3rd endonexin domain: 164-239 and 165-241
- 4th endonexin domain: 241-309 and 244-3111 respectively for cotton and Arabidopsis annexin.

Conserved histidine 40 is in red; methionine and cysteines from C3 cluster are in blue and underlined.

Calcium binding motifs G-X-GTD-{38-40}-D/E are marked by black boxes; potential N-terminal acylation motif is in bold; potential actin-binding domains IRI are in bold and italic;

C-terminal peptide similar to 14-3-3 proteins is marked by pale-green rectangle.

Amino acid residues of high conservation are shown in red, medium - in blue.

Figure S4. Drought tolerant phenotype of transgenic S-7 potato plants.

Each image depicts two WT plants (left side) and two transgenic S-7 plants (right side) subjected to experimental drought. Drought was started on D0 and lasted 21 days. During that time watering was gradually reduced so as to lower the FC to 20%. After reaching that level it was maintained until 21 days after onset of experiment. The soil was than fully saturated with water (rewatering) and FC was maintained at 65% until the end of experiment.

D10 - irrigation withheld for 10 days, D14 - irrigation withheld for 14 days, D21 - irrigation withheld for 21 days, RW5 – rewatered for 5 days.

Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.

In WT symptoms of wilting clearly appeared after 10 days of drought; in S-7 they were apparent only after 2 weeks. On the 21st day WT were severely affected with damaged stems and dry leaves. At the same time in S-7 plants the upper leaves still maintained turgor. After rewatering only a few leaves in WT regenerated; instead, new shoots developed from below-ground parts after at least a week of regular irrigation. In contrast, the S-7 plants preserved their upper leaves and after rewatering returned to a normal healthy look within hours. The exact number of irreversibly damaged leaves varied between experiments, but it was always significantly lower than in WT.

Figure S5. Potato yield during drought.

(A) Irrigated Water Use Efficiency (IWUE) is a quotient of crop produced per unit per amount of water supplied (IWUE = Y / W [g/pot/mL of water])

(B) An exemplary tuber yield per plant. Potato plants WT, S-2, and S-7 were grown in a greenhouse. After 8-10 weeks of growth plants were subjected to drought stress by restricting irrigation to achieve 20% FC and kept at this level until 14th day. After that time plants were rewatered and cultivated in optimal conditions for additional 10 weeks until physiological maturity. Tubers were lifted immediately after withering of haulms. The weight of all fresh tubers from single plant was determined immediately after harvesting. Experiments were repeated twice and gave similar results.

(C) Quantification of tuber yield experiments. Results are shown as mean ±SD (n=10)

Figure S6. Expression of genes coding for PSII proteins and HSPs.

Relative quantification of *PSBS* (A), *LHCB4* (B) *HSP100* (C) and *HSP40* (D) mRNAs in leaves of WT (white bars) and transgenic S-7 (black bars) potato plants during three-week drought and after rewatering. The data represents the mean ±SE from at least four measurements. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test, the same letters designate days which are not significantly different at $P < 0.05$ and belong to the same homogenic group.

Figure S7. The effect of photooxidative stress on potato leaves.

Leaf discs (F ~ 1 cm) were excised from leaves of WT or transgenic plants S-2 and S-7 and immediately infiltrated with (A) 50 mM Tris-Cl, pH 7.5 (B)10 mm MeV or (C) 50 mM MeV in 50 mM Tris-Cl, pH 7.5. Subsequently, leaf discs were exposed to light of 150 PPFD for 30 h.

File S2Tables.

Table S1. Primer pairs used for identification of potato annexins.

Primer pairs corresponding to the predicted 5' (F) and 3' (R) ends of the particular annexin genes were designed on the basis of published potato genome sequence. Gene length refers to the total length of exons and introns. Individual primer pairs (F - forward, R - reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA).

Table S2. Primer pairs used for sq-RT-PCR.

Primers for semi-quantitative analysis of expression of annexins and other genes in potato. Individual primer pairs (F- forward, R- reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA) to span intron–exon boundaries to exclude interference from genomic DNA contamination. Amplified fragments were between 300 and 500 base pairs. The genes were selected from PGSC_DM_v3.4_pep_fasta containing database of potato virtual translation products on the basis of their homology to annotated Arabidopsis genes. Analyzed genes were as follows: annexins: STANN1-9; HSP100 (heat shock protein 100 kDa); HSP40 (heat shock protein 40kDa, DNAJ); PSBS (chlorophyll a/b- binding photosystem II 22kD subunit S); LHCB4 (light-harvesting complex binding protein 4). As a reference the housekeeping gene for Elongation Factor a1 (EF1a) was used.

Table S3. Characterization of putative potato annexin proteins.

chlo – chloroplast; cyto – cytoplasm; cyto_ER – cytoplasm/membrane of endoplasmatic reticulum; cysk – cytoskeleton; ER – endoplasmatic reticulum; extr

– extracellular; mito – mitochondria; nucl – nucleus; plas – plastids; vacu – vacuole

Table S4. Cytokinins in leaves of WT and S-7 potato plants under drought.

S. tuberosum WT and transgenic S-7 plants were subjected to 2-week drought or well-watered. At time points indicated 0.5 g of tissue (without the main vein) was collected 4 hours after beginning of the day from fully expanded leaves. Hormone levels were analyzed by LC-MS as described in Materials and Methods (n=3). Data are shown as pmol g-1 FW.

Abbreviations: tZR, trans-zeatine riboside; tZ, trans-zeatin; iPR, isopentenyl adenosine riboside; iP, isopentenyl adenine; cZR, cis-zeatin riboside; cZ, cis-zeatin.

Potato Annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic *Solanum tuberosum* L. Plants

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Abbreviations:

ABA	abscisic acid
ATANN1	annexin 1 from *Arabidopsis thaliana* L.
Car	carotenoids
Chl*a*, Chl*b*	chlorophyll *a*, chlorophyll *b*
CK	cytokinins
Fv/Fm	the maximum quantum efficiency of photosynthesis
MeV	methyl viologen, paraquat
NPQ	non-photochemical quenching
PCD	programmed cell death
PETC	photosynthetic electron transport chain
PPFD	photosynthetic photon flux density [μmol m^{-2} s^{-1}]
PSI, PSII	photosystem I, photosystem II
PSBS	22-kD photosystem II subunit
STANN1	annexin 1 from *Solanum tuberosum* L.
SA	salicylic acid
Viol	violaxanthine
Y(II)	effective quantum yield of PSII
XCar	xanthophylls
Zea	zeaxanthine

Abstract

Annexins are a family of calcium- and membrane-binding proteins that are important for plant tolerance to adverse environmental conditions. Annexins function to counteract oxidative stress, maintain cell redox homeostasis, and enhance drought tolerance. In the present study, an endogenous annexin, STANN1, was overexpressed to determine whether crop yields could be improved in potato (*Solanum tuberosum* L.) during drought. Nine potential potato annexins were identified and their expression characterized in response to drought treatment. *STANN1* mRNA was constitutively expressed at a high level and drought treatment strongly increased transcription levels. Therefore, *STANN1* was selected for overexpression analysis. Under drought conditions, transgenic potato plants ectopically expressing STANN1 were more tolerant to water deficit in the root zone, preserved more water in green tissues, maintained chloroplast functions, and had higher accumulation of chlorophyll *b* and xanthophylls (especially zeaxanthine) than wild type. Drought-induced reductions in the maximum efficiency of PSII, the quantum yield of photosynthesis, and the electron transport rate were less pronounced in transgenic plants overexpressing STANN1 than in the wild type. This conferred more efficient non-photochemical energy dissipation in the outer antennae of PSII and probably more efficient protection of reaction centers against photooxidative damage in transgenic plants under drought conditions. Consequently, these plants were able to maintain effective photosynthesis during drought, which resulted in greater productivity than wild-type plants despite water scarcity. Although the mechanisms underlying this stress protection are not yet clear, annexin-mediated photoprotection is probably linked to protection against light-induced oxidative stress.

Introduction

Plants have developed passive and active strategies to survive environmental stresses such as drought, salinity, chilling, heat shock, heavy metals, UV radiation, ozone, mechanical stress, nutrient deficiency, hypoxia, and biotic stress [1]. Several stress-response genes have already been targets for bioengineering studies to improve plant stress tolerance [2]. However, ectopic expression of stress-inducible genes often results in developmental aberrations (e.g., stunted growth and irregular leaves) or reduced crop yields under non-stress conditions due to non-specific induction of programmed cell death (PCD) and/or premature senescence [3]. Current knowledge of stress-responsive pathways is based primarily on results obtained by imposing each stress individually, whereas plants in natural settings are generally challenged with multiple concurrent stresses, and the resultant signaling pathways may be superimposed and/or induce/antagonize each another [4-6]. New approaches to bioengineering stress tolerance in crop plants are needed to achieve sustainable improvements in crop biomass production [2].

Recent work shows that changes in redox poise can regulate plant cell function [7-10] by acting as cellular signals [11]. Numerous studies have investigated redox-mediated stress responses [12-16]. Our understanding of the mechanisms that control production and metabolism of reactive oxygen species (ROS) in plants is incomplete. ROS are generated in several cellular compartments. In light, the predominant location of ROS production is in chloroplasts [17, 18], where ROS are continuously formed as photosynthetic by-products under non-stress conditions. Environmental stress can trigger an imbalance in redox homeostasis and affect chloroplast metabolism [17, 18]. The chloroplast antioxidant system has been described as

"loosely tailored" to maintain an endogenous ROS pool [20], which enables plants to quickly respond to fluctuating light levels. Consequently, significantly improving ROS scavenging enhances protection against sustained stress, but also desensitizes plant light responses and impairs environmental fitness. This result suggests that a delicate balance has to be maintained between ROS production and the activity of antioxidant systems. Chloroplasts also are involved in biosynthesis of hormones, carotenoids (Car), amino acids, and lipids. Therefore, the protection of chloroplast structure and function from environmental stresses is crucial for plant stress resistance and recovery.

The two main ROS sources in chloroplasts are the light-driven photosynthetic electron transport chains (PETCs) of photosystem I (PSI) and photosystem II (PSII). Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the PETCs [21]. Under these conditions, oxygen can be utilized instead of $NADP^+$ as an alternative acceptor for excess electrons [22]. Several mechanisms have evolved to dissipate excess excitation energy, including nonphotochemical quenching (NPQ), which dissipates excess electrons as heat in PSII [23] and via the water-water cycle in PSI [21]. The water-water cycle produces hydrogen peroxide (H_2O_2), which is the most stable small ROS that can passively and actively cross membranes [24, 25]. In isolated intact spinach chloroplasts and *Arabidopsis thaliana* (Arabidopsis) protoplasts, H_2O_2 produced under illumination can avoid neutralization by chloroplast antioxidant systems, and escape the organelle in a light-intensity-dependent manner [26-28].

Changes in chloroplast redox poise activate secondary ROS-producing sources in other cellular compartments, such as membrane NADPH oxidases [respiratory burst oxidase homologs (RBOHs)]. Abscisic acid (ABA) induces *RBOH* genes expression

in Arabidopsis leaves and guard cells [29-30], *Hordeum vulgare* aleurone layer [31], and *Zea mays* seedlings [32]. NADPH oxidase-mediated ROS accumulation has been reported in ozone-treated Arabidopsis leaf [33, 34] and salt-treated Arabidopsis root tips [35].

Prolonged drought stress results in CO_2 deficiency due to stomatal closing and activates photorespiration. This rescue reaction dissipates excess reducing equivalents and energy, but generates H_2O_2 during salvage of the toxic byproduct 2-phosphoglycolate in peroxisomes. Under drought conditions, photorespiration is estimated to generate 70% of the H_2O_2 produced [36]. One of the major roles of photorespiration was proposed to be readjustment of redox homeostasis under abiotic stress conditions [37]. Cytokinin (CK)-mediated stimulation of photorespiration in transgenic tobacco plants resulted in substantially enhanced drought resistance [38].

Different buffering systems in discrete subcellular compartments are involved in maintaining cellular redox homeostasis and redox signaling. The primary pathway of H_2O_2 scavenging is the glutathione-ascorbate cycle (GSH-As cycle), which operates in chloroplasts, cytosol, mitochondria, and peroxisomes [21, 39]. Each cellular compartment contains a specific set of H_2O_2 scavenging enzymes, such as peroxidases, catalases (CAT), dismutases, glutaredoxins (GRX), and thioredoxins (TRX) [40, 41]. In Arabidopsis and tobacco plants enhancing the antioxidant capacity of chloroplast and cytosol had a beneficial effect on photosynthesis and stress tolerance [42], whereas removal of any single antioxidant component reduced photosynthesis and stress tolerance [43-47]. There is evidence for cross-talk between antioxidant systems from different compartments [40, 48].

Annexins are a multigene, evolutionarily conserved family of calcium- and phospholipid-binding proteins [49]. They are present in all eukaryotes and are

characterized by a highly conserved tertiary structure. The annexin superfamily is defined by the presence of approximately 70 amino acid endonexin motifs, which contain five α-helices that are repeated four (or eight) times in the protein. The contribution of annexins to plant cell adaptation to adverse environmental conditions is well documented [49-53]. However, an understanding of the primary physiological functions of plant annexins remains elusive.

Annexin 1 was identified in a genome-wide search of Arabidopsis sequences capable of rescuing *Escherichia coli* ΔoxyR growth on high H_2O_2 concentrations [54]. Subsequent analyses confirmed that Arabidopsis annexin 1 (ATANN1), *Brassica juncea* annexins BJANN1 and BJANN3, and *Nelumbo nucifera* annexin NNANN1 ameliorated oxidative stress in homologous or heterologous cells and improved stress tolerance [55-61]. Transgenic tobacco plants expressing BJANN1 were more tolerant of different abiotic stresses and exhibited enhanced resistance to *Phytophthora parasitica* var. *nicotianae*, possibly due to constitutively increased expression of several pathogenesis-related proteins [55]. NNANN1 overexpression in Arabidopsis conferred enhanced tolerance to heat and oxidative stress [58]. Deletion of functional ATANN1 reduced expression of glutathione-*S*-transferase Tau 1 (GSTU1) in Arabidopsis seedlings after H_2O_2 treatment [62]. Under salinity conditions, ATANN1 mediates ROS-dependent Ca^{2+} signaling in roots at low (1.5 mM) external Ca^{2+} concentrations [63]. These studies used seedlings or leaf discs subjected to short-term stress treatments (in hours). There is a lack of information regarding annexin function in cell physiology and metabolism during long-term exposure to environmental stress.

Potato is one the most important vegetable crops. Its global annual production in 2010 exceeded 300 million tons (FAOStat). Potato plants are highly efficient in

terms of water usage (http://www.fao.org/potato-2008/en/potato/water.html), and produce more food per water unit than any other crop [64]. Therefore, potato could be a promising alternative to cereal crops. Modern potato cultivars are susceptible to drought, which is defined as a shortage of water in the root zone [65]. Water deficit affects nearly all stages of potato development, and negatively impacts tuber numbers and quality (crop yield) [66, 67]. Only a few attempts to engineer potato drought tolerance have been reported (reviewed in [68]). These studies had limited success because most transgenic plants did not exhibit good performance and productivity under non-stress and stress conditions. Potato annexin has not been considered for bioengineering applications; however, new proteomics research showed that STANN1 could be a candidate gene to improve stress tolerance. STANN1 was differentially expressed in potato tubers in response to wounding [69, 70], bruising (personal observation; [71]), osmotic stress and salinity [72], and was differentially expressed in potato aerial parts in response to osmotic stress and salinity [73]. In this study, we overexpress potato annexin STANN1 and observe the effects on drought tolerance.

First, we investigated if increased expression of ATANN1 affected potato drought tolerance. We used the *S. tuberosum* genome to identify all potato annexins, and analyzed potential involvement in drought responses using semi-quantitative RT-PCT. Then, we characterized photosynthetic performance in transgenic plants overexpressing ATANN1 during prolonged water deficit around the root zone. We also investigated possible annexin functions in modulating redox signaling, and assessed changes in drought stress responses. Our working hypothesis was that annexin modulated plant stress responses by increasing the cytosolic antioxidant buffering capacity in transgenic plants. Studies on Arabidopsis ecotypes indicate that

ATANN1 mRNA levels differ in ecotypes adapted to very different local climatic condition (TAIR and our non-published data). In potato tubers, STANN1 levels did not differ in proteomes from different genetic backgrounds [74]. Further experiments are necessary to elucidate if drought-tolerant potato landraces and cultivars could be generated by enhancing the level of annexin expression.

Materials and Methods

Generation of transgenic plants, transformation and growth conditions

S. tuberosum cultivar Sante (WT), medium-tolerant to drought, was used for transformation experiment (http://www.europotato.org).The *STANN1* cDNA sequence without the stop codon (957 bp; Acc. No. PGSC0003DMG400017714) was fused at the 3′ end to a 6×His-tag sequence and inserted into the XbaI restriction site of pROK2 [75] between cauliflower mosaic virus 35S promoter and nopaline synthase (Nos) terminator sequences (Fig. S1A in Supporting Information file S1 Figures). This construct was used for *Agrobacterium tumefaciens*-mediated transformation of WT potato plants according to a previously published method [76]. Regenerated transgenic plants were transplanted into separate glass tubes filled with 10 mL of Murashige & Skoog solid medium supplemented with 50 μg/mL kanamycin. The presence of the transgene cassette was verified with genomic PCR (data not shown). Expression of recombinant STANN1_6×His protein was confirmed by purification from leaves of WT and F1 transgenic plants (lines S-2, S-3, S-7, S-83, S91, S-97, and S-123) by Ni-NTA chromatography and detection with anti-HisTag primary antibody (Sigma-Aldrich). Recombinant ATANN1_6×His protein produced by bacterial overexpression was used as a positive control (WT protein extract) (Fig. S1B in Supporting Information file S1Figures).

Potato WT plants (*S. tuberosum* cv Sante) or transgenic lines in the "Sante" background (S-2 and S-7) were used for further experiments. Plants were cultivated in a growth chamber (or an air-conditioned greenhouse when indicated) under standard conditions (21±2°C; 16 h/8 h day/night; light intensity 110 to 130 PPFD (photosynthetic photon flux densities); 60–80% relative humidity.

Water stress

S. tuberosum plantlets sprouted from tubers were grown in plastic pots filled with 1 kg of sterilized soil (mixture of peat and sand, pH 5.5; prepared by the Plant Breeding and Acclimatization Institute) for 160–170 days. The field capacity (FC) was determined gravimetrically (g of water per g of soil). Pots were weighed every 2–3 days and the volume of water necessary to maintain the indicated FC was calculated individually for each plant. For well-watered control plants, FC was maintained at 65% (−0.8 MPa) for the whole experiment. Experimental drought was imposed after 8–10 weeks of growth (tuber initiation) (Fig. S2A in Supporting Information file S1Figures). Irrigation was decreased over 10 days to gradually reduce the FC to ~25% FC (−2.0 MPa) and was then maintained at this level until the end of the water deficit period. Irrigation was subsequently resumed with full soil saturation (rewatering). To estimate the impact of drought on potato productivity, plants were cultivated for an additional 11–12 weeks after rewatering (FC 65%) until physiological maturity. An exemplary schedule of FC changes is shown in Fig. S2B (supporting Information file S1Figures). Samples were collected at the beginning of the water deficit period (D0), and (depending on experiment) at different days of drought, i.e. 3rd (D3), 4th (D4), 6th (D6), 10th (D10), and 14th (D14), and at the first (RW1) and third (RW3) days after rewatering.

Identification of potato annexins

Annexins were identified in silico by searching for the endonexin domain (PFAM definition, PF00191, 66 aa) in six translation frames of the heterozygous diploid potato breeding line, *S. tuberosum* L. group Tuberosum RH89-039-16 genome using the HMMSearch program from the HMMER3 package. According to PFAM, >93% of

proteins from this family contained at least three consecutive repeats of the endonexin domain. By searching with a single repeat, the probability of missing a complete protein due to below-threshold partial hits or incorrectly defined intron-exon boundaries was minimized. Only hits with an E-value ≤0.001 were considered. To verify the presence and sequence of the predicted annexins in WT potato, genome primer sets were designed that corresponded to the 5′ (F) and 3′ (R) ends of the predicted open reading frames (ORFs , Table S1 in Supporting Information file S2Tables). Expression of putative annexin genes was verified using RT-PCR. Briefly, total RNA was isolated from WT leaves and reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania) with poly(T)$_{12\text{-}18}$ primer. Annexins were amplified from cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). PCR products were cloned with pJET Cloning Kit (Thermo Scientific) and their compliance with the predicted sequences was verified.

Semi-quantitative expression of annexins and stress-regulated genes

Gene expression was profiled over 14 days of drought in WT potatoes grown as described above. Samples were taken from the first fully-developed composite leaf at the top of the plant. For each time point, single leaf discs from four independent plants were collected, flash-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated with Trizol (Invitrogen, Scottland). Reverse transcription was performed as described above. Taq DNA Polymerase (Thermo Scientific) was used to amplify specific sequences from cDNA. Genes for semi-quantitative analysis were selected from PGSC_DM_v3.4_pep_fasta, which contains a database of potato virtual translation products predicted according to similarity to annotated Arabidopsis genes. Specific primer sets for expression analysis were designed using PrimerSelect, Laser Gene10.0 DNASTAR

(USA) (Table S2 in Supporting Information file S2Tables). The obtained sq-RT-PCR products were subjected by agarose gel electrophoresis, stained with ethidium bromide, and quantitated by densitometry using MultiGaugeV3.0 (Fuji) software. Expression was normalized with respect to the expression of potato elongation factor 1 alpha mRNA (EF1a; PGSC0003DMT400050664;[77]). Each single experiment included four biological replicates, which were quantitated in three technical replicates. Experiments were repeated three times for each primer set and template.

Relative water content

Relative water content (RWC) was determined as described previously [78] with the slight modification. For full saturation [equivalent to turgor weight (TW)] leaves were incubated in distilled water for 4 hours instead of overnight. Experiments were performed three times on at least five biological replicates for each genotype.

Extraction and determination of plant hormones

Leaf samples of ~0.5 g (without the main vein) from 8–10-week plants subjected to drought (as described above) were collected, immediately frozen in liquid nitrogen, and kept at −80°C until use. Samples were taken from the first fully-developed composite leaf at the top of the plant at the indicated time points. Sampling was performed four hours after the start of daily illumination. Three independent biological replicates were examined. Purification and analysis were performed as described previously [79, 80]. Briefly, leaf samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v) and the following labelled internal standards (10 pmol per sample) were added: $^{2}H_{6}$-ABA, $^{2}H_{5}$-*trans*Z, $^{2}H_{5}$-*trans*ZR, $^{2}H_{5}$-*trans*Z7G, $^{2}H_{5}$-*trans*Z9G, $^{2}H_{5}$-*trans*ZOG, $^{2}H_{5}$-*trans*ZROG, $^{2}H_{5}$-*trans*ZRMP, $^{2}H_{3}$-DHZ, $^{2}H_{3}$-DHZR, $^{2}H_{3}$-DHZ9G, $^{2}H_{6}$-iP, $^{2}H_{6}$-iPR,

$^{2}H_6$-iP7G, $^{2}H_6$-iP9G, and $^{2}H_6$-iPRMP (Olchemim, Czech Republic). Extracts were purified using a SPE-C18 column (SepPak-C18, Waters), and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction (containing ABA) was eluted with methanol and the second fraction (containing CK metabolites) was eluted with 0.35 M NH_4OH in 70% methanol. Both fractions were separated by HPLC (Ultimate 3000, Dionex) and the hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and net photosynthesis were analyzed with a Portable Handheld Photosynthesis System CID 340 device (CID Bio-Science, Camas, WA, USA) according to the manufacturer's instructions. The maximum quantum efficiency of photosynthesis (Fv/Fm) and the effective quantum yield of PSII [Y(II)] were determined with CID 340 (CID Inc., USA) with a CI-510CF Chl fluorescence module and a CI-310LA light attachment (CID Bio-Science) providing actinic light. Measurements were performed 5 h after turning on the light, if not indicated otherwise, on the upper five fully-expanded unwrinkled leaves. Five plants were analyzed per time point. For maximal fluorescence (Fm) determination, plants were dark-adapted for 30 minutes (so all PSII reaction centers were closed) and then stimulated with saturating pulses of light (0.8 seconds, 3,000 PPFD). The minimal fluorescence (Fo) with all PSII reaction centers opened was measured with modulated light of 0.25 PPFD. Fv was calculated from the equation Fv= Fm-Fo. Y(II) was calculated using the equation Y(II) = (Fms-Fs)/Fms. The maximal fluorescence under light (Fms) was determined by allowing plants to adapt to light for 20 minutes and measuring the steady-state of chlorophyll (Chl) fluorescence (Fs).

Next, a saturating pulse (0.8 seconds, 3,000 PPFD) was applied and Fms was determined.

Gross NPQ was estimated with a Dual Pulse Amplitude Modulation device, PAM-100 (Walz, Germany). For a single time point, six composite leaves from three to five control plants were analyzed. NPQ was calculated as (Fm − Fms)/Fms, where Fm represents the fluorescence of a dark-adapted sample and Fms represents a fluorescence of the illuminated sample. Plants were dark-adapted for ~20 minutes and kinetics were measured after repeated light pulses of 94 PPFD for 300 seconds. Leaves were subsequently relaxed in darkness for 240 seconds and fluorescence while continuously measuring and recording fluorescence.

Non-polar lipids extraction and carotenoids/chlorophyll determination

Plant material was collected from 8–10-week-old plants exposed to drought. Samples were collected 4 hours after switching on the light at D0, D6, D14, and RW3. One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully expanded composite leaves, and total six discs harvested from two plants were combined as a single sample. Non-polar lipids were extracted at 4°C. Plant material after shredding in cooled mortar was transferred to a 15 mL Pyrex tube. After the addition of 3 mL acetone-methanol (8:2 v/v), the sample was perfused with argon and mixed vigorously by vortexing for 2 min. For the second and third extractions, hexane (9 mL) was added and the sample was again perfused with argon before capping and shaking in a reciprocating shaker (PROMAX 2020, Heidolph, Germany) for 30 min in the dark. After shaking, the sample was incubated without agitation for 5 min to allow phase separation. The upper hexane phase was collected by aspiration and

transferred to a 100 mL Erlenmeyer flask, perfused with argon, capped and stored in the dark in 4°C. In the second extraction stage, 2 mL of propanol was used in addition to hexane, and perfusion, shaking and phase collection were repeated as before. After removal of hexane, the polar phase was centrifuged for 15 min at 4500 rpm. The supernatant was combined with the two hexane phases, perfused with argon, and filtered through a Milipore syringe filter unit Millex-CV13 Filter Unit (0.22 μm). The combined hexan phases were then transferred to room temperature, evaporated to dryness under argon, and dissolved in 1 mL methanol-propanol-hexane 6:1:3 (v/v/v). Dissolved samples were transferred to 2 mL glass vials, perfused with argon, capped, and stored at −80°C.

Non-polar lipids were analyzed by injecting 5 μL of sample extract onto an ACQUITY UPLC HSS T3 1.0×150 mm 1.8 μm column and eluted with a gradient of solvent A [water and methanol (1:9, v/v)] and solvent B [methanol:isopropanol: hexane (2:1:1, v/v/v)], with a total of 210 minutes to transition from solvent A to B. Separation was monitored in the 300–750 nm range with a photodiode array detector. A single chromatogram at 436 nm was extracted, exported in ASCII format, and used for peak area integration analysis with GRAMS/AI software (Thermo Electro Corp, Finland).

Chl*a* and Chl*b* contents were estimated by recording the absorbance of the aforementioned extract at 663, 652, and 645 nm (Cary 50 Bio UV/VIS spectrophotometer, Varian, Australia) as described previously [81].

ROS levels and lipid peroxidation during high light stress

One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded leaves of potato plants, and for a total of six discs three discs were

harvested from each of two plants were combined as a single sample. Immediately after harvesting, samples were vacuum-infiltrated with methyl viologen (MeV) at the indicated concentrations and then incubated in the dark for 1 hour under normal irradiance (150 PPFD). Images were obtained after 30 hours of incubation.

A similar procedure was used for ROS quantification, with the exception that a single MeV concentration (50 μM) was used and samples were exposed to high irradiance (850 PPFD). Samples were collected at the indicated time points. Superoxide anion ($O_2^{\bullet-}$) content was determined using a colorimetric nitro blue tertrazolium (NBT) assay as described previously [82]. Hydrogen peroxide (H_2O_2), was detected with diaminobenzidine tetrahydrochloride (DAB) and quantified by counting pixels on scanned images using ImageJ software [83]. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA) [84].

Transient expression of STANN1_GFP in *Nicotiana benthamiana*

The *STANN1* sequence (without the stop codon) was introduced between NcoI and BcuI restriction sites at the 5′-end of the monomeric GFP (mGFP) coding sequence in pCAMBIA1302. Intact *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 transformed with empty pCAMBIA1302 expressing mGFP or pCAMBIA1302 expressing STANN1_mGFP as described previously [85]. After 3 days, 1 cm diameter leaf discs were excised and incubated with 50 μM MeV for 1 hour in darkness, and then incubated for 4 hours in high light (850 PPFD). Fluorescence was immediately observed using a Nikon Eclipse TE2000-E inverted C1 confocal laser scanning microscope equipped with a 40× Plan Fluore oil immersion objective (numerical aperture, 1.30). mGFP and chloroplast autofluorescence were excited with a solid-state Coherent Sapphire 488 nm

laser and detected using 515/30 band pass and 610 long pass emission filters, respectively. All samples were analyzed in triplicate. Three independent experiments were performed.

Statistical analyses

Data were analyzed using two-way ANOVA with Duncan's Multiple Range Test (DMRT) (for yield) and MANOVA regression models (for other experiments). Multiple comparisons between means were performed with a HSD Tukey test with a confidence limit of 95%.

Results

Identification of potato annexin genes

Genome-wide examination of the potato sequence database for annexins revealed the presence of 11 DNA segments encoding putative proteins displaying substantial similarity to previously characterized plant annexins. Two of these sequences were classified as pseudogenes due to several defects and a lack of continuity in any of the six ORFs. The remaining nine genes were located on chromosomes I, IV, V, and X and each encoded 5–6 exons (Fig. 1A). The positions and phases of introns in the putative potato annexin genes were consistent with those reported for rice annexins [86] (Fig. 1B). The putative annexin sequences in the *S. tuberosum* genome were verified using genomic PCR (Fig. 1C), and the lengths of the amplified genomic products were as expected (Table S1 in Supporting Information file S2Tables). The degree of nucleotide sequence identity between the putative potato annexins was 41–92%. Sequences identified by bioinformatics approaches were confirmed experimentally. Reverse transcription polymerase chain reaction indicated that all nine genes were expressed in different potato organs (data not shown).

Multiple alignment of the putative potato annexin amino acid sequences with Arabidopsis annexins revealed that all but one of the potato annexins had Arabidopsis homologs (data not shown). The newly-identified potato genes were named accordingly as *STANN1*, *STANN2*, *STANN3.1*, *STANN3.2*, *STANN3.3*, *STANN4*, *STANN5*, *STANN8*, and *STANN9* (data not shown). The potato annexins formed a functionally diverse protein family that was differentially expressed in different plant organs (data not shown). The most striking genomic feature of the potato annexin family was triplication of the annexin 3 gene on chromosome 1 (Fig.

1D). In addition, in an arrangement resembling that in Arabidopsis, potato *STANN3.1* and *STANN4* were adjacently localized and divergently transcribed, possibly from a shared promoter. The Inparanoid database groups all annexins in the same in-paralog cluster; however, we suspect that the annexin 3 variants (*STANN3.1*, *STANN3.2*, and *STANN3.3*) are within-species out-paralogs. Two duplications (ancestral gene → *STANN3.1* and the ancestor of *STANN3.2*; then the ancestor of *STANN3.2* → *STANN3.2* and *STANN3.3*) appear to have occurred prior to potato and tomato speciation, as *S. lycopersicum* contains two orthologs of the annexin STANN4 and STANN3.1. In turn, *STANN4* and *STANN3.1–3.3* are out-paralogs, as *STANN4* is moderately related to all *STANN3* variants but shares high sequence similarity with other annexins from *S. lycopersicum* or Arabidopsis. Multiplication of DNA segments within this region of chromosome 1 during *Solanaceae* evolution apparently took place independently at least twice. In tomato chromosome 1, the entire dyad of *SLANN3*/S*LANN4* was duplicated [87] and gave rise to a tetrad located within a short segment of DNA (21,145 bp) that was not interspersed with other genes. This region of chromosome 1 represents a "hot-spot" in the *Solanaceae* family where duplications of a single gene or gene cluster occurred.

Characteristic of potato annexin proteins

Newly identified potato annexins had similar predicted molecular masses of 34–37 kDa and diverse isoelectric points (5.21–9.02). The overall tertiary structures, which were defined by four endonexin domains containing calcium binding sites, were well preserved (Fig. S3 in Supporting Information file S1Figures, Table S3 in Supporting Information file S2Tables). However, the primary amino acid sequences diverged significantly, with the lowest amino acid identity of 20.9% between STANN4 and

STANN5. Groups with higher similarities were identified, such as STANN3.1, STANN3.2, and STANN3.3. Annexins 3.2 and 3.3 were the most closely related with amino acid identities of 90.5% and 70.1% with STANN3.1, respectively. STANN3.2 and 3.3 differed in length (302 and 317 aa, respectively) due to lack of the 14-3-3 like domain on the C-terminus of STANN3.2. Similarly, the N-terminal end of STANN3.2 and STANN3.3, but not STANN3.1, contained a putative myristoylation motif (MG). To date, a myristoylation-mediating membrane localization has been confirmed only for mammalian AnxA13b. With respect to plant annexins, a myristoylation motif was found in poplar annexin EEE95606.1, but the functionality of this motif was not experimentally verified. In summary, despite extensive similarities, there were substantial differences between members of the STANN3 subfamily. This suggested that family might be unique to *Solanaceae*, and that distinct cellular functions evolved for each of the annexins.

The potato annexins contained canonical type II calcium binding sites G-X-GTD-{30-40}D/E solely in the first and occasionally fourth endonexin domains (Fig. S3 in Supporting Information file S1Figures). STANN4 and STANN8 appear to have lost calcium responsiveness as a result of substantial mutations (substitutions and insertions) in these regions. The calcium binding site in the fourth endonexin repeat was probably the only one preserved in STANN5. Tryptophan residues within the first endonexin repeat (G-W-GT) were conserved in potato annexins 1, 2, 8, and 9, but were replaced with phenylalanine (STANN5) or lysine (STANN3.1-3.3 and STANN4) in other annexins (Fig. S3 in Supporting Information file S1Figures). This phenylalanine modification is not predicted to interfere with calcium binding because phenylalanine and tryptophan residues are both hydrophobic and possess aromatic rings. By contrast, the lysine modification may impede membrane translocation of

annexin because introducing a positive charge into the calcium coordination site has the potential to disrupt calcium binding. Other amino acids or motifs important for the plant annexin tertiary structure were preserved in the potato proteins, such as histidine 40 (except in STANN3.2 and STANN4), cysteine 111 (except in STANN4), and cysteine 239 (except in STANN1).

Potato annexin gene expression during drought

To generate drought-tolerant potato, the genes whose products confer drought tolerance have to be identified. Potato annexins are a multigene family; therefore, we characterized the expression of all annexins during drought. Only five annexin genes (*STANN1*, *STANN4*, *STANN5*, *STANN9*, and *STANN2*) were expressed in the leaves of well-watered control WT plants (Fig. 2). At the onset of drought (D0), *STANN1* mRNA was the most abundant transcript (relative to the *EF1a* mRNA). Over time, the level of *STANN1* mRNA increased whereas *STANN4*, *STANN5*, *STANN9*, and *STANN2* mRNA levels remained unchanged. The difference in the accumulation of *STANN1* mRNA from D0 to D14 was statistically significant (Fig. 2). Concurrently, additional annexins were expressed that were not detected under control conditions. The levels of *STANN3.1* and *STANN3.2* mRNA (relative to *EF1a*) increased on D6 and remained elevated until the end of the drought period. The level of *STANN8* mRNA increased continuously during the whole period of water deficit (Fig. 2). However, these induced annexins were expressed at levels at least ten-fold lower than that of annexin 1. This strongly suggested that STANN1 was the key annexin involved in the plant cell response to drought.

Tolerance to soil water deficit

Drought is one of the most devastating environmental stresses in modern agriculture; it reduces global crop yields in developed and developing countries [88]. Continued efforts are required to obtain new crop varieties that assure food security. Annexins were shown to be a promising target in model plants; thus we wanted to verify if they can be used to improve stress tolerance in crop plants. To investigate the effect of STANN1 on drought tolerance in potato transgenic plants overexpressing STANN1 were generated by introducing the STANN1coding sequence under control of the 35S promoter. Transgenic plants displayed normal morphology without any discernible abnormalities and/or growth aberrations under well-watered conditions in growth chamber and in the greenhouse. Leaf turgidity was similar between transgenic and WT plants, which indicated that the leaf water status of WT and transgenic lines was comparable (Fig. 3A, upper panel; Fig. S4A in Supporting Information file S1Figures). During soil water deficit, STANN1 overexpression conferred sustained turgor maintenance, whereas leaf wilting was clearly visible on D8 in WT plants (Fig. 3A, middle panel). In WT the effect of drought was more apparent by D9, and leaves began to shrivel, roll, and curl up. Younger leaves near the top the of the plant were most severely affected (Fig. 3A, lower panel). Leaves of the transgenic lines S-2 and S-7 maintained turgor and did not show signs of dehydration. Rewatering restored leaf turgor and normal growth resumed within 1 day for transgenic plants and 3 days for WT (Fig. 3B). After 2 weeks of drought, S-2 and S-7 leaves were less damaged then those of WT. Experiments were repeated four times in succeeding years, under greenhouse and growth chamber conditions, and in all cases similar results were obtained (Fig. S4 in Supporting Information file S1Figures). The exact number of irreversibly damaged leaves varied between experiments depending on the drought severity (intensity and length). Damage was

consistently significantly lower in transgenic lines than in WT. For example, survival rates after a 3-week drought were 12% and 82% for WT and S-7, respectively.

The ability to preserve turgor in leaves is closely related to drought tolerance. To further characterize drought responses in transgenic plants overexpressing STANN1, RWC changes under water deficit were analyzed. RWC was comparable in WT and transgenic plants. Drought reduced RWC in WT and transgenic plants. However, differences between lines became apparent with increasing drought severity and this became statistically significant at D12 (Fig. 4A). Rewatering after 2 weeks of drought treatment restored control RWC values in WT and transgenic plants. The effect of drought on stomatal conductance (a measure of water and carbon dioxide vapor through the leaf stomata) was apparent by the third day after reducing watering, but the difference between WT and transgenic lines was statistically insignificant (Fig. 4B). Conductance remained low during the whole period of water deficit and only partially returned to control levels on the third day after the resumption of watering.

The real goal for any genetic engineering efforts in crop plants is to improve crop yield. We examined the productivity of transgenic plants under control conditions and under drought. STANN1 overexpression improved plant yield both in terms of the total tuber mass (Fig. S5A in Supporting Information file S1Figures) and consistency of the tuber size (Fig. S5B–C in Supporting Information file S1Figures). The net productivity of well-irrigated WT and transgenic S-7 and S-2 lines was almost identical but tuber quality (size and uniformity) was enhanced in the transgenic lines. A 14-day drought decreased the tuber yield of the WT plants by half, whereas yield loss for S-2 and S-7 lines under comparable conditions was statistically less significant. Tuber quality in the transgenic lines was less impaired

after drought compared with WT. On the basis of these results, we concluded that increasing STANN1 levels is a promising strategy to improve drought tolerance in potato.

Plant photosynthetic activity during drought

We showed that elevation of ATANN1 levels enhanced drought tolerance in potato, but the mechanism of this process was unknown. There is some indication that this could be due to annexin-mediated modulation of redox homeostasis. During drought, ROS accumulation in chloroplasts leads to oxidative damage of photosystems [89, 90]. PSII catalyzes water oxidation and provides electrons for all further photosynthetic reactions; thus, its efficiency is crucial for the entire pathway. The PSII complex is a highly vulnerable structure that is constantly photodamaged; to maintain activity, constant repair and reassembly of reaction centers with newly synthesized proteins is necessary [91]. PSII activity is involved in mediating plant adaptation to environmental conditions. Drought impairs photosynthetic capacity and reduces leaf netto carbon uptake due to increased photorespiration activity (another sink for the absorbed energy) [92]. To directly estimate the effect of STANN1 on drought-induced PSII damage, the photosynthetic performance of PSII in transgenic plants overexpressing STANN1 was characterized under drought conditions. Several physiological parameters related to plant vigor were analyzed to assess the effect of STANN1 overexpression. These included, net photosynthesis (Pnet, associated with plant vitality and biomass production) (Fig. 5A), maximum efficiency of PSII in the dark-adapted state (a measure of the organization and vitality of PSII) (Fig. 5B), and effective quantum yield of PSII in illuminated samples (Fig. 5C). Under control conditions, STANN1 overexpression did not influence any of these parameters. By

contrast, essentially all photosynthetic functions were disturbed during drought, and changes in the two overexpression lines were consistent.

Wild-type Pnet declined to zero by D3 (Fig. 5A). Subsequently, it dropped to negative values by D3 and D10. In the two transgenic plant lines, Pnet remained positive until D10. After rewatering, Pnet increased in all three lines (Fig. 5A). Under control conditions, Fv/Fm values (Fig. 5B) were similar in the all three plant lines (~0.79), and was in the same range as in most investigated plant species. Drought negatively affected Fv/Fm in our experiments; this was observed by D3 in WT, but become apparent in transgenic plants arent on D6. In all three lines, Fv/Fm recovered to baseline within 3 days of rewatering. Measurements were performed on upper non-wrinkled leaves, indicating that apical shoot meristems were not irreversibly damaged by dehydration. In WT and transgenic plants Y(II) (Fig. 5C) declined steadily from the onset of drought, but the reduction appeared on D6 in transgenic plants, and the effect was significantly reduced compared to WT. Y(II) fully recovered in S-2 and S-7;however, even on the third day after soil resaturation, the physiological efficiency of PSII was not restored in WT. This suggested that photorespiration was activated later in STANN1 overexpressing plants then in WT. Thus, PETC was protected for a longer time against irreversible damage and diminished photorespiration-induced H_2O_2 accumulation in cytosol. These results show that PSII impairment in transgenic plants was fully reversible

Photosynthetic pigments content in transgenic plants

Drought activates premature senescence in plants [93] and stimulates catabolism of photosynthetic pigments [94], particularly chlorophyll (Chl) and carotenoids (Car). We determined the photosynthetic pigment contents under drought conditions to better understand the effect of STANN1 on photosynthetic machinery.

Chla and Chlb accumulation

In WT and transgenic lines under well-watered conditions, the total Chl content (11.2±0.01 and 10.6±2.29 mg mL^{-1}, respectively) and the ratio of Chl*a* to Chl*b* were similar. In WT accumulation of Chl*a* (Fig. 6A) and Chl*b* (Fig. 6B) did not change during drought and after rewatering, the level of Chl*a* increased to 180% of the control value at D0. During water deficit in S-7 line, the Chl*a* level was stable; however, Chl*b* levels increased and reached 168% at D14 compared to D0. Consequently, the Chl*a*/*b* ratio rose to 2.0. After rewatering, Chl*a* levels doubled and Chl*b* levels remained stable.

Xanthophyll accumulation

The xanthophyll (XCar) cycle is essential for harmless dissipation of excess excitation energy in PSII as heat (NPQ). The relative XCar abundance in the total Car pool changes during the day depending on the incident light [95]. To exclude diurnal fluctuations, samples were collected at the same time (approximately 4 hours after the start of daily illumination). Under non-stress conditions, STANN1 overexpression did not significantly affect the total Car level, but the XCar content increased [zeaxanthin (Zea), 188%; violaxanthin (Viol), 144%] compared with that in WT plants (Fig. 6C–D). This result indicates that the xanthophyll cycle activity was higher in transgenic plants than in WT plants under the same light conditions. In WT plants, Zea content increased progressively during drought and reached a similar level to that in transgenic S-7 plants only after rewatering [0.35±0.01 pmol/g fresh weight (FW)]. In transgenic S-7 plants, the Zea level remained largely stable and fluctuated in the range of 0.31–0.34 pmol/g FW. Viol declined significantly during drought in both plant lines. The most significant reduction was observed during the

first 6 days of drought, and was more pronounced in S-7 than in WT (57% and 10.5% reduction, respectively). At subsequent time points, the differences between lines disappeared and Viol remained at a stable level after rewatering (0.45±0.01 pmol/g FW in WT and 0.44±0.06 g^{-1} FW in S-7).

Nonphotochemical quenching activity

The observed differences in XCar accumulation prompted us to analyze gross NPQ performance in attached leaves of control, well-watered WT, and transgenic plants (Fig. 7). As expected, NPQ of S-7 and WT differed. Maximal NPQ occurred in S-7 plants after the start of daily illumination, and NPQ amplitude was ~25% higher in S-7 than in WT. The steady-state NPQ level was elevated and saturation was delayed in S-7 compared with those of WT. The *PSBS* (another key NPQ factor) mRNA level during drought was higher in S-7 than in WT plants (Fig. S6A in Supporting Information file S1Figures). These results indicate that the NPQ capacity in transgenic line S-7 was greater than that of WT, which likely conferred better protection of PSII against photooxidative damage. Electrons were redirected to H_2O_2 in transgenic plants and subsequent ROS accumulation was lower than in WT.

Annexin overexpression affects hormonal homeostasis in plants subjected to drought

The drought phenotype of transgenic potato plants overexpressing STANN1resembled that of plants overproducing CK. Compelling evidence indicates that the redox signaling network integrates with phytohormone-activated pathways [96]. ROS are positioned upstream and downstream of at least some hormone-signaling pathways [14].We therefore stress-hormone levels [pro-senescing: ABA

and salicylic acid (SA); anti-senescing: CK] in leaves of WT and S-7 plants subjected to drought. Under well-watered conditions, the level of biologically active ABA in transgenic plants was significantly lower than in WT (Fig. 8A). However, this difference was insignificant by D6 after the initiation of drought This suggested that biosynthesis of ABA in transgenic plants during the first week of drought was more active than that in WT, which is consistent with a more pronounced reduction of Viol (ABA precursor) levels in transgenic plants (Fig. 6D). During the second week of water deficit, only a slight increase in ABA level was observed and maximum levels on D14 were similar WT and S-7 (3.21±264.01 and 3.02±101.59 nmol g^{-1} FW, respectively) (Fig. 8A). As expected, ABA levels declined to control values on resumption of watering.

Under control conditions, annexin overexpression had no significant effect on CK levels (Fig. 8B; Table S4 in Supporting Information file S1Figures). The contents of active and total CK were similar and amounted to 6.35 and 6.90 pmol g^{-1} FW, and 506.34 and 542.08 pmol g^{-1} FW, in WT and S-7 plants, respectively. Drought stress was associated with down-regulation of *trans*-zeatin (tZ), the most physiologically active CK involved in the stimulation of cell division. During early drought stages (RWC ~85%, only minor difference from control conditions), the level of active CK in WT increased, especially compared to the less active isopentenyladenosine (iPR) levels. Active CK declined under severe drought conditions, with the exception of *cis*-zeatin (cZ) and its riboside (cZR), both of which were CK species associated with stress responses. After rewatering, active CK content strongly increased, especially that of *trans*-zeatin (tZ), whereas cZ and cZR levels substantially declined. High levels of active CK (including high levels of cZ) were maintained in S-7 even under severe drought conditions. These levels were

substantially higher than in parental plants. After rewatering, active CK elevation was much more pronounced in S-7 than in WT. The level of storage compounds (CK O-glucosides) was generally low. By contrast, levels of deactivation products (CK N-glucosides) substantially increased during drought, probably as a result of the enhanced deactivation of CK (data not shown).

SA accumulation was reported in response to different abiotic stresses [97]. STANN1 overexpression had no effect on SA levels under well-watered conditions. SA accumulation in WT and S-7 did not change significantly under moderate drought (D6). During the second week of water limitation, the SA level increased in both lines, and SA accumulation in S-7 was approximately twice that in WT (Fig. 8C). The SA level declined in S-7 during recovery, but remained slightly higher than that observed at D0. By contrast, SA continued to increase in WT and exceeded the level observed in S-7. These data indicate that ROS-modulating systems are activated more rapidly and to a higher extent in transgenic plants overexpressing STANN1 than in WT plants.

In summary, genetic modification did not influence ABA synthesis andABA-dependent responses. The elevation in CK metabolism upon rewatering was consistent with phenotypic observations. SA levels in S-7 increased rapidly during drought and peaked by D14 but declined rapidly after rewatering. This suggested that SA-mediated activation of antioxidant systems during drought was faster in STANN1 overexpressing plants. In WT plants, delayed SA-mediated effects such as induction of PCD might be induced.

STANN1 mitigates drought-mediated oxidative stress in cytosol and chloroplasts

Although the experimental plants were grown under constant temperature conditions, heat stress response (HSR) was induced in WT and transgenic plants during drought. In WT plants, water deficit increased the accumulation of chloroplast-specific *HSP100* and cytosol-targeted *HSP40* mRNAs (compared to the EF1a normalization control), which peaked during the second week of drought. In transgenic plants, only *HSP100* expression was induced under water deficit (Fig. S6C–D). This result suggests that STANN1 overexpression mitigates cytosolic oxidative stress.

STANN1 mitigates photooxidative stress induced by MeV

Enhanced stress tolerance frequently reduces plant responsiveness to light [98]. To verify if annexin-mediated drought tolerance influenced light stress responses, we analyzed the effect of the photosensitizer MeV on transgenic plants overexpressing STANN1. MeV induces oxidative stress, which enables studies of oxidative tolerance and stress cross-tolerance in plants [99]. MeV induces an oxidative burst by accepting electrons from PSI and transferring them to molecular oxygen, which results in massive H_2O_2 accumulation in light and generates oxidative stress in chloroplasts.

Leaf disc senescence assay

Leaf discs from WT, S-2 and S-7 plants were exposed to normal light (150 PPFD) in the presence of 10 and 50 μM MeV. The damage caused by MeV was visualized as the degree of leaf tissue bleaching. In the absence of MeV, exposure to light for up to 30 hours had no significant effect on leaf discs. By contrast, exposure to light during MeV treatment induced leaf tissue bleaching, which increased according to MeV concentration (Fig. S7 in Supporting Information file S1Figures). Transgenic plants

S-2 and S-7 had higher tolerance to MeV, and exhibited lower levels of leaf disc bleaching in light.

Quantification of ROS and lipid peroxidation

To further analyze STANN1-mediated protection against light stress, leaf discs from WT and S-7 plants were subjected to the combination of relative excess light (850 PPDF) and 50 μM MeV. The levels of superoxide anion, hydrogen peroxide, and malonyldialdehyde (MDA) were quantified at the indicated time points (Fig. 9).

Exposure of WT to excess light and high MeV concentration induced biphasic accumulation of superoxide anions, with an initial peak at 30 minutes after induction and a second, more substantial and long-lasting, peak beginning at 9 hours after induction. In S-7, an initial increase in superoxide anion level was observed, which was significantly lower than that in WT.. The maximum level of O_2^- was the same in WT and S-7, but the kinetics of the second peak differed (Fig. 9A). In WT, the level of superoxide increased steadily from 6 to12 h after induction. In S-7 superoxide anion accumulation occurred during 6-9 hours after induction, reaching a similar maximal level as in WT at this time point, and the superoxide level then remained unchanged until 12 h after induction.

In WT, light-induced changes in H_2O_2 level were biphasic, with a second higher and sustained peak (Fig. 9B). The first peak occurred within 30 minutes and the second peak occurred by 12 hours after induction. In S-7, the first peak had a similar magnitude to that in WT. After several hours, no further accumulation of H_2O_2 was observed in S-7, and overall levels were significantly lower than in WT.

Lipid peroxidation, measured as an MDA equivalent, was apparent in WT only after 30 minutes and 12 hours. No statistically significant changes in the lipid peroxidation state were observed under high light stress in S-7 (Fig. 9C).

Annexin 1 attenuates cell death and protects chloroplast structure against oxidative stress

In our experiments, the annexin STANN1 attenuated both phases of chloroplast-derived oxidative stress. In transgenic plants overexpressing STANN1, the expression of nucleus-encoded PSII proteins (Fig. S6A–B) and HSPs was modified correspondingly (Fig. S6C–D). A transient mGFP expression assay was performed to confirm that tolerance to photooxidative stress was due to elevated STANN1 levels. In this experiment, STANN1 was produced as an in-frame C-terminal fusion with mGFP. *N. benthamiana* leaf discs were transformed with STANN1_mGFP (experiment) or mGFP (control) constructs. Leaf discs were then subjected to high light or to the combination of high light and MeV as described above. Leaves with similar fluorescent protein expression levels were used for analysis. Exposure to high light alone had no effect on cell structure, regardless of the construct used (mGFP-alone, Fig. 10A–D; STANN1_mGFP, Fig. 10E–H). High light plus MeV induced cytosol condensation and chloroplast damage (as determined by a decline in chloroplast autofluorescence) in mGFP-expressing cells (Fig. 10I–L). Annexin 1 overexpression attenuated both of these effects, and the cell morphology resembled that of control samples (Fig. 10M–P). Chloroplast fluorescence intensity was quantified and, there was no significant difference in mGFP fluorescence between plants transiently expressing mGFP and STANN1_mGFP. The difference in chloroplast autofluorescence (red) between the mGFP and STANN1_mGFP

expressing leaves was statistically significant. This strongly suggests that the chloroplast structure was maintained in the presence of STANN1 protein.

Discussion

This study clearly demonstrate that elevation of endogenous STANN1 expression can be successfully employed to improve potato tolerance to water deficit. Under optimal conditions, genetic modification had no negative effects on plant phenotype, growth, or productivity. Reduction of the photosynthetic rate in response to water deficit is usually attributed to ROS-induced damage of lipids, pigments, and proteins in the photosynthetic apparatus. Overexpressed STANN1 relieved the negative effects of drought stress, such as degradation of photosynthetic pigments, reduction of photosynthetic activity, and loss of productivity. In transgenic plants NPQ was induced more rapidly and had higher capacity in STANN1-overexpressing plants, which contributed to increased tolerance to photooxidative stress. Exposure to MeV reduced ROS accumulation and membrane lipid damage, so STANN1-overexpressing plants were not desensitized to light. Consequently, we assume that maintenance of photosynthesis during water deficit was due to protection against drought-induced oxidative stress and/or modification of redox/hormonal signaling in STANN1-overexpressing plants. We propose that manipulation of annexin expression is a valuable new approach for crop improvement that focuses on delay and/or attenuation of leaf senescence and maintenance of physiological processes when plants are exposed to challenging environmental conditions.

Annexin selection for transgenic experiment

Potato annexins form a multigene family that encodes proteins with similar tertiary structures but different primary amino acid similarities. Despite some extensive similarities, the individual annexins displayed unique expression patterns in the different plant organs (data not shown) and in response to drought. This suggests the

specialization of individual family members towards unique roles in growth/development and adaptation to environmental conditions. Recently it was shown that functional knock-out of annexin 5 (At1g68090) in Arabidopsis was male-sterile due to the abortion of pollen grains before mature pollen stage; however, on the basis of primary amino acid sequence, no specialized functions could be predicted for annexin 5. Detailed investigations revealed that ATANN5 was expressed primarily during microsporogenesis. The observed pollen lethality was due to impairment in pollen development [100, 101]. This suggests that the specialization of individual annexin family members for unique roles in growth/development may be reflected in specific expression patterns. This also may be observed for adaptation to environmental conditions. It will be necessary to test if ectopic expression of any other Arabidopsis annexin under the ATANN5 promoter restores pollen development.

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Expression of four potato annexins was induced during drought. However, STANN1 expression was several-fold higher than other annexins. This strongly suggested that STANN1 was involved primarily in stress responses rather than complementation within the annexin family, at least at the transcriptional level. *STANN1* is expressed in all plant organs (data not shown); therefore the risk of inducing developmental aberrations due to ectopic *STANN1* expression during

development is minimal. Hence, we considered STANN1 as a good candidate to improve drought tolerance in potato and possibly other crops.

STANN1 affects NPQ and mitigates chloroplast-induced oxidative stress in cytosol

Chloroplasts are one of the major stress-induced ROS sources in plant cells. Disruption of chloroplast redox poise permeates throughout the cell and activates secondary ROS sources in other compartments. In mesophyll cells of *Eupatorium adenophorum*, tenuazonic acid (TeA) inhibits electron flow along PSI and PSII and induces H_2O_2 accumulation in chloroplasts within 1 hour. By 4 hours, H_2O_2 spread to the cell walls facing intercellular spaces [102]. We provided evidence that photooxidative stress in potato leaves induced a biphasic oxidative burst, with the first transient peak after 1 hour and the second more significant peak occurring by 12 hours. In STANN1-overexpressing plants, both phases of ROS accumulation were reduced. Biphasic ROS accumulation with a similar kinetics was reported in response to ozone and salt stress treatments [33-35]. In Arabidopsis and tobacco, the first transient ROS peak occurring after O_3 treatment originated in the chloroplast, whereas the second required NADPH oxidase activity and undisturbed functioning of PETC [34].

We assume that the first peak of ROS accumulation during photooxidative stress in potato is due primarily to chloroplast-generated ROS, whereas the second resulted from activation of secondary ROS sources. The question arises how proteins such as annexins that contain no specific signal sequences that target them to chloroplasts can modulate processes inside chloroplasts. Annexins are found occasionally in chloroplast proteomes of some plants (reviewed in [49]). For example, a mustard

(*Sinapis alba* L.) annexin was identified as a component of a multisubunit chloroplast RNA polymerase A complex [103]; however, these results were not confirmed in a subsequent study [104]. Overall, these chloroplast localizations remain exceptions. We believe that annexin-mediated protection of chloroplasts and photosynthesis could be an indirect effect of increased redox homeostasis buffering in the cytosol. In Arabidopsis, the cross-talk between different ROS-scavenging systems in distinct cellular compartments was reported. During light stress, cytosolic antioxidant capacity had an essential role in protecting chloroplasts. Deletion of cytosolic ascorbate peroxidase APX1 induced degradation of thylakoid and stromal/mitochondrial APXs, a cytochrome b6f complex subunit protein, and the small subunit protein of Rubisco [43]. This result is in agreement with observations from other studies [26, 105], which reported that ROS accumulation rapidly reduced chloroplast antioxidant capacity due to APX inhibition.

Manipulation of chloroplast/cytosol antioxidant capacity was successfully used to modulate potato tolerance to adverse conditions. Transgenic potato lines engineered to express cytosolic or chloroplast Cu/Zn-superoxide dismutase (SOD) from tomato displayed enhanced tolerance to MeV [106]. Overexpression of cytosolic Cu/Zn-SOD from *Potentilla atrosanguinea* improved drought stress tolerance and enhanced net photosynthetic rates [107]. Co-expression of Cu/Zn-SOD and APX in chloroplasts enhanced potato tolerance to multiple abiotic stresses, including chilling, high temperature, photooxidative stress, and drought [108]. Accordingly, the lack of chloroplast thioredoxin CDSP32 resulted in greater susceptibility of potato plants to oxidative stress [109]. Taken together, these data show that elevating cytosolic antioxidant capacity is a promising way to enhance stress tolerance in potato. STANN1 overexpression improved drought tolerance and mitigated photooxidative

stress, similarly to that observed for plants with overexpression of ROS-scavenging enzymes. The accumulation of mRNAs coding for cytosolic HSP40 and chloroplast HSP100 was entirely or partially reduced during drought, suggesting that the HSR in transgenic lines developed more slowly and to a lesser extent. This is in agreement with a previous report [78] for *Solanum andigeanum*, in which the expression level of respective *HSP* mRNAs during drought was higher in less tolerant lines than in more resistant landraces.

In summary, chloroplasts are the site of cross-talk between basic metabolic pathways and stress responses, which places them in a key position with respect to coordination of defense responses [110]. This suggests that protection of chloroplasts from ROS-induced damage is of utmost importance.

Cross-talk between redox signaling and phytohormone-mediated pathways in transgenic plants overexpressing STANN1

Cross-talk between ABA, SA, CK, JA and other phytohormone pathways modulates plant development and stress adaptation. Our results showed that increased STANN1 expression modified drought-induced hormone accumulation. We assume that this is an indirect consequence of STANN1-mediated modulation of cellular redox homeostasis. Accumulating data indicate that multi-faceted and multi-level feedback interactions orchestrate hormone- and ROS-mediated signaling networks. Alterations in the cellular redox state were sufficient to modify hormone accumulation and downstream effects [96]. ROS signaling is positioned upstream and downstream of hormone-signaling pathways [13, 14]. Redox cues integrate with the action of

different phytohormones such as ABA and SA in the coordination of plant growth and stress tolerance [111, 112].

Annexin elevation reduced the ABA steady-state level, but drought-induced ABA accumulation had similar kinetics in transgenic and WT plants, and levels similar to those in WT were eventually achieved in STANN1-overexpressing plants. Stress-dependent ABA accumulation typically results from elevated ABA synthesis [113]. ABA biosynthesis is induced by protonation of the thylakoid lumen and accumulation of oxidized ascorbic acid as a result of a reduction in PETC efficiency. Oxidized AA cannot function as a cofactor for violaxanthin de-epoxidase, which deactivates the XCar cycle in favor of ABA biosynthesis [114]. In leaves of ascorbate-deficient *vte1* Arabidopsis plants, the ABA level increased by 60% [115]. In transgenic potato plants overexpressing STANN1, the ABA biosynthetic pathway was fully functional. ABA is a key factor in abiotic stress responses (induces stomatal closing and transcriptional reprograming); therefore, it is of utmost importance that ABA content and ABA-dependent stress signaling pathways are similarly activated in WT and transgenic plants. However, the reduced steady-state ABA level indicated that biosynthetic pathways in STANN1-overexpressing plants under control conditions were directed toward XCar activity at the expense of ABA synthesis.

The control SA levels in transgenic plants were not significantly different than those in WT plants, and were similar to previously reported values for potato cv Desiree [116]. *S. tuberosum* has higher basal SA levels than Arabidopsis, maize, tobacco, or tomato [117]. Increases in SA levels in potato are relatively moderate (e.g., two-fold) after infection with *Phytophthora infestans*, compared with a 20-fold increase in Arabidopsis [118]. There is a lack of data on SA accumulation in potato

leaves during drought, although it has been shown that SA functions as a regulatory signal mediating drought stress responses in several plant species [119, 120]. In our experiments, SA increases during water deficit in both plant lines were similar (6-fold in WT and 5-fold in S-7 over basal level), which was in perfect agreement with observations in *Phillyrea angustifolia* [121]. However, the SA peak was observed in WT plants only after rewatering, whereas in transgenic potato it occurred earlier, even during drought.

Recently, it became clear that SA is an important regulator of photosynthesis. In Arabidopsis, SA influences plant photosynthetic performance, and properly balanced SA levels are necessary for acclimation to changing light [122, 123]. The SA-mediated signaling pathway in Arabidopsis is involved in optimal photosynthetic activity under stress conditions by modulating redox homeostasis [124]. SA enhances the cell antioxidant capacity during drought, although the mechanism of this process is unclear. Endogenous SA deficiency in potato results in ineffective induction of stress defense system and enhances stress sensitivity [116, 125, 126]. During plant response to pathogen infection, SA inhibits the ROS-scavenging enzymes CAT and APX [127-129] and stabilizes H_2O_2 levels. SA and H_2O_2 function as a positive-feedback amplifying loop; if not properly balanced, this loop exerts detrimental effects for cell survival. In rice, reduced endogenous SA levels enhanced H_2O_2 accumulation and the appearance of spontaneous necrotic lesions during senescence and development of oxidative damage and in response to high light intensities [130]. SA accumulation induces different responses depending on the timing and accumulation level; it induces stress-responsive defense systems such as antioxidant enzymes, or induces PCD in response to long-term elevations of SA levels. In WT plants, slow and prolonged SA accumulation despite the resumption watering may

ultimately lead to PCD. Rapid SA accumulation in transgenic plants appears to indicate more efficient mobilization of SA-induced stress responses, and accounts for improved photosynthetic performance.

Recent work shows that CK has an important role in plant adaptation to environmental stresses such as drought, cold, osmotic stress, and light stress [131-134]. In our experiments, CK species and their levels under control conditions were similar to those previously reported for potato cv Desiree [135]. STANN1 overexpression did not influence CK profiles or steady-state levels, but CK levels were maintained during water deficit and rapidly increased after rewatering.

CK antagonizes many ABA-induced physiological responses to drought such as stomatal closure or leaf senescence [136]. Maintaining CK biosynthesis during drought improves stress tolerance, confers protection against photooxidative stress, and mitigates reductions in photosynthesis [38, 83, 137-141]. CK activity is anti-senescent and associated with maintenance of greater antioxidant activity. In creeping bentgrass, elevated CK levels due to senescence-driven expression of isoprenyl transferase (IPT), a key enzyme in CK biosynthesis pathways, conferred drought resistance, increased the levels and activity of scavenging enzymes such as APX and CAT1, and reduced MDA accumulation [142]. Similarly, elevated CK levels in tobacco plant leaves and chloroplasts conferred higher physiological parameters than those in controls [143], and increased APX and dehydroascorbate reductase (DHAR) activity, which prevented over-oxidation of the chloroplastic ascorbate pool. CK regulates stress responses on several levels, such as inducing stress-inducible gene expression [144-145], including peroxidases, GRX, and glutathione S-transferases. Plants with reduced CK levels had lower ROS-scavenging capacity, exhibited more severe photodamage after high-light treatment, and had

reduced neoxanthin and Zea levels under control conditions, which declined further during photooxidative stress. Similar effects were observed in scavenging enzyme activities, and a strong reduction in APX and SOD activities were observed under control conditions and in response to light stress [146]. We assume that sustained biosynthesis of CK during drought in transgenic potato plants overexpressing STANN1 remediates oxidative stress and improves photosynthetic performance.

STANN1 affects redox homeostasis

Different hypotheses have been proposed to explain the molecular basis of annexin-mediated alleviation of oxidative stress, including innate peroxidase activity [54, 147, 148], calcium-induced stabilization of peroxidases activity [149], and modulation of calcium influx [62, 150]. Based on the results of our experiments, we assume that annexin-mediated reduction in oxidative stress in transgenic potato overexpressing STANN1 results from the annexin effect on thiol-disulfide homeostasis.

Downstream transmission of several environmental cues for H_2O_2 accumulation is sensed and mediated by several ROS-neutralizing systems, which are low-molecular-weight antioxidant buffers such as ascorbate (ASC) and glutathione (GSH), and oxidoreductases such as GRX, TRX, and scavenging enzymes [39]. The most prominent ROS-scavenging and redox-signal perception system is GSH accumulation and GSH oxidation to disulfide (GSSG) during ascorbate regeneration in the glutathione-ascorbate cycle [151, 152]. This type of redox imbalance is transduced downstream by reversible formation of a mixed disulfide between GSH and a target protein (*S*-glutathionylation). An increased GSH:GSSG ratio was observed in plants exposed to chilling, heat stress, heavy metals, xenobiotics,

drought, ozone, pathogen [153-159], and during oxidative stress resulting from deficiency in the H_2O_2-scavenging photorespiratory enzymes CAT or APX [36, 47, 160-164]. In maize and rice, the ability to maintain higher GSH:GSSG ratios was associated with greater stress tolerance [164]. GSSG accumulation is a key determinant of cell death and growth arrest [165, 166].

Immunolocalization studies revealed that stress-triggered GSSG accumulation occurred in discrete subcellular compartments. Localization studies in Arabidopsis detected little or no accumulation in mitochondria, slight but significant accumulation in the cytosol, and prominent accumulation in vacuole and chloroplasts [162]. GSSG sequestration in metabolically inert vacuole is thought to initiate catabolism, whereas accumulation in chloroplasts could have functional consequences for photosynthetic efficiency. Increased GSSG level is sufficient to trigger protein *S*-glutathionylation [167], which is thought to regulate enzymatic protein activity [168]. A large number of unidentified targets of this posttranslational modification represent chloroplast proteins (e.g., RuBisCO or glucose-6-phosphate dehydrogenase) [167]. However, it is not clear if GSSG accumulation in chloroplast results from import or *in situ* synthesis. Isolated wheat chloroplasts can take up GSSG from the medium [36]. Re-engineering of compartment-specific glutathione synthesis pathways suggested that cytosol-to-chloroplast GSSG transport also occurs *in vivo* [169]. Specific GSSG transporters have been identified in tonoplast but not in the chloroplast envelope [170].

The presence of redox-sensitive cysteines has been shown for mammalian ANXA2 [171] and ANXA1 [172]. They are located in the extended C- or N -terminal end (Cys324 aa for ANXA1, and Cys8 and Cys334 for ANXA2), which confer structural diversity to proteins from this family. It cannot be easily generalized

if other annexins contain cysteines susceptible to oxidation. ANXA2 was proposed to directly neutralize H_2O_2 with accompanying oxidation of only Cys8 [173-175]; subsequently, it would be reduced via the NADPH-dependent thioredoxin system (NTS) being thus an ultimate acceptor of electrons from NADPH. Oxidative damage in annexin A2-depleted cells enhanced oxidation of the ANXA2-binding proteins actin and transcription factor JunD. This suggests that ANXA2 can function directly as a protein reductase [174].

The presence of reactive cysteines was confirmed in Arabidopsis annexin ATANN1. Implicated amino acids are localized within the endonexin domains and are highly evolutionarily conserved [53] (Fig. S3 in Supporting Information file S1Figures). ATANN1 Cys underwent *in planta S*-nitrosylation within 20 minutes after NO treatment [176] and *S*-glutathionylation within 30 minutes after ABA induction [56]. MeV treatment resulted in oxidation of both cysteines residues of ATANN1 (Cys111 and Cys 239), although the exact type of modification (mixed disulfide bonds with GSH, or intramolecular disulfide bond) has not been defined [177]. The closest ATANN1 homolog in *Brassica rapa*, BRANN1, appears to form a complex with peroxidase in floral buds [178].

STANN1 contains two cysteine residues, Cys17 and Cys111. Among potato annexins, the former is unique for STANN1 and STANN3.2, whereas the latter is homologous to Arabidopsis Cys111. This arrangement resembles that of ANXA1, with reactive Cys8 in the N-terminal amino acid. Elevated STANN1 levels mitigated photooxidative stress and diminished ROS accumulation, which suggested that STANN1 enhanced the capacity of cytosolic antioxidant buffer. Therefore, it appears that the evolutionarily conserved cysteine homologous to ATANN1 Cys239 is not necessary for such activity. Plant annexins can prevent ROS over-accumulation in a similar way to that previously shown for ANXA2: by direct ROS neutralization and further regeneration by NADPH-dependent thioredoxin/glutaredoxin system. Therefore, annexin would

function as an ultimate acceptor of excess electrons leaking from over-reduced PETC. Alternatively, STANN1 may be used as an acceptor for ROS diminishing thus GSSG formation (Fig. 11). Annexins are abundant cytosolic proteins (accounting for up to 2% of the total soluble proteins). They possess redox-sensitive cysteines and could participate significantly in the cellular protein thiol pool. In transgenic plants overexpressing STANN1, annexin levels are higher and the antioxidant protective effect is increased. Reduced GSSG accumulation prevents a decline in the GSH:GSSG ratio and over-oxidation of the cellular environment. The latter mechanism could explain the broad-specificity of annexin-mediated protection, which is functional in bacteria and photosynthetic and non-photosynthetic eukaryotic cells. Glutathione is one of the most abundant non-protein thiols; it is present in cyanobacteria and proteobacteria, and in all mitochondria- or chloroplast-containing eukaryotes [179, 180]. The mechanism of GSH-mediated regulation and maintenance of cellular redox status is similar in all living organisms. Reversible oxidative thiol modifications modulate the function of proteins involved in many different pathways, including gene transcription, translation, protein folding, metabolism, signal transduction, and apoptosis**.**

Conclusions

The results obtained in this study clearly indicate that annexin overexpression has potential application for developing drought-tolerant crops. Enhanced drought tolerance in transgenic potato overexpressing STANN1 confers greater tolerance to high-light stresses, stomatal closure, and diminished CO_2 supply. ROS accumulation was attenuated, which improved chloroplast function; genetically modified plants maintained efficient PSII under stress conditions. Maintenance of a high photosynthetic yield under sub-optimal conditions had a beneficial effect on crop

yields and biomass production. Annexins are a promising target for manipulation of plant tolerance to environmental conditions.

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Figure legends.Figure 1. Annexin genes in potato genome.

(A) Localization of annexin genes on potato chromosomes. The Roman numerals at the top denote the chromosome, digits in brackets indicate chromosome size.

(B) Intron-exon organization of potato annexin genes.

(C) Genomic PCR confirming the presence of predicted annexin genes in WT potato. Specific primers anneal to the 5'- and 3'- ends of coding sequence of certain annexin gene, hence the length of the resulting PCR product is a sum of the respective coding sequence with introns.

(D) Schematic arrangement of *STANN3.1*, *STANN3.2*, *STANN3.3* and *STANN4* on chromosome I.

Figure 2. Profiling of annexin expression in WT potato leaves during drought.

Potato WT plants grew in the walk-in growth chamber under controlled conditions. After 8-10 weeks irrigation was gradually reduced to decrease the field capacity (FC) to 25% (which took approximately 10 days) and then maintained at this level till 14th day. Samples were collected from the first fully developed composite leaf from the top at indicated time points (D0 – beginning of drought, D6 – sixth day of drought, and D14 – fourteenth day of drought). RNA was isolated with Trizol and sq-RT-PCR was performed with primer sets specific for certain annexins. The level of expression was normalized against *EF1a* mRNA. Results are means ±SE ($n \leq 4$). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 3. Drought tolerant phenotype of transgenic plants.

Potato WT plants and transgenic lines (S-2, S-7) was subjected to drought as described above in Fig. 2. (A) Drought stress phenotype of WT (left column), S-2 (middle column) and S-7 (right column) plants. Photographs were taken on the beginning (D0), on eighth (D-8) and ninth (D-9) day of drought. Experiments were repeated twice in greenhouse and twice in growth chamber and gave similar results.

(B) Regeneration of potato plants after prolonged drought. The procedure of drought imposition was the same as described above but the FC was maintained at 25% until the twenty first day of drought (D21). On D22 plants were rewatered and after draining of gravitationally bound water FC was kept up at 65%. Photograph was taken on the third day after rewatering. Left side - two WT plants; middle – two S-2 plants, and right – two S-7 plants. Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.

Figure 4. Examination of leaf water status.

Potato WT plants (white bars) and transgenic lines: S-2 (gray bars) and S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. (A) Relative water content (RWC) analysis. Samples from the first fully developed undamaged leaf from the top of plant were collected at D0, D4, D7, D12 and 3 days after rewatering (RW3) and relative water content (RWC) was determined. Results are means ±SE (n=3). (B) Stomatal conductance were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results.

Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated 3 times and gave comparable results.

Figure 5. Netto photosynthesis and photosynthetic performance of PSII in potato plants during drought.

Potato WT (white bars) and transgenic lines: S-2 (grey bars) and S-7 (black bars) were subjected to drought as described in Fig. 2. (A) Netto photosynthesis, (B) maximum quantum yield of photosystem II (Fv/Fm) and (C) effective quantum yield of photosystem II,Y(II) were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values which are not significantly different at $p<0.05$ and belong to the same homogenic group.

Figure 6. Photosynthetic pigment content during drought.

WT (white bars) and transgenic line S-7 (black bars) were exposed to drought as described in Fig. 2. Samples were collected at the same time during the day at D0, D6, D14 and RW3 from third, fourth and fifth fully expanded leaves from top at 4 hours after turning the light. The level (A) chlorophyll *a*; (B) chlorophyll *b*; (C) zeaxanthine; and (D) violaxanthine were determined Non-polar lipids were separated on an ACQUITY UPLC system (Waters) and peaks were integrated at 436 nm. The level of xanthophylls is expressed as percent of

the total carotenoids. The level of chlorophyll is expressed as mg mL^{-1}. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 7. NPQ assayed in leaf of well-watered potato plants.

Potato WT (dashed line) and transgenic S-7 (solid line) grew in the walk-in growth chamber under controlled conditions and were watered to maintained FC at 65%. Performance of gross non-photochemical quenching (NPQ) were assayed on the first fully developed composite leaf from the top of plant at 4 hours after turning the light with Dual PAM-100. For measurement plants were adapted to dark for 20 minutes and then stimulated with repeated light pulses of actinic light (94 PPFD) for 5 minutes and once again subjected to dark for 6 minutes. Each point represents the mean ±SD (*n*=3-4). Experiment was repeated three times and gave comparable results.

Figure 8. Accumulation of stress-related hormones during drought.

WT (white bars) and transgenic line S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. The level of (A) absicisic acid ABA; (B) sum of active cytokinins, CK; (C) salicylic acid, SA were determined at D0, D6, D14 and RW1. Samples (0.5g of fresh leaf tissue without the midrib) were collected from the first fully developed, undamaged leaf from the top of plant at 4 hours after turning the light. Labeled internal standards were added to the leaf samples before homogenization. Hormones were then extracted, purified using a SPE-C18 column and separated on a reverse phase-cation exchange SPE column. Hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer. The level of ABA and SA is expressed as nmol g^{-1} of fresh weight; the levels of cytokinins – as pmol g^{-1} of fresh weight. Results are means ±SE (n=3). Homogenic

groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 9. Accumulation of ROS (hydrogen peroxide and superoxide anion) and lipid peroxidation.

Potato WT (white bars) and transgenic line S-7 (black bars) grew in walk-in growth chamber under controlled conditions. Leaf discs were expunged from the third, fourth and fifth upper fully expanded leaves and immediately vacuum infiltrated with methyl viologen (50 μM). After 1 hour incubation in dark discs were exposed to high light irradiance (850 PPFD) for indicated times (0.5 – 24 hours). Superoxide anion was determined colorimetrically with nitro blue tetrazolium chloride 9NBT). Hydrogen peroxide was stained in tissue with diaminobenzidine tetrahydrochloride (DAB) and quantified using the ImageJ. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA). Results are means ±SE (n=5). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 10. STANN1 attenuated MeV-induced photooxidative stress.

Confocal laser scanning image of the leaf epidermis of tobacco plant transiently expressing GFP (A-D and I-L) or STANN1_GFP (E-H and M-P). 3 days after infiltration leaf discs were excised and subjected to high light (850 PPFD) (A-H) or the combine treatment of high light (850 PPFD) and 50 μM MeV (G-L). The fluorescence was monitored with Nikon TE-2000E EZ-C1 exc. 488 nm and emission 515/30 and 605/75 for GFP and chloroplast, respectively. First column represent single focal plane, second – chloroplast autofluorescence acquired with the same

excitation parameter for each construction to visualized the difference between responses to the same treatment, third – overlay of green and red fluorescence channels with GFP enhanced to visualized cells; right column – stack obtained with Volume Render program EZ-C1 combined with chloroplasts. Scale bar is 20 μm. Experiment was performed 3 times.

Figure 11. A simplify scheme depicting the interactions between cellular redox state and participation in ROS scavenging mechanisms.

Oxidative stress is an unavoidable consequence of environmental stresses. ROS accumulation begins in chloroplasts and then it spreads throughout the whole cell. Activation of a secondary ROS sources e.g. NADPH oxidase complex or photorespiration resulted in substantial H_2O_2 accumulation in cytosol. To avoid deleterious effects of ROS several compartment-specific mechanisms evolved, including accumulation of low-molecular-weight antioxidants (glutathione, ascorbate), scavenging enzymes (CAT, APX, SOD) and protein thiols (PRX, GRX and TRX) that undergoes a reversible cycles the thiol-disulphide exchange.

The redox-sensitive proteins sense, transduce, and translate ROS signals into appropriate cellular responses. Thus, precise regulation of size and redox status of the thiol pool is of essential importance for induction of appropriate responses. In plant cells glutathione is present in different compartments in milimolar concentrations and in quiescents it maintained largely in reduced state due to activity of glutathione reductases (GR) at expense of NADPH. Stress-induced ROS accumulation stimulates oxidation of glutathione (GSSG) and in the same time *de novo* synthesis of GSH. Disturbances in GSH/GSSG ratio might non-specifically influence several downstream pathways, e.g. by induction of thiol-disulfide exchange

on target proteins. Cellular redox potential depends primarily on the total concentration of the total glutathione and the extend of its oxidation. GSSG accumulation did not disturb the redox potential if it is compensated by increasing the total glutathione concentration. However, if size of total pool remains unchanged when the GSH:GSSG ratio increased the cell redox potential in the cytosol become more positive.

We propose that the improved stress tolerance of annexin STANN1-overexpressing potato plants results from amelioration of oxidative shift of the cytosolic glutathione redox potential. Elevation of STANN1 level had a pleiotropic effect on plant metabolism and physiology what suggested that not one specific but several downstream signaling pathways were touched. Disruption of the glutathione redox potential is sufficient to induce such effect; e.g., in transgenic tobacco with constitutive upregulation of glutathione content MAPK and SA signaling pathways were modified. Annexin posses oxidation-sensitive cysteines and can act as a reductant influencing thus the redox potential. During stress in transgenic plants the capacity of cytosol redox buffer was more reducing compared to WT what prevents oxidation of downstream targets and modulate timing as well as magnitude of stress response. It had a beneficial effect on cell survival, photosynthesis and delay senescence. Similar effects were observed in tobacco and Arabidopsis plants and over-expressing particular elements of antioxidant systems.

Supporting information legends:

File S1Figures.

Figure S1. Construction of transgenic plants.

A) Structure of the T-DNA region from pROK2 carrying STANN1_His6x that was used for *Agrobacterium* -mediated transformation. LB – left border; RB – right border; NPTII – neomycin phosphotransferase II, CaMV – cauliflower mosaic virus 35S promoter; NOS – nopaline synthase terminator;
(B) Expression of STANN1_His6x protein in F1 transgenic potato lines. Proteins were isolated from leaves of WT and F1 transgenic lines S-2, S-3, S-7, S-83, S-91, S-97 and S-123 grown *in vitro*. His-tagged proteins were purified with Ni-NTA agarose, subjected to SDS_PAGE and blotting followed by detection with anti-His primary Ab. The band detected in WT represents *Arabidopsis* annexin ATANN1_His6x (molecular weight *ca* 36 kD) produced in *Escherichia coli* that was added before purification to the ground protein to STANN1_His6x easily dimerized hence the two bands were detected, the lower with molecular weight corresponding to monomer and the upper corresponding to dimer.

Figure S2. Characteristics of experimental drought.

(A) Potato WT and S-7 plants after 8 week of growth at the phase of experimental drought implementation. Transgenesis has no impact on tuber development. Formation of stolon hooks and stolon swelling as well as first tubers are visible.
(B) Field capacity (FC) was normalized at the beginning of experiment and maintained at constant level (app. 65%); for control (well-irrigated plants) FC was maintained at this level throughout the whole experiment. For experimental drought FC was gradually lowered to 20% and kept at this level un till the end of drought. Rewatering was applied by full water saturation of the soil and after gravity draining of excess water FC was kept at the 65% untill the end of experiment.

Figure S3. Multiple alignment of amino acid sequences of putative annexins from potato and selected annexins from human, Arabidopsis and cotton.

The alignment was done with Cobalt (Constrain-based Multiple Alignment Tool). Gene Bank Acc Nos of employed sequences are as follows: human AnxA5 (NP_001145.1), Gossypium hirsutum GHANN1 (1N00), Arabidopsis thaliana ATANN1 (2Q4C) and for potato annexins: STANN1 PGSC0003DMG4000177114, STANN2, STANN3.1 PGSC0003DMG4000221817, STANN3.2 PGSC0003DMG401019427, STANN3.3 PGSC0003DMG402019427, STANN4 PGSC0003DMG400019446, STANN5 PGSC0003DMG400007966, STANN8 PGSC0003DMG400007482 and STANN9 PGSC0003DMG40001879.

The boundaries of endonexin repeats were marked on the basis of crystal structures obtained for GHANN1 (Hofmann et al., 2003) and ATANN1 (Levin et al., 2007) and are, respectively:

- 1st endonexin domain: 14-80 and 13-81;
- 2nd endonexin domain: 83-153 and 84-154;
- 3rd endonexin domain: 164-239 and 165-241
- 4th endonexin domain: 241-309 and 244-3111 respectively for cotton and Arabidopsis annexin.

Conserved histidine 40 is in red; methionine and cysteines from C3 cluster are in blue and underlined.

Calcium binding motifs G-X-GTD-{38-40}-D/E are marked by black boxes; potential N-terminal acylation motif is in bold; potential actin-binding domains IRI are in bold and italic;

C-terminal peptide similar to 14-3-3 proteins is marked by pale-green rectangle.

Amino acid residues of high conservation are shown in red, medium - in blue.

Figure S4. Drought tolerant phenotype of transgenic S-7 potato plants.

Each image depicts two WT plants (left side) and two transgenic S-7 plants (right side) subjected to experimental drought. Drought was started on D0 and lasted 21 days. During that time watering was gradually reduced so as to lower the FC to 20%. After reaching that level it was maintained until 21 days after onset of experiment. The soil was than fully saturated with water (rewatering) and FC was maintained at 65% until the end of experiment.

D10 - irrigation withheld for 10 days, D14 - irrigation withheld for 14 days, D21 - irrigation withheld for 21 days, RW5 – rewatered for 5 days.
Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.
In WT symptoms of wilting clearly appeared after 10 days of drought; in S-7 they were apparent only after 2 weeks. On the 21st day WT were severely affected with damaged stems and dry leaves. At the same time in S-7 plants the upper leaves still maintained turgor. After rewatering only a few leaves in WT regenerated; instead, new shoots developed from below-ground parts after at least a week of regular irrigation. In contrast, the S-7 plants preserved their upper leaves and after rewatering returned to a normal healthy look within hours. The exact number of irreversibly damaged leaves varied between experiments, but it was always significantly lower than in WT.

Figure S5. Potato yield during drought.

(A) Irrigated Water Use Efficiency (IWUE) is a quotient of crop produced per unit per amount of water supplied (IWUE = Y / W [g/pot/mL of water])
(B) An exemplary tuber yield per plant. Potato plants WT, S-2, and S-7 were grown in a greenhouse. After 8-10 weeks of growth plants were subjected to drought stress by restricting irrigation to achieve 20% FC and kept at this level until 14th day. After that time plants were rewatered and cultivated in optimal conditions for additional 10 weeks until physiological maturity. Tubers were lifted immediately after withering of haulms. The weight of all fresh tubers from single plant was determined immediately after harvesting. Experiments were repeated twice and gave similar results.
(C) Quantification of tuber yield experiments. Results are shown as mean ±SD (n=10)

Figure S6. Expression of genes coding for PSII proteins and HSPs.

Relative quantification of *PSBS* (A), *LHCB4* (B) *HSP100* (C) and *HSP40* (D) mRNAs in leaves of WT (white bars) and transgenic S-7 (black bars) potato plants during three-week drought and after rewatering. The data represents the mean ±SE from at least four measurements. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test, the same letters designate

days which are not significantly different at $P < 0.05$ and belong to the same homogenic group.

Figure S7. The effect of photooxidative stress on potato leaves.

Leaf discs (F ~ 1 cm) were excised from leaves of WT or transgenic plants S-2 and S-7 and immediately infiltrated with (A) 50 mM Tris-Cl, pH 7.5 (B)10 mm MeV or (C) 50 mM MeV in 50 mM Tris-Cl, pH 7.5. Subsequently, leaf discs were exposed to light of 150 PPFD for 30 h.

File S2Tables.

Table S1. Primer pairs used for identification of potato annexins.

Primer pairs corresponding to the predicted 5' (F) and 3' (R) ends of the particular annexin genes were designed on the basis of published potato genome sequence. Gene length refers to the total length of exons and introns. Individual primer pairs (F - forward, R - reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA).

Table S2. Primer pairs used for sq-RT-PCR.

Primers for semi-quantitative analysis of expression of annexins and other genes in potato. Individual primer pairs (F- forward, R- reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA) to span intron–exon boundaries to exclude interference from genomic DNA contamination. Amplified fragments were between 300 and 500 base pairs. The genes were selected from PGSC_DM_v3.4_pep_fasta containing database of potato virtual translation products on the basis of their homology to annotated Arabidopsis genes. Analyzed genes were as follows: annexins: STANN1-9; HSP100 (heat shock protein 100 kDa); HSP40 (heat shock protein 40kDa, DNAJ); PSBS (chlorophyll a/b- binding photosystem II 22kD subunit S); LHCB4 (light-harvesting complex binding protein 4). As a reference the housekeeping gene for Elongation Factor a1 (EF1a) was used.

Table S3. Characterization of putative potato annexin proteins.

chlo – chloroplast; cyto – cytoplasm; cyto_ER – cytoplasm/membrane of endoplasmatic reticulum; cysk – cytoskeleton; ER – endoplasmatic reticulum; extr – extracellular; mito – mitochondria; nucl – nucleus; plas – plastids; vacu – vacuole

Table S4. Cytokinins in leaves of WT and S-7 potato plants under drought.

S. tuberosum WT and transgenic S-7 plants were subjected to 2-week drought or well-watered. At time points indicated 0.5 g of tissue (without the main vein) was collected 4 hours after beginning of the day from fully expanded leaves. Hormone levels were analyzed by LC-MS as described in Materials and Methods (n=3). Data are shown as pmol g-1 FW.

Abbreviations: tZR, trans-zeatine riboside; tZ, trans-zeatin; iPR, isopentenyl adenosine riboside; iP, isopentenyl adenine; cZR, cis-zeatin riboside; cZ, cis-zeatin.

Potato Annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic *Solanum tuberosum* L. Plants

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Abbreviations:

ABA	abscisic acid
APX	ascorbate peroxidase
ATANN1	annexin 1 from *Arabidopsis thaliana* L.
ASC	ascorbate
Car	carotenoids
Chl*a*, Chl*b*	chlorophyll *a*, chlorophyll *b*
CK	cytokinins
Fv/Fm	the maximum quantum efficiency of photosynthesis
MeV	methyl viologen, paraquat
NPQ	non-photochemical quenching
PCD	programmed cell death
PETC	photosynthetic electron transport chain
PPFD	photosynthetic photon flux density [μmol m^{-2} s^{-1}]
PSI, PSII	photosystem I, photosystem II
PSBS	22-kD photosystem II subunit
SOD	superoxide dysmutase
STANN1	annexin 1 from *Solanum tuberosum* L.
SA	salicylic acid
Viol	violaxanthin
ViolDE	violaxanthine deepoxidase
XCar	xanthophylls
Y(II)	effective quantum yield of PSII
Zea	zeaxanthin

Abstract

Annexins are a family of calcium- and membrane-binding proteins that are important for plant tolerance to adverse environmental conditions. Annexins function to counteract oxidative stress, maintain cell redox homeostasis, and enhance drought tolerance. In the present study, an endogenous annexin, STANN1, was overexpressed to determine whether crop yields could be improved in potato (*Solanum tuberosum* L.) during drought. Nine potential potato annexins were identified and their expression characterized in response to drought treatment. *STANN1* mRNA was constitutively expressed at a high level and drought treatment strongly increased transcription levels. Therefore, *STANN1* was selected for overexpression analysis. Under drought conditions, transgenic potato plants ectopically expressing STANN1 were more tolerant to water deficit in the root zone, preserved more water in green tissues, maintained chloroplast functions, and had higher accumulation of chlorophyll *b* and xanthophylls (especially zeaxanthine) than wild type (WT). Drought-induced reductions in the maximum efficiency and the electron transport rate of photosystem II (PSII), as well as the quantum yield of photosynthesis, were less pronounced in transgenic plants overexpressing STANN1 than in the WT. This conferred more efficient non-photochemical energy dissipation in the outer antennae of PSII and probably more efficient protection of reaction centers against photooxidative damage in transgenic plants under drought conditions. Consequently, these plants were able to maintain effective photosynthesis during drought, which resulted in greater productivity than WTplants despite water scarcity. Although the mechanisms underlying this stress

protection are not yet clear, annexin-mediated photoprotection is probably linked to protection against light-induced oxidative stress.

Introduction

Plants have developed passive and active strategies to survive environmental stresses such as drought, salinity, chilling, heat shock, heavy metals, UV radiation, ozone, mechanical stress, nutrient deficiency, hypoxia, and biotic stress [1]. Several stress-response genes have already been targets for bioengineering studies to improve plant stress tolerance [2]. However, ectopic expression of stress-inducible genes often results in developmental aberrations (e.g., stunted growth and irregular leaves) or reduced crop yields under non-stress conditions due to non-specific induction of programmed cell death (PCD) and/or premature senescence [3]. Current knowledge of stress-responsive pathways is based primarily on results obtained by imposing each stress individually, whereas plants in natural settings are generally challenged with multiple concurrent stresses, and the resultant signaling pathways may be superimposed and/or induce/antagonize each another [4-6]. New approaches to bioengineering stress tolerance in crop plants are needed to achieve sustainable improvements in crop biomass production [2].

Recent work shows that changes in redox poise can regulate plant cell function [7-9] by acting as cellular signals [10]. In light, the predominant location of ROS production is in chloroplasts [11]. The two main ROS sources there are the light-driven photosynthetic electron transport chains (PETCs) of photosystem I (PSI) and photosystem II (PSII). Environmental stress can trigger an imbalance in redox homeostasis and affect chloroplast metabolism [12]. Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the photosynthetic electron transfer chain (PETC) and utilization of oxygen as an alternative acceptor for excess electrons [13]. Changes in chloroplast redox poise activate secondary ROS-producing sources,

such as membrane NADPH oxidase complex [respiratory burst oxidase homologs (RBOHs)] [14] or photorespiration [15].

Annexins are a multigene, evolutionarily conserved family of calcium- and phospholipid-binding proteins [16] that is present in all eukaryotes. They have a highly conserved tertiary structure defined by the presence in the molecule of four (or eight) approximately 70 amino acid motifs, each consisting of five α-helices. The contribution of annexins to plant cell adaptation to adverse environmental conditions is well documented [16-20]. However, an understanding of the primary physiological functions of plant annexins remains elusive. Initially, based on their prototypical characteristics, annexins were thought to participate primarily in membrane-related events, such as cellular transport, membrane-cytoskeleton interactions, or endo-/exocytosis [16; 20]. Later, it became clear that their cellular functions go far beyond this and include regulation of cellular redox poise and modulation of calcium transients upon stress.

Annexin 1 was identified in a genome-wide search of *Arabidopsis thaliana* (Arabidopsis) sequences capable of rescuing *Escherichia coli* ΔoxyR growth on high H_2O_2 concentrations [21]. Further experiments showed that Arabidopsis annexin 1 (ATANN1) is an element of the ROS signaling network in Arabidopsis. Deletion of functional ATANN1 reduced expression of glutathione-*S*-transferase Tau 1 (GSTU1) in seedlings after H_2O_2 treatment [22]. It is well accepted that upon salinity stress, ROS trigger increases in Ca^{2+}, and ATANN1 was proposed to mediate calcium conductance activated by NADPH oxidases in root epidermal cells [22]. These data suggest that ATANN1 can function at a cross-road of calcium and ROS signaling [23; 24]. It is still an open question if other proteins from this family can function in a similar way.

Subsequent analyses confirmed that not only ATANN1, but also *Brassica juncea* annexins BJANN1 and BJANN3, and *Nelumbo nucifera* annexin NNANN1 ameliorated oxidative stress in homologous or heterologous cells and improved stress tolerance of tobacco, cotton, and Arabidopsis [23; 25-30]. In some cases, overexpression of annexins resulted in multi-stress tolerance. Transgenic tobacco plants expressing BJANN1 exhibited enhanced resistance to different abiotic stresses and infection with *Phytophthora parasitica* var. *nicotianae*, the latter possibly due to constitutively increased expression of several pathogenesis-related proteins [25]. NNANN1 overexpression in Arabidopsis conferred enhanced tolerance to heat and oxidative stress [27]. These studies generally used seedlings or leaf discs subjected to short-term stress treatments (in hours). There is a lack of information regarding annexin function in cell physiology, hormonal homeostasis, and metabolism during long-term exposure to environmental stress.

Potato is one the most important vegetable crops. Its global annual production in 2010 exceeded 300 million tons (FAOStat). Potato plants are highly efficient in terms of water usage (http://www.fao.org/potato-2008/en/potato/water.html), and produce more food per water unit than any other crop [31]. Therefore, potato could be a promising alternative to cereal crops. Modern potato cultivars are susceptible to drought, which is defined as a shortage of water in the root zone [32]. Water deficit affects nearly all stages of potato development, and negatively impacts tuber numbers and quality (crop yield) [33; 34]. Only a few attempts to engineer potato drought tolerance have been reported (reviewed in [35]). These studies had limited success because most transgenic plants did not exhibit good performance and productivity under non-stress and stress conditions. Potato annexin has not been considered for bioengineering applications;

however, new proteomics research showed that STANN1 could be a candidate gene to improve stress tolerance. STANN1 was differentially expressed in potato tubers in response to wounding [36; 37], bruising (personal observation; [38]), osmotic stress and salinity [39], and was differentially expressed in potato aerial parts in response to osmotic stress and salinity [40]. We overexpressed potato annexin STANN1 and observed the effects on plant biochemistry and physiology during drought.

First, we investigated if increased expression of ATANN1 affected potato drought tolerance. We used the *S. tuberosum* genome to identify all potato annexins, and analyzed potential involvement in drought responses using semi-quantitative RT-PCT. Then, we characterized photosynthetic performance in transgenic plants overexpressing ATANN1 during prolonged water deficit around the root zone. One of the plant strategies to cope with environmental stresses is premature induction of the senescence program [41]; therefore, we analyzed the influence of STANN1 on long-lasting changes in hormonal homeostasis during drought. We also investigated possible annexin functions in modulating redox signaling, and assessed changes in drought stress responses. Our working hypothesis was that annexin modulated plant stress responses by increasing the cytosolic antioxidant buffering capacity in transgenic plants. Studies on Arabidopsis ecotypes indicate that *ATANN1* mRNA levels differ in ecotypes adapted to very different local climatic condition (TAIR and our non-published data). In potato tubers, STANN1 levels did not differ in proteomes from different genetic backgrounds [42]. Further experiments are necessary to elucidate if drought-tolerant potato landraces and cultivars could be generated by enhancing the level of annexin expression.

Materials and Methods

Generation of transgenic plants, transformation and growth conditions

S. tuberosum cultivar Sante (WT), medium-tolerant to drought, was used for transformation experiment (http://www.europotato.org). The *STANN1* cDNA sequence without the stop codon (957 bp; Acc. No. PGSC0003DMG400017714) was fused at the 3′ end to a 6×His-tag sequence and inserted into the XbaI restriction site of pROK2 [43] between cauliflower mosaic virus 35S promoter and nopaline synthase (Nos) terminator sequences (Fig. S1A in Supporting Information file S1 Figures). This construct was used for *Agrobacterium tumefaciens*-mediated transformation of WT potato plants according to a previously published method [44]. Regenerated transgenic plants were transplanted into separate glass tubes filled with 10 mL of Murashige & Skoog solid medium supplemented with 50 μg/mL kanamycin. The presence of the transgene cassette was verified with genomic PCR (data not shown). Expression of recombinant STANN1_6×His protein was confirmed by purification from leaves of WT and F1 transgenic plants (lines S-2, S-3, S-7, S-83, S91, S-97, and S-123) by Ni-NTA chromatography and detection with anti-HisTag primary antibody (Sigma-Aldrich). Recombinant ATANN1_6×His protein produced by bacterial overexpression was used as a positive control (WT protein extract) (Fig. S1B in Supporting Information file S1Figures).

Potato WT plants (*S. tuberosum* cv Sante) or transgenic lines in the "Sante" background (S-2 and S-7) were used for further experiments. Plants were cultivated in a growth chamber (or an air-conditioned greenhouse when indicated) under standard conditions (21±2°C; 16 h/8 h day/night; light intensity 110 to 130 PPFD (photosynthetic photon flux densities); 60–80% relative humidity.

Water stress

S. tuberosum plantlets sprouted from tubers were grown in plastic pots filled with 1 kg of sterilized soil (mixture of peat and sand, pH 5.5; prepared by the Plant Breeding and Acclimatization Institute) for 160–170 days. The field capacity (FC) was determined gravimetrically (g of water per g of soil). Pots were weighed every 2–3 days and the volume of water necessary to maintain the indicated FC was calculated individually for each plant. For well-watered control plants, FC was maintained at 65% (−0.8 MPa) for the whole experiment. Experimental drought was imposed after 8–10 weeks of growth (tuber initiation) (Fig. S2A in Supporting Information file S1Figures). Irrigation was decreased over 10 days to gradually reduce the FC to ~25% FC (−2.0 MPa) and was then maintained at this level until the end of the water deficit period. Irrigation was subsequently resumed with full soil saturation (rewatering). To estimate the impact of drought on potato productivity, plants were cultivated for an additional 11–12 weeks after rewatering (FC 65%) until physiological maturity. An exemplary schedule of FC changes is shown in Fig. S2B (supporting Information file S1Figures). Samples were collected at the beginning of the water deficit period (D0), and (depending on experiment) at different days of drought, i.e. 3rd (D3), 4th (D4), 6th (D6), 10th (D10), and 14th (D14), and at the first (RW1) and third (RW3) days after rewatering.

Identification of potato annexins

Annexins were identified in silico by searching for the endonexin domain (PFAM definition, PF00191, 66 aa) in six translation frames of the heterozygous diploid potato breeding line, *S. tuberosum* L. group Tuberosum RH89-039-16 genome using the HMMSearch program from the HMMER3 package. According to PFAM, >93% of proteins from this family contained at

least three consecutive repeats of the endonexin domain. By searching with a single repeat, the probability of missing a complete protein due to below-threshold partial hits or incorrectly defined intron-exon boundaries was minimized. Only hits with an E-value ≤ 0.001 were considered. To verify the presence and sequence of the predicted annexins in WT potato, genome primer sets were designed that corresponded to the 5′ (F) and 3′ (R) ends of the predicted open reading frames (ORFs, Table S1 in Supporting Information file S2Tables). Expression of putative annexin genes was verified using RT-PCR. Briefly, total RNA was isolated from WT leaves and reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania) with poly(T)$_{12-18}$ primer. Annexins were amplified from cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). PCR products were cloned with pJET Cloning Kit (Thermo Scientific) and their compliance with the predicted sequences was verified.

Semi-quantitative expression of annexins and stress-regulated genes

Gene expression was profiled over 14 days of drought in WT potatoes grown as described above. Samples were taken from the first fully-developed composite leaf at the top of the plant. For each time point, single leaf discs from four independent plants were collected, flash-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated with Trizol (Invitrogen, Scottland). Reverse transcription was performed as described above. Taq DNA Polymerase (Thermo Scientific) was used to amplify specific sequences from cDNA. Genes for semi-quantitative analysis were selected from PGSC_DM_v3.4_pep_fasta, which contains a database of potato virtual translation products predicted according to similarity to annotated Arabidopsis genes. Specific primer sets for expression analysis were designed using

PrimerSelect, Laser Gene10.0 DNASTAR (USA) (Table S2 in Supporting Information file S2Tables). The obtained sq-RT-PCR products were subjected by agarose gel electrophoresis, stained with ethidium bromide, and quantitated by densitometry using MultiGaugeV3.0 (Fuji) software. Expression was normalized with respect to the expression of potato elongation factor 1 alpha mRNA (EF1a; PGSC0003DMT400050664; [45]). Each single experiment included four biological replicates, which were quantitated in three technical replicates. Experiments were repeated three times for each primer set and template.

Relative water content

Relative water content (RWC) was determined as described previously [46] with the slight modification. For full saturation [equivalent to turgor weight (TW)] leaves were incubated in distilled water for 4 h instead of overnight. Experiments were performed three times on at least five biological replicates for each genotype.

Extraction and determination of plant hormones

Leaf samples of ~0.5 g (without the main vein) from 8–10-week plants subjected to drought (as described above) were collected, immediately frozen in liquid nitrogen, and kept at −80°C until use. Samples were taken from the first fully-developed composite leaf at the top of the plant at the indicated time points. Sampling was performed 4 h after the start of daily illumination. Three independent biological replicates were examined. Purification and analysis were performed as described previously [47, 48]. Briefly, leaf samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v) and the following labeled internal standards (10 pmol per sample) were added: $^{2}H_{6}$-ABA, $^{2}H_{5}$-*trans*Z, $^{2}H_{5}$-*trans*ZR, $^{2}H_{5}$-*trans*Z7G, $^{2}H_{5}$-*trans*Z9G, $^{2}H_{5}$-*trans*ZOG, $^{2}H_{5}$-*trans*ZROG, $^{2}H_{5}$-*trans*ZRMP,

$^{2}H_3$-DHZ, $^{2}H_3$-DHZR, $^{2}H_3$-DHZ9G, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, and $^{2}H_6$-iPRMP (Olchemim, Czech Republic). Extracts were purified using a SPE-C18 column (SepPak-C18, Waters), and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction [containing abscisic acid (ABA) and its metabolites] was eluted with methanol and the second fraction (containing CK metabolites)] was eluted with 0.35 M NH_4OH in 70% methanol. Both fractions were separated by HPLC (Ultimate 3000, Dionex) and the hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and net photosynthesis were analyzed with a Portable Handheld Photosynthesis System CID 340 device (CID Bio-Science, Camas, WA, USA) according to the manufacturer's instructions. The maximum quantum efficiency of photosynthesis (Fv/Fm) and the effective quantum yield of PSII [Y(II)] were determined with CID 340 (CID Inc., USA) with a CI-510CF Chl fluorescence module and a CI-310LA light attachment (CID Bio-Science) providing actinic light. Measurements were performed 5 h after turning on the light, if not indicated otherwise, on the upper five fully-expanded unwrinkled leaves. Five plants were analyzed per time point. For maximal fluorescence (Fm) determination, plants were dark-adapted for 30 min (so all PSII reaction centers were closed) and then stimulated with saturating pulses of light (0.8 sec, 3,000 PPFD). The minimal fluorescence (Fo) with all PSII reaction centers opened was measured with modulated light of 0.25 PPFD. Fv was calculated from the equation Fv= Fm-Fo. Y(II) was calculated using the equation Y(II) = (Fms-Fs)/Fms. The maximal fluorescence under light (Fms) was determined by allowing

plants to adapt to light for 20 min and measuring the steady-state of chlorophyll (Chl) fluorescence (Fs). Next, a saturating pulse (0.8 sec, 3,000 PPFD) was applied and Fms was determined.

Gross non-photochemical quenching (NPQ) was estimated with a Dual Pulse Amplitude Modulation device, PAM-100 (Walz, Germany). For a single time point, six composite leaves from three to five control plants were analyzed. NPQ was calculated as (Fm − Fms)/Fms, where Fm represents the fluorescence of a dark-adapted sample and Fms represents a fluorescence of the illuminated sample. Plants were dark-adapted for ~20 min and kinetics were measured after repeated light pulses of 94 PPFD for 300 sec. Leaves were subsequently relaxed in darkness for 240 sec and fluorescence while continuously measuring and recording fluorescence.

Non-polar lipids extraction and carotenoids (Car)/Chl determination

Plant material was collected from 8–10-week-old plants exposed to drought. Samples were collected 4 h after switching on the light at D0, D6, D14, and RW3. One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded composite leaves, and total six discs harvested from two plants were combined as a single sample. Non-polar lipids were extracted at 4°C. Plant material after shredding in cooled mortar was transferred to a 15 mL Pyrex tube. After the addition of 3 mL acetone-methanol (8:2 v/v), the sample was perfused with argon and mixed vigorously by vortexing for 2 min. For the second and third extractions, hexane (9 mL) was added and the sample was again perfused with argon before capping and shaking in a reciprocating shaker (PROMAX 2020, Heidolph, Germany) for 30 min in the dark. After shaking, the sample

was incubated without agitation for 5 min to allow phase separation. The upper hexane phase was collected by aspiration and transferred to a 100 mL Erlenmeyer flask, perfused with argon, capped and stored in the dark in 4°C. In the second extraction stage, 2 mL of propanol was used in addition to hexane, and perfusion, shaking and phase collection were repeated as before. After removal of hexane, the polar phase was centrifuged for 15 min at 4500 rpm. The supernatant was combined with the two hexane phases, perfused with argon, and filtered through a Milipore syringe filter unit Millex-CV13 Filter Unit (0.22 µm). The combined hexan phases were then transferred to room temperature, evaporated to dryness under argon, and dissolved in 1 mL methanol-propanol-hexane 6:1:3 (v/v/v). Dissolved samples were transferred to 2 mL glass vials, perfused with argon, capped, and stored at −80°C.

Non-polar lipids were analyzed by injecting 5 µL of sample extract onto an ACQUITY UPLC HSS T3 1.0×150 mm 1.8 µm column and eluted with a gradient of solvent A [water and methanol (1:9, v/v)] and solvent B [methanol:isopropanol: hexane (2:1:1, v/v/v)], with a total of 210 min to transition from solvent A to B. Separation was monitored in the 300–750 nm range with a photodiode array detector. A single chromatogram at 436 nm was extracted, exported in ASCII format, and used for peak area integration analysis with GRAMS/AI software (Thermo Electro Corp, Finland).

Chl*a* and Chl*b* contents were estimated by recording the absorbance of the aforementioned extract at 663, 652, and 645 nm (Cary 50 Bio UV/VIS spectrophotometer, Varian, Australia) as described previously [49].

ROS levels and lipid peroxidation during high light stress

One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded leaves of potato plants, and for a total of six discs three discs were harvested from each of two plants were combined as a single sample. Immediately after harvesting, samples were vacuum-infiltrated with methyl viologen (MeV) at the indicated concentrations and then incubated in the dark for 1 h under normal irradiance (150 PPFD). Images were obtained after 30 h of incubation.

A similar procedure was used for ROS quantification, with the exception that a single MeV concentration (50 μM) was used and samples were exposed to high irradiance (850 PPFD), well in excess of the levels that saturate photosynthesis in Arabidopsis (high light stress, HL). Samples were collected at the indicated time points. Superoxide anion ($O_2^{\bullet -}$) content was determined using a colorimetric nitro blue tertrazolium (NBT) assay as described previously [50]. Hydrogen peroxide (H_2O_2), was detected with diaminobenzidine tetrahydrochloride (DAB) and quantified by counting pixels on scanned images using ImageJ software [51]. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA) [52].

Transient expression of STANN1_GFP in *Nicotiana benthamiana*

The *STANN1* sequence (without the stop codon) was introduced between NcoI and BcuI restriction sites at the 5′-end of the monomeric GFP (mGFP) coding sequence in pCAMBIA1302. Intact *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 transformed with empty pCAMBIA1302 expressing mGFP or pCAMBIA1302 expressing STANN1_mGFP as described previously [53]. After 3 days, 1 cm diameter leaf discs were excised and incubated with 50 μM MeV for 1 hh in darkness, and then incubated for 4 h in high light (850 PPFD). Fluorescence was immediately observed using a Nikon Eclipse

TE2000-E inverted C1 confocal laser scanning microscope equipped with a 40× Plan Fluore oil immersion objective (numerical aperture, 1.30). mGFP and chloroplast autofluorescence were excited with a solid-state Coherent Sapphire 488 nm laser and detected using 515/30 band pass and 610 long pass emission filters, respectively. All samples were analyzed in triplicate. Three independent experiments were performed.

Statistical analyses

Data were analyzed using two-way ANOVA with Duncan's Multiple Range Test (DMRT) (for yield) and MANOVA regression models (for other experiments). Multiple comparisons between means were performed with a HSD Tukey test with a confidence limit of 95%.

Results

Identification of potato annexin genes

Genome-wide examination of the potato sequence database for annexins revealed the presence of 11 DNA segments encoding putative proteins displaying substantial similarity to previously characterized plant annexins. Two of these sequences were classified as pseudogenes due to several defects and a lack of continuity in any of the six ORFs. The remaining nine genes were located on chromosomes I, IV, V, and X and each encoded 5–6 exons (Fig. 1A). The positions and phases of introns in the putative potato annexin genes were consistent with those reported for rice annexins [54] (Fig. 1B). The putative annexin sequences in the *S. tuberosum* genome were verified using genomic PCR (Fig. 1C), and the lengths of the amplified genomic products were as expected (Table S1 in Supporting Information file S2Tables). The degree of nucleotide sequence identity between the putative potato annexins was 41–92%. Sequences identified by bioinformatics approaches were confirmed experimentally. Reverse transcription polymerase chain reaction indicated that all nine genes were expressed in different potato organs (data not shown).

Multiple alignment of the putative potato annexin amino acid sequences with Arabidopsis annexins revealed that all but one of the potato annexins had Arabidopsis homologs (data not shown). The newly-identified potato genes were named accordingly as *STANN1*, *STANN2*, *STANN3.1*, *STANN3.2*, *STANN3.3*, *STANN4*, *STANN5*, *STANN8*, and *STANN9* (data not shown). The potato annexins formed a functionally diverse protein family that was differentially expressed in different plant organs (data not shown). The most striking genomic feature of the potato annexin family was triplication

of the annexin 3 gene on chromosome 1 (Fig. 1D). In addition, in an arrangement resembling that in Arabidopsis, potato *STANN3.1* and *STANN4* were adjacently localized and divergently transcribed, possibly from a shared promoter. The Inparanoid database groups all annexins in the same in-paralog cluster; however, we suspect that the annexin 3 variants (*STANN3.1*, *STANN3.2*, and *STANN3.3*) are within-species out-paralogs. Two duplications (ancestral gene → *STANN3.1* and the ancestor of *STANN3.2*; then the ancestor of *STANN3.2* → *STANN3.2* and *STANN3.3*) appear to have occurred prior to potato and tomato speciation, as *S. lycopersicum* contains two orthologs of the annexin STANN4 and STANN3.1. In turn, *STANN4* and *STANN3.1–3.3* are out-paralogs, as *STANN4* is moderately related to all *STANN3* variants but shares high sequence similarity with other annexins from *S. lycopersicum* or Arabidopsis. Multiplication of DNA segments within this region of chromosome 1 during *Solanaceae* evolution apparently took place independently at least twice. In tomato chromosome 1, the entire dyad of *SLANN3*/S*LANN4* was duplicated [55] and gave rise to a tetrad located within a short segment of DNA (21,145 bp) that was not interspersed with other genes. This region of chromosome 1 represents a "hot-spot" in the *Solanaceae* family where duplications of a single gene or gene cluster occurred.

Characteristic of potato annexin proteins

Newly-identified potato annexins had similar predicted molecular masses of 34–37 kDa and diverse isoelectric points (5.21–9.02). The overall tertiary structures, which were defined by four endonexin domains containing calcium binding sites, were well preserved (Fig. S3 in Supporting Information file S1Figures, Table S3 in Supporting Information file S2Tables). However, the primary amino acid sequences diverged

significantly, with the lowest amino acid identity of 20.9% between STANN4 and STANN5. Groups with higher similarities were identified, such as STANN3.1, STANN3.2, and STANN3.3. Annexins 3.2 and 3.3 were the most closely related with amino acid identities of 90.5% and 70.1% with STANN3.1, respectively. STANN3.2 and 3.3 differed in length (302 and 317 aa, respectively) due to lack of the 14-3-3 like domain on the C-terminus of STANN3.2. Similarly, the N-terminal end of STANN3.2 and STANN3.3, but not STANN3.1, contained a putative myristoylation motif (MG). To date, a myristoylation-mediating membrane localization has been confirmed only for mammalian AnxA13b. With respect to plant annexins, a myristoylation motif was found in poplar annexin EEE95606.1, but the functionality of this motif was not experimentally verified. In summary, despite extensive similarities, there were substantial differences between members of the STANN3 subfamily. This suggested that family might be unique to *Solanaceae*, and that distinct cellular functions evolved for each of the annexins.

The potato annexins contained canonical type II calcium binding sites G-X-GTD-{30-40}D/E solely in the first and occasionally fourth endonexin domains (Fig. S3 in Supporting Information file S1Figures). STANN4 and STANN8 appear to have lost calcium responsiveness as a result of substantial mutations (substitutions and insertions) in these regions. The calcium binding site in the fourth endonexin repeat was probably the only one preserved in STANN5. Tryptophan residues within the first endonexin repeat (G-W-GT) were conserved in potato annexins 1, 2, 8, and 9, but were replaced with phenylalanine (STANN5) or lysine (STANN3.1-3.3 and STANN4) in other annexins (Fig. S3 in Supporting Information file S1Figures). This phenylalanine modification is not predicted to interfere with calcium binding because phenylalanine

and tryptophan residues are both hydrophobic and possess aromatic rings. By contrast, the lysine modification may impede membrane translocation of annexin because introducing a positive charge into the calcium coordination site has the potential to disrupt calcium binding. Other amino acids or motifs important for the plant annexin tertiary structure were preserved in the potato proteins, such as histidine 40 (except in STANN3.2 and STANN4), cysteine 111 (except in STANN4), and cysteine 239 (except in STANN1).

Potato annexin gene expression during drought

To generate drought-tolerant potato, the genes whose products confer drought tolerance have to be identified. Potato annexins are a multigene family; therefore, we characterized the expression of all annexins during drought. Only five annexin genes (*STANN1*, *STANN4*, *STANN5*, *STANN9*, and *STANN2*) were expressed in the leaves of well-watered control WT plants (Fig. 2). At the onset of drought (D0), *STANN1* mRNA was the most abundant transcript (relative to the *EF1a* mRNA). Over time, the level of *STANN1* mRNA increased whereas *STANN4*, *STANN5*, *STANN9*, and *STANN2* mRNA levels remained unchanged. The difference in the accumulation of *STANN1* mRNA from D0 to D14 was statistically significant (Fig. 2). Concurrently, additional annexins were expressed that were not detected under control conditions. The levels of *STANN3.1* and *STANN3.2* mRNA (relative to *EF1a*) increased on D6 and remained elevated until the end of the drought period. The level of *STANN8* mRNA increased continuously during the whole period of water deficit (Fig. 2). However, these induced annexins were expressed at levels at least ten-fold lower than that of annexin 1. This

strongly suggested that STANN1 was the key annexin involved in the plant cell response to drought.

Tolerance to soil water deficit

Drought is one of the most devastating environmental stresses in modern agriculture; it reduces global crop yields in developed and developing countries [56]. Continued efforts are required to obtain new crop varieties that assure food security. Annexins were shown to be a promising target in model plants; thus we wanted to verify if they can be used to improve stress tolerance in crop plants. To investigate the effect of STANN1 on drought tolerance in potato transgenic plants overexpressing STANN1 were generated by introducing the STANN1coding sequence under control of the 35S promoter. Transgenic plants displayed normal morphology without any discernible abnormalities and/or growth aberrations under well-watered conditions in growth chamber and in the greenhouse. Leaf turgidity was similar between transgenic and WT plants, which indicated that the leaf water status of WT and transgenic lines was comparable (Fig. 3A, upper panel; Fig. S4A in Supporting Information file S1Figures). During soil water deficit, STANN1 overexpression conferred sustained turgor maintenance, whereas leaf wilting was clearly visible on D8 in WT plants (Fig. 3A, middle panel). In WT the effect of drought was more apparent by D9, and leaves began to shrivel, roll, and curl up. Younger leaves near the top the of the plant were most severely affected (Fig. 3A, lower panel). Leaves of the transgenic lines S-2 and S-7 maintained turgor and did not show signs of dehydration. Rewatering restored leaf turgor and normal growth resumed within 1 day for transgenic plants and 3 days for WT (Fig. 3B). After 2 weeks of drought, S-2 and S-7 leaves were less damaged then those of

WT. Experiments were repeated four times in succeeding years, under greenhouse and growth chamber conditions, and in all cases similar results were obtained (Fig. S4 in Supporting Information file S1Figures). The exact number of irreversibly damaged leaves varied between experiments depending on the drought severity (intensity and length). Damage was consistently significantly lower in transgenic lines than in WT. For example, survival rates after a 3-week drought were 12% and 82% for WT and S-7, respectively.

The ability to preserve turgor in leaves is closely related to drought tolerance. To further characterize drought responses in transgenic plants overexpressing STANN1, RWC changes under water deficit were analyzed. RWC was comparable in WT and transgenic plants. Drought reduced RWC in WT and transgenic plants. However, differences between lines became apparent with increasing drought severity and this became statistically significant at D12 (Fig. 4A). Rewatering after 2 weeks of drought treatment restored control RWC values in WT and transgenic plants. The effect of drought on stomatal conductance (a measure of water and carbon dioxide vapor through the leaf stomata) was apparent by the third day after reducing watering, but the difference between WT and transgenic lines was statistically insignificant (Fig. 4B). Conductance remained low during the whole period of water deficit and only partially returned to control levels on the third day after the resumption of watering.

The real goal for any genetic engineering efforts in crop plants is to improve crop yield. We examined the productivity of transgenic plants under control conditions and under drought. STANN1 overexpression improved plant yield both in terms of the total tuber mass (Fig. S5A in Supporting Information file S1Figures) and consistency of the tuber size (Fig. S5B–C in Supporting Information file S1Figures). The net productivity

of well-irrigated WT and transgenic S-7 and S-2 lines was almost identical but tuber quality (size and uniformity) was enhanced in the transgenic lines. A 14-day drought decreased the tuber yield of the WT plants by half, whereas yield loss for S-2 and S-7 lines under comparable conditions was statistically less significant. Tuber quality in the transgenic lines was less impaired after drought compared with WT. On the basis of these results, we concluded that increasing STANN1 levels is a promising strategy to improve drought tolerance in potato.

Plant photosynthetic activity during drought

We showed that elevation of ATANN1 levels enhanced drought tolerance in potato, but the mechanism of this process was unknown. There is some indication that this could be due to annexin-mediated modulation of redox homeostasis. During drought, ROS accumulation in chloroplasts leads to oxidative damage of photosystems 57; 58]. PSII catalyzes water oxidation and provides electrons for all further photosynthetic reactions; thus, its efficiency is crucial for the entire pathway. Drought impairs photosynthetic capacity and reduces leaf netto carbon uptake due to increased photorespiration activity (another sink for the absorbed energy) [59]. To directly estimate the effect of STANN1 on drought-induced PSII damage, the photosynthetic performance of PSII in transgenic plants overexpressing STANN1 was characterized under drought conditions. Several physiological parameters related to plant vigor were analyzed to assess the effect of STANN1 overexpression. These included, net photosynthesis (Pnet, associated with plant vitality and biomass production) (Fig. 5A), maximum efficiency of PSII in the dark-adapted state (a measure of the organization and vitality of PSII) (Fig. 5B), and effective quantum yield of PSII in illuminated samples (Fig. 5C). Under control

conditions, STANN1 overexpression did not influence any of these parameters. By contrast, essentially all photosynthetic functions were disturbed during drought, and changes in the two overexpression lines were consistent.

WT Pnet declined to zero by D3 (Fig. 5A). Subsequently, it dropped to negative values by D3 and D10. In the two transgenic plant lines, Pnet remained positive until D10. After rewatering, Pnet increased in all three lines (Fig. 5A). Under control conditions, Fv/Fm values (Fig. 5B) were similar in the all three plant lines (~0.79), and was in the same range as in most investigated plant species. Drought negatively affected Fv/Fm in our experiments; this was observed by D3 in WT, but become apparent in transgenic plants arent on D6. In all three lines, Fv/Fm recovered to baseline within 3 days of rewatering. Measurements were performed on upper non-wrinkled leaves, indicating that apical shoot meristems were not irreversibly damaged by dehydration. In WT and transgenic plants Y(II) (Fig. 5C) declined steadily from the onset of drought, but the reduction appeared on D6 in transgenic plants, and the effect was significantly reduced compared to WT. Y(II) fully recovered in S-2 and S-7; however, even on the third day after soil resaturation, the physiological efficiency of PSII was not restored in WT. This suggested that photorespiration was activated later in STANN1 overexpressing plants then in WT. Thus, PETC was protected for a longer time against irreversible damage and diminished photorespiration-induced H_2O_2 accumulation in cytosol. These results show that PSII impairment in transgenic plants was fully reversible

Photosynthetic pigments content in transgenic plants

Drought activates premature senescence in plants [60] and stimulates catabolism of photosynthetic pigments [61], particularly Chl and Car. We determined the

photosynthetic pigment contents under drought conditions to better understand the effect of STANN1 on photosynthetic machinery.

Chla and Chlb accumulation

In WT and transgenic lines under well-watered conditions, the total Chl content (11.2±0.01 and 10.6±2.29 mg mL^{-1}, respectively) and the ratio of Chl*a* to Chl*b* were similar. In WT accumulation of Chl*a* (Fig. 6A) and Chl*b* (Fig. 6B) did not change during drought and after rewatering, the level of Chl*a* increased to 180% of the control value at D0. During water deficit in S-7 line, the Chl*a* level was stable; however, Chl*b* levels increased and reached 168% at D14 compared to D0. Consequently, the Chl*a*/*b* ratio rose to 2.0. After rewatering, Chl*a* levels doubled and Chl*b* levels remained stable.

Xanthophyll (XCar)accumulation

The relative XCarabundance in the total Car pool changes during the day depending on the incident light [62]. To exclude diurnal fluctuations, samples were collected at the same time (approximately 4 h after the start of daily illumination). Under non-stress conditions, STANN1 overexpression did not significantly affect the total Car level, but the XCar content increased [zeaxanthin (Zea), 188%; violaxanthin (Viol), 144%] compared with that in WT plants (Fig. 6C–D). This result indicates that the XCar cycle activity was higher in transgenic plants than in WT plants under the same light conditions. In WT plants, Zea content increased progressively during drought and reached a similar level to that in transgenic S-7 plants only after rewatering [0.35±0.01 pmol/g fresh weight (FW)]. In transgenic S-7 plants, the Zea level remained largely stable and fluctuated in the range of 0.31–0.34 pmol/g FW. Viol declined significantly during drought in both plant lines. The most significant reduction was observed during

the first 6 days of drought, and was more pronounced in S-7 than in WT (57% and 10.5% reduction, respectively). At subsequent time points, the differences between lines disappeared and Viol remained at a stable level after rewatering (0.45±0.01 pmol/g FW in WT and 0.44±0.06 g^{-1} FW in S-7).

NPQ activity

The PSII complex is a highly vulnerable structure that undergoes constant photodamage even under optimal conditions and mediates plant adaptation to the dynamic light environment. NPQ is an effective short-term mechanism that provides protection for PSII against excessive irradiation and allows excess excitation energy to be harmlessly dissipated as heat [63]. For efficient NPQ, XCar cycle effectiveness and the level of PsbS protein are essential. The observed differences in XCar accumulation prompted us to analyze gross NPQ performance in attached leaves of control, well-watered WT, and transgenic plants (Fig. 7). As expected, NPQ of S-7 and WT differed. Maximal NPQ occurred in S-7 plants after the start of daily illumination, and NPQ amplitude was ~25% higher in S-7 than in WT. The steady-state NPQ level was elevated and saturation was delayed in S-7 compared with those of WT. The *PSBS* (another key NPQ factor) mRNA level during drought was higher in S-7 than in WT plants (Fig. S6A in Supporting Information file S1Figures). These results indicate that the NPQ capacity in transgenic line S-7 was greater than that of WT, which likely conferred better protection of PSII against photooxidative damage. While excess absorbed electrons were redirected to H_2O_2 in WT plants, they were more efficiently dissipated as heat in transgenic plants, which prevented subsequent ROS accumulation.

Annexin overexpression affects hormonal homeostasis in plants subjected to drought

The drought phenotype of transgenic potato plants overexpressing STANN1resembled that of plants overproducing cytokinins (CK). Compelling evidence indicates that the redox signaling network integrates with phytohormone-activated pathways [64]. ROS are positioned upstream and downstream of at least some hormone-signaling pathways [65]. We therefore stress-hormone levels [pro-senescing: ABA and salicylic acid (SA); anti-senescing: CK] in leaves of WT and S-7 plants subjected to drought. Under well-watered conditions, the level of biologically active ABA in transgenic plants was significantly lower than in WT (Fig. 8A). However, this difference was insignificant by D6 after the initiation of drought This suggested that biosynthesis of ABA in transgenic plants during the first week of drought was more active than that in WT, which is consistent with a more pronounced reduction of Viol (ABA precursor) levels in transgenic plants (Fig. 6D). During the second week of water deficit, only a slight increase in ABA level was observed and maximum levels on D14 were similar WT and S-7 (3.21±264.01 and 3.02±101.59 nmol g^{-1} FW, respectively) (Fig. 8A). As expected, ABA levels declined to control values on resumption of watering.

Under control conditions, annexin overexpression had no significant effect on CK levels (Fig. 8B; Table S4 in Supporting Information file S1Figures). The contents of active and total CK were similar and amounted to 6.35 and 6.90 pmol g^{-1} FW, and 506.34 and 542.08 pmol g^{-1} FW, in WT and S-7 plants, respectively. Drought stress was associated with down-regulation of *trans*-zeatin (tZ), the most physiologically active CK involved in the stimulation of cell division. During early drought stages (RWC

~85%, only minor difference from control conditions), the level of active CK in WT increased, especially compared to the less active isopentenyladenosine (iPR) levels. Active CK declined under severe drought conditions, with the exception of *cis*-zeatin (cZ) and its riboside (cZR), both of which were CK species associated with stress responses. After rewatering, active CK content strongly increased, especially that of *trans*-zeatin (tZ), whereas cZ and cZR levels substantially declined. High levels of active CK (including high levels of cZ) were maintained in S-7 even under severe drought conditions. These levels were substantially higher than in parental plants. After rewatering, active CK elevation was much more pronounced in S-7 than in WT. The level of storage compounds (CK O-glucosides) was generally low. By contrast, levels of deactivation products (CK N-glucosides) substantially increased during drought, probably as a result of the enhanced deactivation of CK (data not shown).

SA accumulation was reported in response to different abiotic stresses [66]. STANN1 overexpression had no effect on SA levels under well-watered conditions. SA accumulation in WT and S-7 did not change significantly under moderate drought (D6). During the second week of water limitation, the SA level increased in both lines, and SA accumulation in S-7 was approximately twice that in WT (Fig. 8C). The SA level declined in S-7 during recovery, but remained slightly higher than that observed at D0. By contrast, SA continued to increase in WT and exceeded the level observed in S-7. These data indicate that ROS-modulating systems are activated more rapidly and to a higher extent in transgenic plants overexpressing STANN1 than in WT plants.

In summary, genetic modification influence neither ABA synthesis no ABA-dependent responses. The elevation in CK metabolism upon rewatering was consistent with phenotypic observations. SA levels in S-7 increased rapidly during drought and

peaked by D14 but declined rapidly after rewatering. This suggested that SA-mediated activation of antioxidant systems during drought was faster in STANN1 overexpressing plants. In WT plants, delayed SA-mediated effects such as induction of PCD might be induced.

STANN1 mitigates drought-mediated oxidative stress in cytosol and chloroplasts

Although the experimental plants were grown under constant temperature conditions, heat stress response (HSR) was induced in WT and transgenic plants during drought. In WT plants, water deficit increased the accumulation of chloroplast-specific *HSP100* and cytosol-targeted *HSP40* mRNAs (compared to the EF1a normalization control), which peaked during the second week of drought. In transgenic plants, only *HSP100* expression was induced under water deficit (Fig. S6C–D). This result suggests that STANN1 overexpression mitigates cytosolic oxidative stress.

STANN1 mitigates photooxidative stress induced by MeV

Enhanced stress tolerance frequently reduces plant responsiveness to light [67]. The chloroplast antioxidant system is "loosely tailored" to maintain an endogenous ROS pool under control conditions [12], which enables plants to quickly respond to fluctuating light levels. Consequently, significantly improving ROS scavenging enhances protection against sustained stress, but also desensitizes plant light responses and impairs environmental fitness. To verify if annexin-mediated drought tolerance influenced light responses, we analyzed the effect of the photosensitizer MeV on

transgenic plants overexpressing STANN1. MeV induces oxidative stress, which enables studies of oxidative tolerance and stress cross-tolerance in plants [68]. MeV induces an oxidative burst by accepting electrons from PSI and transferring them to molecular oxygen, which results in massive H_2O_2 accumulation in light and generates oxidative stress in chloroplasts.

Leaf disc senescence assay

Leaf discs from WT, S-2 and S-7 plants were exposed to normal light (150 PPFD) in the presence of 10 and 50 μM MeV. The damage caused by MeV was visualized as the degree of leaf tissue bleaching. In the absence of MeV, exposure to light for up to 30 h had no significant effect on leaf discs. By contrast, exposure to light during MeV treatment induced leaf tissue bleaching, which increased according to MeV concentration (Fig. S7 in Supporting Information file S1Figures). Transgenic plants S-2 and S-7 had higher tolerance to MeV, and exhibited lower levels of leaf disc bleaching in light.

Quantification of ROS and lipid peroxidation

To further analyze STANN1-mediated protection against light stress, leaf discs from WT and S-7 plants were subjected to the combination of relative excess light (850 PPDF) and 50 μM MeV. The levels of superoxide anion, hydrogen peroxide, and malonyldialdehyde (MDA) were quantified at the indicated time points (Fig. 9).

Exposure of WT to excess light and high MeV concentration induced biphasic accumulation of superoxide anions, with an initial peak at 30 min after induction and a second, more substantial and long-lasting, peak beginning at 9 h after induction. In S-7, an initial increase in superoxide anion level was observed, which was significantly

lower than that in WT. The maximum level of O_2^- was the same in WT and S-7, but the kinetics of the second peak differed (Fig. 9A). In WT, the level of superoxide increased steadily from 6 to12 h after induction. In S-7 superoxide anion accumulation occurred during 6–9 h after induction, reaching a similar maximal level as in WT at this time point, and the superoxide level then remained unchanged until 12 h after induction.

In WT, light-induced changes in H_2O_2 level were biphasic, with a second higher and sustained peak (Fig. 9B). The first peak occurred within 30 min and the second peak occurred by 12 h after induction. In S-7, the first peak had a similar magnitude to that in WT. After several hours, no further accumulation of H_2O_2 was observed in S-7, and overall levels were significantly lower than in WT.

Lipid peroxidation, measured as an MDA equivalent, was apparent in WT only after 30 min and 12 h. No statistically significant changes in the lipid peroxidation state were observed under high light stress in S-7 (Fig. 9C).

Annexin 1 attenuates cell death and protects chloroplast structure against oxidative stress

In our experiments, the annexin STANN1 attenuated both phases of chloroplast-derived oxidative stress. In transgenic plants overexpressing STANN1, the expression of nucleus-encoded PSII proteins (Fig. S6A–B) and HSPs was modified correspondingly (Fig. S6C–D). A transient mGFP expression assay was performed to confirm that tolerance to photooxidative stress was due to elevated STANN1 levels. In this experiment, STANN1 was produced as an in-frame C-terminal fusion with mGFP. *N. benthamiana* leaf discs were transformed with STANN1_mGFP (experiment) or mGFP (control) constructs. Leaf discs were then subjected to high light or to the combination

of high light and MeV as described above. Leaves with similar fluorescent protein expression levels were used for analysis. Exposure to high light alone had no effect on cell structure, regardless of the construct used (mGFP-alone, Fig. 10A–D; STANN1_mGFP, Fig. 10E–H). High light plus MeV induced cytosol condensation and chloroplast damage (as determined by a decline in chloroplast autofluorescence) in mGFP-expressing cells (Fig. 10I–L). Annexin 1 overexpression attenuated both of these effects, and the cell morphology resembled that of control samples (Fig. 10M–P). Chloroplast fluorescence intensity was quantified and, there was no significant difference in mGFP fluorescence between plants transiently expressing mGFP and STANN1_mGFP. The difference in chloroplast autofluorescence (red) between the mGFP and STANN1_mGFP expressing leaves was statistically significant. This strongly suggests that the chloroplast structure was maintained in the presence of STANN1 protein.

Discussion

This study clearly demonstrates that elevation of endogenous STANN1 expression can be successfully employed to improve potato tolerance to water deficit. Under optimal conditions, genetic modification had no negative effects on plant phenotype, growth, or productivity. Reduction of the photosynthetic rate in response to water deficit is usually attributed to ROS-induced damage of lipids, pigments, and proteins in the photosynthetic apparatus. Overexpressed STANN1 relieved the negative effects of drought stress, such as degradation of photosynthetic pigments, reduction of photosynthetic activity, and loss of productivity. In transgenic plants, NPQ was induced more rapidly and had higher capacity in STANN1-overexpressing plants, which contributed to increased tolerance to photooxidative stress. Exposure to MeV reduced ROS accumulation and membrane lipid damage, so STANN1-overexpressing plants were not desensitized to light. Consequently, we assume that maintenance of photosynthesis during water deficit was due to protection against drought-induced oxidative stress and/or modification of redox/hormonal signaling in STANN1-overexpressing plants. We propose that manipulation of annexin expression is a valuable new approach for crop improvement that focuses on delay and/or attenuation of leaf senescence and maintenance of physiological processes when plants are exposed to challenging environmental conditions.

Annexin selection for transgenic experiment

Potato annexins have similar tertiary structures but display different levels of primary amino acid sequence similarity. Despite some extensive structural similarities, the individual annexins displayed unique expression patterns in the different plant organs

(data not shown) and in response to drought. This suggests that the specialization of individual family members towards unique roles in growth/development or adaptation to environmental conditions. Indeed, recently it was shown that functional knock-out of annexin 5 (At1g68090) in Arabidopsis was male-sterile due to the abortion of pollen grains before mature pollen stage; however, on the basis of primary amino acid sequence, no specialized functions could be predicted for annexin 5. Detailed investigations revealed that ATANN5 is the most abundantly expressed annexin during microsporogenesis [69; 70]. It will be necessary to test if ectopic expression of any other Arabidopsis annexin under the ATANN5 promoter restores pollen development.

Expression of four potato annexins was induced during drought. However, STANN1 expression was several-fold higher than other annexins. This strongly suggested that STANN1 was involved primarily in stress responses and argued against complementation within the annexin family, at least at the transcriptional level. *STANN1* is expressed in all plant organs (data not shown); therefore, the risk of inducing developmental aberrations due to ectopic *STANN1* expression during development is minimal. Hence, we considered STANN1 as a good candidate to improve drought tolerance in potato and possibly other crops.

STANN1 mitigates chloroplast-induced oxidative stress in cytosol

In light, chloroplasts are one of the major stress-induced ROS sources in plant cells [11]. Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the PETC [71]. Under these conditions, oxygen can be utilized instead of $NADP^+$ as an alternative acceptor for excess electrons [13]. Disruption of chloroplast redox poise

permeates throughout the cell and activates secondary ROS sources in other compartments. In mesophyll cells of *Eupatorium adenophorum*, tenuazonic acid (TeA) inhibits electron flow along PSI and PSII and induces H_2O_2 accumulation in chloroplasts within 1 h. By 4 h, H_2O_2 spread to the cell walls facing intercellular spaces [72]. The most prominent secondary ROS source is the membrane NADPH oxidase complex. ABA induces RBOH gene expression in Arabidopsis leaves and guard cells [14; 73], the *Hordeum vulgar*e aleurone layer [74], and *Zea mays* seedlings [75]. Furthermore, NADPH oxidase-mediated ROS accumulation has been reported in ozone-treated Arabidopsis leaves [76; 77] and salt-treated Arabidopsis root tips [78].

We provided evidence that photooxidative stress in potato leaves induced a biphasic oxidative burst, with the first transient peak after 1 h and the second more significant peak occurring by 12 h. In STANN1-overexpressing plants, both phases of ROS accumulation were reduced. Biphasic ROS accumulation with a similar kinetics was reported in response to ozone and salt stress treatments [76-78]. In Arabidopsis and tobacco, the first transient ROS peak occurring after O_3 treatment originated in the chloroplast, whereas the second required NADPH oxidase activity and undisturbed functioning of PETC [77].

The question arises as to how annexins that contain no specific chloroplasts signal sequences can modulate processes inside chloroplasts and attenuate the first peak. Annexins are found occasionally in chloroplast proteomes of some plants (reviewed in [16]). For example, a mustard (*Sinapis alba* L.) annexin was identified as a component of a multisubunit chloroplast RNA polymerase A complex [79]; however, these results were not confirmed in a subsequent study [80]. Overall, these chloroplast localizations remain exceptions. We believe that annexin-mediated protection of chloroplasts and

photosynthesis could be an indirect effect of increased redox homeostasis buffering in the cytosol. We proposed that the first peak of ROS accumulation during photooxidative stress in potato is primarily due to chloroplast-generated ROS, whereas the second peak results from activation of secondary ROS sources, and STANN1 overexpression protects chloroplasts by improving ROS-scavenging systems in the cytosol.

In Arabidopsis, deletion of cytosolic ascorbate peroxidase 1 (APX1) resulted in collapse of chloroplast ROS-scavenging system; this induced degradation of thylakoid and stromal/mitochondrial APXs, a cytochrome b6f complex subunit protein, and the small subunit protein of Rubisco [81]. At the same time, chloroplast APX is one of the very first targets for ROS-mediated inhibition, and ROS accumulation rapidly reduced chloroplast antioxidant capacity [82; 83]. In Arabidopsis and tobacco plants, enhancing the antioxidant capacity of chloroplasts and cytosol has a beneficial effect on photosynthesis and stress tolerance [84], whereas removal of any single antioxidant component reduces photosynthesis and stress tolerance [81; 85-88]. Manipulation of chloroplast/cytosol antioxidant capacity was successfully used to modulate potato tolerance to adverse conditions. Transgenic potato lines engineered to express cytosolic or chloroplast Cu/Zn-superoxide dismutase (Cu/Zn-SOD) from tomato displayed enhanced tolerance to MeV [89]. Overexpression of cytosolic Cu/Zn-SOD from *Potentilla atrosanguinea* improved drought stress tolerance and enhanced net photosynthetic rates [90]. Co-expression of Cu/Zn-SOD and APX in chloroplasts enhanced potato tolerance to multiple abiotic stresses, including chilling, high temperature, photooxidative stress, and drought [91]. Accordingly, the lack of chloroplast thioredoxin CDSP32 resulted in greater susceptibility of potato plants to oxidative stress [92]. Taken together, these data show that elevating cytosolic

antioxidant capacity is a promising way to enhance stress tolerance in potato. STANN1 overexpression improved drought tolerance and mitigated photooxidative stress, similarly to that observed for plants with overexpression of ROS-scavenging enzymes. The accumulation of mRNAs coding for cytosolic HSP40 and chloroplast HSP100 was entirely or partially reduced during drought, suggesting that the HSR in transgenic lines developed more slowly and to a lesser extent. This is in agreement with a previous report [46] for *Solanum andigeanum*, in which the expression level of respective *HSP* mRNAs during drought was higher in less tolerant lines than in more resistant landraces.

In summary, we assume that annexin-mediated protection of chloroplasts from ROS-induced damage is of utmost importance for plant stress resistance and recovery. Chloroplasts host crucial biosynthesis pathways (e.g., of hormones, Car, amino acids, and lipids) and are the site of cross-talk between basic metabolic pathways and stress responses, which places them in a key position with respect to coordination of defense responses [93; 94].

Cross-talk between redox signaling and phytohormone-mediated pathways in transgenic plants overexpressing STANN1

Cross-talk between ABA, SA, CK, JA and other phytohormone pathways modulates plant development and stress adaptation. Our results showed that increased STANN1 expression modified drought-induced hormone accumulation. We assume that this is an indirect consequence of STANN1-mediated modulation of cellular redox homeostasis.

Accumulating data indicate that multi-faceted and multi-level feedback interactions orchestrate hormone- and ROS-mediated signaling networks. Alterations in the cellular redox state were sufficient to modify hormone accumulation and their downstream effects [64]. ROS signaling is positioned upstream and downstream of hormone-signaling pathways [65; 95]. Redox cues integrate with the action of different phytohormones such as ABA and SA in the coordination of plant growth and stress tolerance [95; 96].

The control SA levels in transgenic plants were not significantly different than those in WT plants, and were similar to previously reported values for potato cv Desiree [97]. *S. tuberosum* has higher basal SA levels than Arabidopsis, maize, tobacco, or tomato [98]. Increases in SA levels in potato are relatively moderate (e.g., two-fold) after infection with *Phytophthora infestans*, compared with a 20-fold increase in Arabidopsis [99]. There is a lack of data on SA accumulation in potato leaves during drought, although it has been shown that SA functions as a regulatory signal mediating drought stress responses in several plant species [100; 101]. In our experiments, SA increases during water deficit in both plant lines were similar (6-fold in WT and 5-fold in S-7 over basal level), which was in perfect agreement with observations in *Phillyrea angustifolia* [102]. However, the SA peak was observed in WT plants only after rewatering, whereas in transgenic potato it occurred earlier, even during drought.

Recently, it became clear that SA is an important regulator of photosynthesis. In Arabidopsis, SA influences plant photosynthetic performance, and properly balanced SA levels are necessary for acclimation to changing light [103; 104]. The SA-mediated signaling pathway in Arabidopsis is involved in optimal photosynthetic activity under stress conditions by modulating redox homeostasis [105]. SA enhances the cell

antioxidant capacity during drought, although the mechanism of this process is unclear. Endogenous SA deficiency in potato results in ineffective induction of stress defense system and enhances stress sensitivity [97; 106; 107]. During plant response to pathogen infection, SA inhibits the ROS-scavenging enzymes CAT and APX [108-110] and stabilizes H_2O_2 levels. SA and H_2O_2 function as a positive-feedback amplifying loop; if not properly balanced, this loop exerts detrimental effects for cell survival. In rice, reduced endogenous SA levels enhanced H_2O_2 accumulation and the appearance of spontaneous necrotic lesions during senescence and development of oxidative damage and in response to high light intensities [111]. SA accumulation induces different responses depending on the timing and accumulation level; it induces stress-responsive defense systems such as antioxidant enzymes, or induces PCD in response to long-term elevations of SA levels. In WT plants, slow and prolonged SA accumulation despite the resumption watering may ultimately lead to PCD. Rapid SA accumulation in transgenic plants appears to indicate more efficient mobilization of SA-induced stress responses, and accounts for improved photosynthetic performance.

Recent work shows that CK has an important role in plant adaptation to environmental stresses such as drought, cold, osmotic stress, and light stress [112-115]. In our experiments, CK species and their levels under control conditions were similar to those previously reported for potato cv Desiree [116]. STANN1 overexpression did not influence CK profiles or steady-state levels, but CK levels were maintained during water deficit and rapidly increased after rewatering.

CK antagonizes many ABA-induced physiological responses to drought such as stomatal closure or leaf senescence [117]. Maintaining CK biosynthesis during drought improves stress tolerance, confers protection against photooxidative stress, and

mitigates reductions in photosynthesis [118-123]. CK activity is anti-senescent and associated with maintenance of greater antioxidant activity. In creeping bentgrass, elevated CK levels due to senescence-driven expression of isoprenyl transferase (IPT), a key enzyme in CK biosynthesis pathways, conferred drought resistance, increased the levels and activity of scavenging enzymes such as APX and CAT1, and reduced MDA accumulation [124]. Similarly, elevated CK levels in tobacco plant leaves and chloroplasts conferred higher physiological parameters than those in controls [125], and increased APX and dehydroascorbate reductase (DHAR) activity, which prevented over-oxidation of the chloroplastic ascorbate (ASC) pool. CK regulates stress responses on several levels, such as inducing stress-inducible gene expression [126; 127], including peroxidases, GRX, and glutathione S-transferases (GSTs). Plants with reduced CK levels had lower ROS-scavenging capacity, exhibited more severe photodamage after high light treatment, and had reduced neoxanthin and Zea levels under control conditions, which declined further during photooxidative stress [128]. Similar effects were observed in scavenging enzyme activities, and a strong reduction in APX and SOD activities were observed under control conditions and in response to light stress [128]. We assume that sustained biosynthesis of CK during drought in transgenic potato plants overexpressing STANN1 remediates oxidative stress and improves photosynthetic performance.

STANN1 overexpression affects ABA accumulation and NPQ

ABA is a key factor in abiotic stress responses (induces stomatal closing and transcriptional reprograming); therefore, it is of utmost importance that ABA content and ABA-dependent stress signaling pathways in WT and transgenic plants are

similarly activated. The ABA biosynthetic pathway is a side-branch of the Car biosynthetic pathway, with Viol being a direct precursor [129]. Viol is synthesized from Zea by zeaxanthin epoxidase, which is constitutively active in darkness and sub-saturating light; hence, under such conditions, the level of Viol far exceeds the level of its precursor. Instead, under saturating light (when the proton gradient produced is too high to be entirely consumed for CO_2 assimilation), the level of Zea increases as a consequence of violaxanthin deepoxidase (ViolDE) activation, which requires reduced ASC as an electron donor. Hence, the actual level of Zea is determined mainly by the processivity of ViolDE [130]. Elevation of the annexin level resulted in reduced the ABA steady-state level and concomitantly increased the relative content of photoprotective Zea and Viol, which suggests that the ABA synthetic pathway was disabled. However, drought-induced ABA accumulation had similar kinetics, and similar maximal ABA levels were eventually achieved in transgenic and WT plants.

Both in control conditions and during drought, the levels of Viol and Zea were higher in STANN1-overexpressing plants than in WT plants. Partitioning of Viol into competing biosynthetic pathways (reconversion to Zea or ABA biosynthesis) depends on the chloroplast ASC status. The accessibility of reduced ASC promotes ViolDE activity resulting in Zea accumulation, whereas depletion of reduced ASC activates ABA biosynthesis. In leaves of ASC-deficient *vtc1* Arabidopsis plants, the ABA level is increased by 60% [131]. The Arabidopsis mutant *npq1* with no functional ViolDE does not accumulate Zea in HL. This is accompanied by increased photodamage of photosynthetic apparatus (reduction in CO_2 assimilation and elevated lipid peroxidation) and strongly inhibited NPQ [132].

Overall, the plant capacity to dissipate excess light energy in a non-photochemical manner is also affected by redox poise. In leaves of two Arabidopsis ASC-deficient lines that over-accumulate ABA, *vtc1* and *vtc2-2*, NPQ at HL is decreased [133; 134]. Regeneration of the ASC pool is maintained mainly by DHAR [135] and suppression of DHAR in Arabidopsis results in lower induction of NPQ, while increased DHAR expression enlarges the size of the XCar pool [135]. For activity, DHAR requires glutathione as an electron donor, and in Arabidopsis *pad2-1* mutants, a shortage of glutathione also impairs NPQ and compromises adaptation to severe drought stress [136]. Similarly, an increased level of another lipid-soluble antioxidant, α-tocopherol, restores the control level of NPQ in the Arabidopsis *npq1* mutant lacking Zea [137]. Over-accumulation of α-tocopherol in the *Chlamydomonas reinhardtii* double mutant *npq1 lor1* (lacking both Zea and lutein) restores tolerance to HL and tolerance to oxidative stress [138]. These results suggest that NPQ is dependent on redox homeostasis, probably due to the effect on the xanthophyll cycle and Zea accumulation. Hence, an enhanced buffering capacity in the cytosol upon overexpression of STANN1 could result in improved NPQ.

STANN1 affects redox homeostasis

Different hypotheses have been proposed to explain the molecular basis of annexin-mediated alleviation of oxidative stress, including innate peroxidase activity [21; 139; 140], calcium-induced stabilization of peroxidases activity [141], and modulation of calcium influx [142; 143]. Based on the results of our experiments, we assume that annexin-mediated reduction in oxidative stress in transgenic potato overexpressing STANN1 results from the annexin effect on thiol-disulfide homeostasis.

Downstream transmission of several environmental cues for H_2O_2 accumulation is sensed and mediated by several ROS-neutralizing systems, which are low-molecular-weight antioxidant buffers such as ASC and glutathione (GSH), and oxidoreductases such as GRX, TRX, and scavenging enzymes [144]. The most prominent ROS-scavenging and redox-signal perception system is GSH accumulation and GSH oxidation to disulfide (GSSG) during ASC regeneration in the glutathione-ASC cycle [145; 146]. This type of redox imbalance is transduced downstream by reversible formation of a mixed disulfide between GSH and a target protein (*S*-glutathionylation). An increased GSH:GSSG ratio was observed in plants exposed to chilling, heat stress, heavy metals, xenobiotics, drought, ozone, pathogen [147-153], and during oxidative stress resulting from deficiency in the H_2O_2-scavenging photorespiratory enzymes CAT or APX [88; 155-158]. In maize and rice, the ability to maintain higher GSH:GSSG ratios was associated with greater stress tolerance [158]. GSSG accumulation is a key determinant of cell death and growth arrest [159; 160].

Immunolocalization studies revealed that stress-triggered GSSG accumulation occurred in discrete subcellular compartments. Localization studies in Arabidopsis detected little or no accumulation in mitochondria, slight but significant accumulation in the cytosol, and prominent accumulation in vacuole and chloroplasts [157]. GSSG sequestration in metabolically inert vacuole is thought to initiate catabolism, whereas accumulation in chloroplasts could have functional consequences for photosynthetic efficiency. Increased GSSG level is sufficient to trigger protein *S*-glutathionylation [160], which is thought to regulate enzymatic protein activity [161]. A large number of unidentified targets of this posttranslational modification represent chloroplast proteins (e.g., RuBisCO or glucose-6-phosphate dehydrogenase) [161]. However, it is not clear

if GSSG accumulation in chloroplast results from import or *in situ* synthesis. Isolated wheat chloroplasts can take up GSSG from the medium [154]. Re-engineering of compartment-specific glutathione synthesis pathways suggested that cytosol-to-chloroplast GSSG transport also occurs *in vivo* [162]. Specific GSSG transporters have been identified in tonoplast but not in the chloroplast envelope [163].

The presence of redox-sensitive cysteines has been shown for mammalian ANXA2 [164] and ANXA1 [165]. They are located in the extended C- or N-terminal end (Cys324 aa for ANXA1, and Cys8 and Cys334 for ANXA2), which confer structural diversity to proteins from this family. It cannot be easily generalized if other annexins contain cysteines susceptible to oxidation. ANXA2 was proposed to directly neutralize H_2O_2 with accompanying oxidation of only Cys8 [166; 167]; subsequently, it would be reduced via the NADPH-dependent thioredoxin system (NTS) being thus an ultimate acceptor of electrons from NADPH. Oxidative damage in annexin A2-depleted cells enhanced oxidation of the ANXA2-binding proteins actin and transcription factor JunD. This suggests that ANXA2 can function directly as a protein reductase [168].

The presence of reactive cysteines was confirmed in Arabidopsis annexin ATANN1. Implicated amino acids are localized within the endonexin domains and are highly evolutionarily conserved [20] (Fig. S3 in Supporting Information file S1Figures). ATANN1 Cys underwent *in planta S*-nitrosylation within 20 min after NO treatment [169] and *S*-glutathionylation within 30 min after ABA induction [23]. MeV treatment resulted in oxidation of both cysteines residues of ATANN1 (Cys111 and Cys 239), although the exact type of modification (mixed disulfide bonds with GSH, or intramolecular disulfide bond) has not been defined [170]. The closest ATANN1 homolog in *Brassica rapa*, BRANN1, appears to form a complex with peroxidase in floral buds [171].

STANN1 contains two cysteine residues, Cys17 and Cys111. Among potato annexins, the former is unique for STANN1 and STANN3.2, whereas the latter is homologous to Arabidopsis Cys111. This arrangement resembles that of ANXA1, with reactive Cys8 in the N-terminal amino acid. Elevated STANN1 levels mitigated photooxidative stress and diminished ROS accumulation, which suggested that STANN1 enhanced the capacity of cytosolic antioxidant buffer. Therefore, it appears that the evolutionarily conserved cysteine homologous to ATANN1 Cys239 is not necessary for such activity. Plant annexins can prevent ROS over-accumulation in a similar way to that previously shown for ANXA2: by direct ROS neutralization and further regeneration by NADPH-dependent thioredoxin/glutaredoxin system. Therefore, annexin would function as an ultimate acceptor of excess electrons leaking from over-reduced PETC. Alternatively, STANN1 may be used as an acceptor for ROS diminishing thus GSSG formation (Fig. 11). Annexins are abundant cytosolic proteins (accounting for up to 2% of the total soluble proteins). They possess redox-sensitive cysteines and could participate significantly in the cellular protein thiol pool. In transgenic plants overexpressing STANN1, annexin levels are higher and the antioxidant protective effect is increased. Reduced GSSG accumulation prevents a decline in the GSH:GSSG ratio and over-oxidation of the cellular environment. The latter mechanism could explain the broad-specificity of annexin-mediated protection, which is functional in bacteria and photosynthetic and non-photosynthetic eukaryotic cells. Glutathione is one of the most abundant non-protein thiols; it is present in cyanobacteria and proteobacteria, and in all mitochondria- or chloroplast-containing eukaryotes [172; 173]. The mechanism of GSH-mediated regulation and maintenance of cellular redox status is similar in all living organisms. Reversible oxidative thiol modifications modulate the function of proteins involved in many different pathways, including gene transcription, translation, protein folding, metabolism, signal transduction, and apoptosis.

Conclusions

The results obtained in this study clearly indicate that annexin overexpression has potential application for developing drought-tolerant crops. Enhanced drought tolerance in transgenic potato overexpressing STANN1 confers greater tolerance to high light stresses, stomatal closure, and diminished CO_2 supply. ROS accumulation was attenuated, which improved chloroplast function; genetically modified plants maintained efficient PSII under stress conditions. Maintenance of a high photosynthetic yield under sub-optimal conditions had a beneficial effect on crop yields and biomass production. Annexins are a promising target for manipulation of plant tolerance to environmental conditions.

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Figure legends.Figure 1. Annexin genes in potato genome.

(A) Localization of annexin genes on potato chromosomes. The Roman numerals at the top denote the chromosome, digits in brackets indicate chromosome size.

(B) Intron-exon organization of potato annexin genes.

(C) Genomic PCR confirming the presence of predicted annexin genes in WT potato. Specific primers anneal to the 5'- and 3'- ends of coding sequence of certain annexin gene, hence the length of the resulting PCR product is a sum of the respective coding sequence with introns.

(D) Schematic arrangement of *STANN3.1*, *STANN3.2*, *STANN3.3* and *STANN4* on chromosome I.

Figure 2. Profiling of annexin expression in WT potato leaves during drought.

Potato WT plants grew in the walk-in growth chamber under controlled conditions. After 8-10 weeks irrigation was gradually reduced to decrease the field capacity (FC) to 25% (which took approximately 10 days) and then maintained at this level till 14th day. Samples were collected from the first fully developed composite leaf from the top at indicated time points (D0 – beginning of drought, D6 – sixth day of drought, and D14 – fourteenth day of drought). RNA was isolated with Trizol and sq-RT-PCR was performed with primer sets specific for certain annexins. The level of expression was normalized against *EF1a* mRNA. Results are means ±SE ($n \leq 4$). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 3. Drought tolerant phenotype of transgenic plants.

Potato WT plants and transgenic lines (S-2, S-7) was subjected to drought as described above in Fig. 2. (A) Drought stress phenotype of WT (left column), S-2 (middle column) and S-7 (right column) plants. Photographs were taken on the beginning (D0), on eighth (D-8) and ninth (D-9) day of drought. Experiments were repeated twice in greenhouse and twice in growth chamber and gave similar results.

(B) Regeneration of potato plants after prolonged drought. The procedure of drought imposition was the same as described above but the FC was maintained at 25% until the twenty first day of drought (D21). On D22 plants were rewatered and after draining of gravitationally bound water FC was kept up at 65%. Photograph was taken on the third day after rewatering. Left side - two WT plants; middle – two S-2 plants, and right – two S-7 plants. Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.

Figure 4. Examination of leaf water status.

Potato WT plants (white bars) and transgenic lines: S-2 (gray bars) and S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. (A) Relative water content (RWC) analysis. Samples from the first fully developed undamaged leaf from the top of plant were collected at D0, D4, D7, D12 and 3 days after rewatering (RW3) and relative water content (RWC) was determined. Results are means ±SE (n=3). (B) Stomatal conductance were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-

310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results.

Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated 3 times and gave comparable results.

Figure 5. Netto photosynthesis and photosynthetic performance of PSII in potato plants during drought.

Potato WT (white bars) and transgenic lines: S-2 (grey bars) and S-7 (black bars) were subjected to drought as described in Fig. 2. (A) Netto photosynthesis, (B) maximum quantum yield of photosystem II (Fv/Fm) and (C) effective quantum yield of photosystem II,Y(II) were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values which are not significantly different at $p<0.05$ and belong to the same homogenic group.

Figure 6. Photosynthetic pigment content during drought.

WT (white bars) and transgenic line S-7 (black bars) were exposed to drought as described in Fig. 2. Samples were collected at the same time during the day at D0, D6, D14 and RW3 from third, fourth and fifth fully expanded leaves from top at 4 hours after turning the light. The level (A) chlorophyll *a*; (B) chlorophyll *b*; (C) zeaxanthine; and (D) violaxanthine were

determined Non-polar lipids were separated on an ACQUITY UPLC system (Waters) and peaks were integrated at 436 nm. The level of xanthophylls is expressed as percent of the total carotenoids. The level of chlorophyll is expressed as mg mL^{-1}. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 7. NPQ assayed in leaf of well-watered potato plants.

Potato WT (dashed line) and transgenic S-7 (solid line) grew in the walk-in growth chamber under controlled conditions and were watered to maintained FC at 65%. Performance of gross non-photochemical quenching (NPQ) were assayed on the first fully developed composite leaf from the top of plant at 4 hours after turning the light with Dual PAM-100. For measurement plants were adapted to dark for 20 minutes and then stimulated with repeated light pulses of actinic light (94 PPFD) for 5 minutes and once again subjected to dark for 6 minutes. Each point represents the mean ±SD (*n*=3-4). Experiment was repeated three times and gave comparable results.

Figure 8. Accumulation of stress-related hormones during drought.

WT (white bars) and transgenic line S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. The level of (A) absicisic acid ABA; (B) sum of active cytokinins, CK; (C) salicylic acid, SA were determined at D0, D6, D14 and RW1. Samples (0.5g of fresh leaf tissue without the midrib) were collected from the first fully developed, undamaged leaf from the top of plant at 4 hours after turning the light. Labeled internal standards were added to the leaf samples before homogenization. Hormones were then extracted, purified using a SPE-C18 column and separated on a reverse phase-cation exchange SPE column. Hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer. The level of

ABA and SA is expressed as nmol g^{-1} of fresh weight; the levels of cytokinins – as pmol g^{-1} of fresh weight. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 9. Accumulation of ROS (hydrogen peroxide and superoxide anion) and lipid peroxidation.

Potato WT (white bars) and transgenic line S-7 (black bars) grew in walk-in growth chamber under controlled conditions. Leaf discs were expunged from the third, fourth and fifth upper fully expanded leaves and immediately vacuum infiltrated with methyl viologen (50 μM). After 1 hour incubation in dark discs were exposed to high light irradiance (850 PPFD) for indicated times (0.5 – 24 hours). Superoxide anion was determined colorimetrically with nitro blue tetrazolium chloride 9NBT). Hydrogen peroxide was stained in tissue with diaminobenzidine tetrahydrochloride (DAB) and quantified using the ImageJ. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA). Results are means ±SE (n=5). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 10. STANN1 attenuated MeV-induced photooxidative stress.

Confocal laser scanning image of the leaf epidermis of tobacco plant transiently expressing GFP (A-D and I-L) or STANN1_GFP (E-H and M-P). 3 days after infiltration leaf discs were excised and subjected to high light (850 PPFD) (A-H) or the combine treatment of high light (850 PPFD) and 50 μM MeV (G-L). The fluorescence was monitored with Nikon TE-2000E EZ-C1 exc. 488 nm and emission 515/30 and

605/75 for GFP and chloroplast, respectively. First column represent single focal plane, second – chloroplast autofluorescence acquired with the same excitation parameter for each construction to visualized the difference between responses to the same treatment, third – overlay of green and red fluorescence channels with GFP enhanced to visualized cells; right column – stack obtained with Volume Render program EZ-C1 combined with chloroplasts. Scale bar is 20 μm. Experiment was performed 3 times.

Figure 11. A simplify scheme depicting the interactions between cellular redox state and participation in ROS scavenging mechanisms.

Oxidative stress is an unavoidable consequence of environmental stresses. ROS accumulation begins in chloroplasts and then it spreads throughout the whole cell. Activation of a secondary ROS sources e.g. NADPH oxidase complex or photorespiration resulted in substantial H_2O_2 accumulation in cytosol. To avoid deleterious effects of ROS several compartment-specific mechanisms evolved, including accumulation of low-molecular-weight antioxidants (glutathione, ascorbate), scavenging enzymes (CAT, APX, SOD) and protein thiols (PRX, GRX and TRX) that undergoes a reversible cycles the thiol-disulphide exchange.

The redox-sensitive proteins sense, transduce, and translate ROS signals into appropriate cellular responses. Thus, precise regulation of size and redox status of the thiol pool is of essential importance for induction of appropriate responses. In plant cells glutathione is present in different compartments in milimolar concentrations and in quiescents it maintained largely in reduced state due to activity of glutathione reductases (GR) at expense of NADPH. Stress-induced ROS accumulation stimulates oxidation of glutathione (GSSG) and in the same time *de novo* synthesis of GSH. Disturbances in

GSH/GSSG ratio might non-specifically influence several downstream pathways, e.g. by induction of thiol-disulfide exchange on target proteins. Cellular redox potential depends primarily on the total concentration of the total glutathione and the extend of its oxidation. GSSG accumulation did not disturb the redox potential if it is compensated by increasing the total glutathione concentration. However, if size of total pool remains unchanged when the GSH:GSSG ratio increased the cell redox potential in the cytosol become more positive.

We propose that the improved stress tolerance of annexin STANN1-overexpressing potato plants results from amelioration of oxidative shift of the cytosolic glutathione redox potential. Elevation of STANN1 level had a pleiotropic effect on plant metabolism and physiology what suggested that not one specific but several downstream signaling pathways were touched. Disruption of the glutathione redox potential is sufficient to induce such effect; e.g., in transgenic tobacco with constitutive upregulation of glutathione content MAPK and SA signaling pathways were modified. Annexin posses oxidation-sensitive cysteines and can act as a reductant influencing thus the redox potential. During stress in transgenic plants the capacity of cytosol redox buffer was more reducing compared to WT what prevents oxidation of downstream targets and modulate timing as well as magnitude of stress response. It had a beneficial effect on cell survival, photosynthesis and delay senescence. Similar effects were observed in tobacco and Arabidopsis plants and over-expressing particular elements of antioxidant systems.

Supporting information legends:

File S1Figures.

Figure S1. Construction of transgenic plants.

A) Structure of the T-DNA region from pROK2 carrying STANN1_His6x that was used for *Agrobacterium* -mediated transformation. LB – left border; RB – right border; NPTII – neomycin phosphotransferase II, CaMV – cauliflower mosaic virus 35S promoter; NOS – nopaline synthase terminator;

(B) Expression of STANN1_His6x protein in F1 transgenic potato lines. Proteins were isolated from leaves of WT and F1 transgenic lines S-2, S-3, S-7, S-83, S-91, S-97 and S-123 grown *in vitro*. His-tagged proteins were purified with Ni-NTA agarose, subjected to SDS_PAGE and blotting followed by detection with anti-His primary Ab. The band detected in WT represents *Arabidopsis* annexin ATANN1_His6x (molecular weight *ca* 36 kD) produced in *Escherichia coli* that was added before purification to the ground protein to STANN1_His6x easily dimerized hence the two bands were detected, the lower with molecular weight corresponding to monomer and the upper corresponding to dimer.

Figure S2. Characteristics of experimental drought.

(A) Potato WT and S-7 plants after 8 week of growth at the phase of experimental drought implementation. Transgenesis has no impact on tuber development. Formation of stolon hooks and stolon swelling as well as first tubers are visible.

(B) Field capacity (FC) was normalized at the beginning of experiment and maintained at constant level (app. 65%); for control (well-irrigated plants) FC was maintained at this level throughout the whole experiment. For experimental drought FC was gradually lowered to 20% and kept at this level un till the end of drought. Rewatering was applied by full water saturation of the soil and after gravity draining of excess water FC was kept at the 65% untill the end of experiment.

Figure S3. Multiple alignment of amino acid sequences of putative annexins from potato and selected annexins from human, Arabidopsis and cotton.

The alignment was done with Cobalt (Constrain-based Multiple Alignment Tool). Gene Bank Acc Nos of employed sequences are as follows: human AnxA5 (NP_001145.1), Gossypium hirsutum GHANN1 (1N00), Arabidopsis thaliana ATANN1 (2Q4C) and for potato annexins: STANN1 PGSC0003DMG4000177114, STANN2, STANN3.1 PGSC0003DMG4000221817, STANN3.2 PGSC0003DMG401019427, STANN3.3 PGSC0003DMG402019427, STANN4 PGSC0003DMG400019446, STANN5 PGSC0003DMG400007966, STANN8 PGSC0003DMG400007482 and STANN9 PGSC0003DMG40001879.

The boundaries of endonexin repeats were marked on the basis of crystal structures obtained for GHANN1 (Hofmann et al., 2003) and ATANN1 (Levin et al., 2007) and are, respectively:

- 1st endonexin domain: 14-80 and 13-81;
- 2nd endonexin domain: 83-153 and 84-154;
- 3rd endonexin domain: 164-239 and 165-241
- 4th endonexin domain: 241-309 and 244-3111 respectively for cotton and Arabidopsis annexin.

Conserved histidine 40 is in red; methionine and cysteines from C3 cluster are in blue and underlined.

Calcium binding motifs G-X-GTD-{38-40}-D/E are marked by black boxes; potential N-terminal acylation motif is in bold; potential actin-binding domains IRI are in bold and italic;

C-terminal peptide similar to 14-3-3 proteins is marked by pale-green rectangle.

Amino acid residues of high conservation are shown in red, medium - in blue.

Figure S4. Drought tolerant phenotype of transgenic S-7 potato plants.

Each image depicts two WT plants (left side) and two transgenic S-7 plants (right side) subjected to experimental drought. Drought was started on D0 and lasted 21 days. During that time watering was gradually reduced so as to lower the FC to 20%. After reaching that level it was maintained until 21 days after onset of experiment. The soil was than fully saturated with water (rewatering) and FC was maintained at 65% until the end of experiment.

D10 - irrigation withheld for 10 days, D14 - irrigation withheld for 14 days, D21 - irrigation withheld for 21 days, RW5 – rewatered for 5 days.
Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.
In WT symptoms of wilting clearly appeared after 10 days of drought; in S-7 they were apparent only after 2 weeks. On the 21st day WT were severely affected with damaged stems and dry leaves. At the same time in S-7 plants the upper leaves still maintained turgor. After rewatering only a few leaves in WT regenerated; instead, new shoots developed from below-ground parts after at least a week of regular irrigation. In contrast, the S-7 plants preserved their upper leaves and after rewatering returned to a normal healthy look within hours. The exact number of irreversibly damaged leaves varied between experiments, but it was always significantly lower than in WT.

Figure S5. Potato yield during drought.

(A) Irrigated Water Use Efficiency (IWUE) is a quotient of crop produced per unit per amount of water supplied (IWUE = Y / W [g/pot/mL of water])
(B) An exemplary tuber yield per plant. Potato plants WT, S-2, and S-7 were grown in a greenhouse. After 8-10 weeks of growth plants were subjected to drought stress by restricting irrigation to achieve 20% FC and kept at this level until 14th day. After that time plants were rewatered and cultivated in optimal conditions for additional 10 weeks until physiological maturity. Tubers were lifted immediately after withering of haulms. The weight of all fresh tubers from single plant was determined immediately after harvesting. Experiments were repeated twice and gave similar results.
(C) Quantification of tuber yield experiments. Results are shown as mean ±SD (n=10)

Figure S6. Expression of genes coding for PSII proteins and HSPs.

Relative quantification of *PSBS* (A), *LHCB4* (B) *HSP100* (C) and *HSP40* (D) mRNAs in leaves of WT (white bars) and transgenic S-7 (black bars) potato plants during three-week drought and after rewatering. The data represents the mean ±SE from at least four measurements. Homogenic groups are determined by Tukey HSD

(Honestly Significant Differences) test, the same letters designate days which are not significantly different at $P < 0.05$ and belong to the same homogenic group.

Figure S7. The effect of photooxidative stress on potato leaves.

Leaf discs (F ~ 1 cm) were excised from leaves of WT or transgenic plants S-2 and S-7 and immediately infiltrated with (A) 50 mM Tris-Cl, pH 7.5 (B)10 mm MeV or (C) 50 mM MeV in 50 mM Tris-Cl, pH 7.5. Subsequently, leaf discs were exposed to light of 150 PPFD for 30 h.

File S2Tables.

Table S1. Primer pairs used for identification of potato annexins.

Primer pairs corresponding to the predicted 5' (F) and 3' (R) ends of the particular annexin genes were designed on the basis of published potato genome sequence. Gene length refers to the total length of exons and introns. Individual primer pairs (F - forward, R - reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA).

Table S2. Primer pairs used for sq-RT-PCR.

Primers for semi-quantitative analysis of expression of annexins and other genes in potato. Individual primer pairs (F- forward, R- reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA) to span intron–exon boundaries to exclude interference from genomic DNA contamination. Amplified fragments were between 300 and 500 base pairs. The genes were selected from PGSC_DM_v3.4_pep_fasta containing database of potato virtual translation products on the basis of their homology to annotated Arabidopsis genes. Analyzed genes were as follows: annexins: STANN1-9; HSP100 (heat shock protein 100 kDa); HSP40 (heat shock protein 40kDa, DNAJ); PSBS (chlorophyll a/b- binding photosystem II 22kD subunit S); LHCB4 (light-harvesting complex binding protein 4). As a reference the housekeeping gene for Elongation Factor a1 (EF1a) was used.

Table S3. Characterization of putative potato annexin proteins.

chlo – chloroplast; cyto – cytoplasm; cyto_ER – cytoplasm/membrane of endoplasmatic reticulum; cysk – cytoskeleton; ER – endoplasmatic reticulum; extr – extracellular; mito – mitochondria; nucl – nucleus; plas – plastids; vacu – vacuole

Table S4. Cytokinins in leaves of WT and S-7 potato plants under drought.

S. tuberosum WT and transgenic S-7 plants were subjected to 2-week drought or well-watered. At time points indicated 0.5 g of tissue (without the main vein) was collected 4 hours after beginning of the day from fully expanded leaves. Hormone levels were analyzed by LC-MS as described in Materials and Methods (n=3). Data are shown as pmol g-1 FW.

Abbreviations: tZR, trans-zeatine riboside; tZ, trans-zeatin; iPR, isopentenyl adenosine riboside; iP, isopentenyl adenine; cZR, cis-zeatin riboside; cZ, cis-zeatin.

Fig. 1
Click here to download Figure: Fig1.tif

Figure 1. Annexin genes in potato genome.

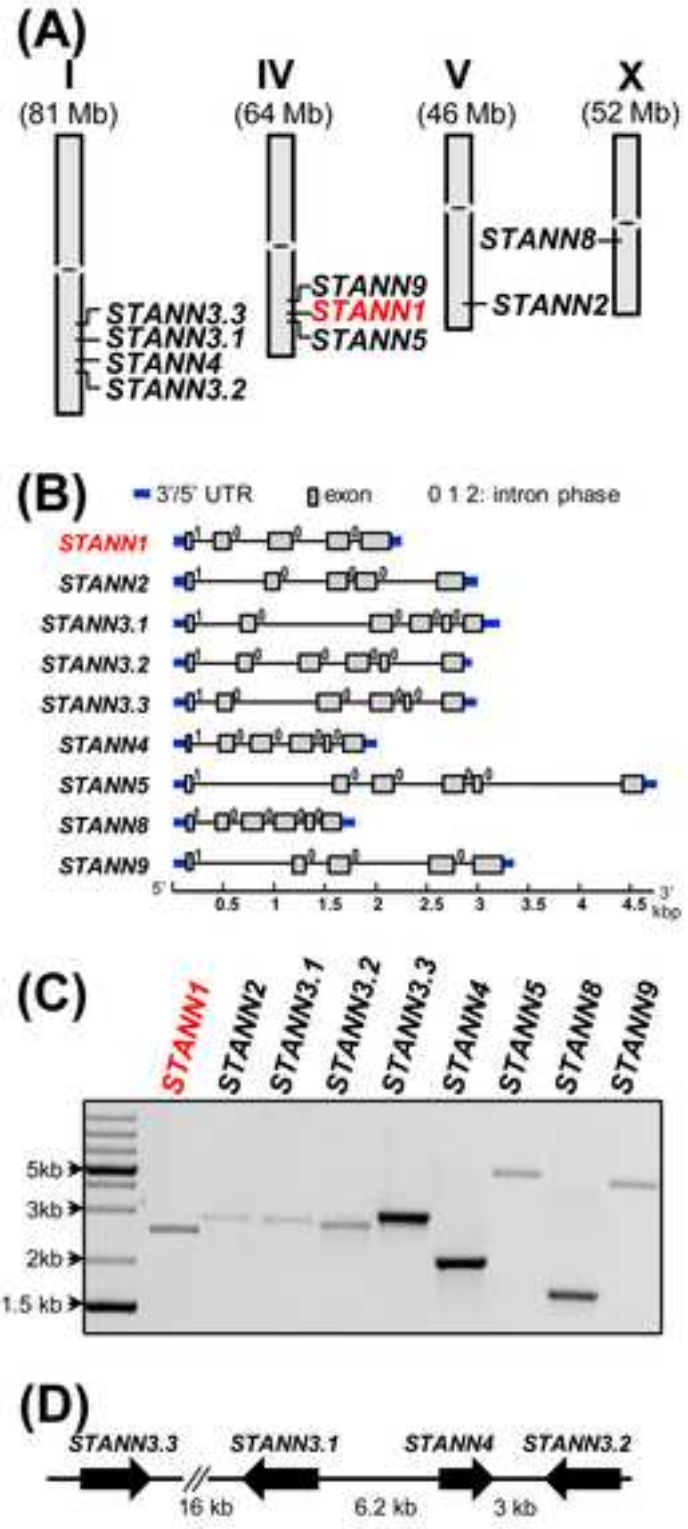

Fig. 2
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Figure 2. Profiling of annexins expression in WT potato leaves during drought.

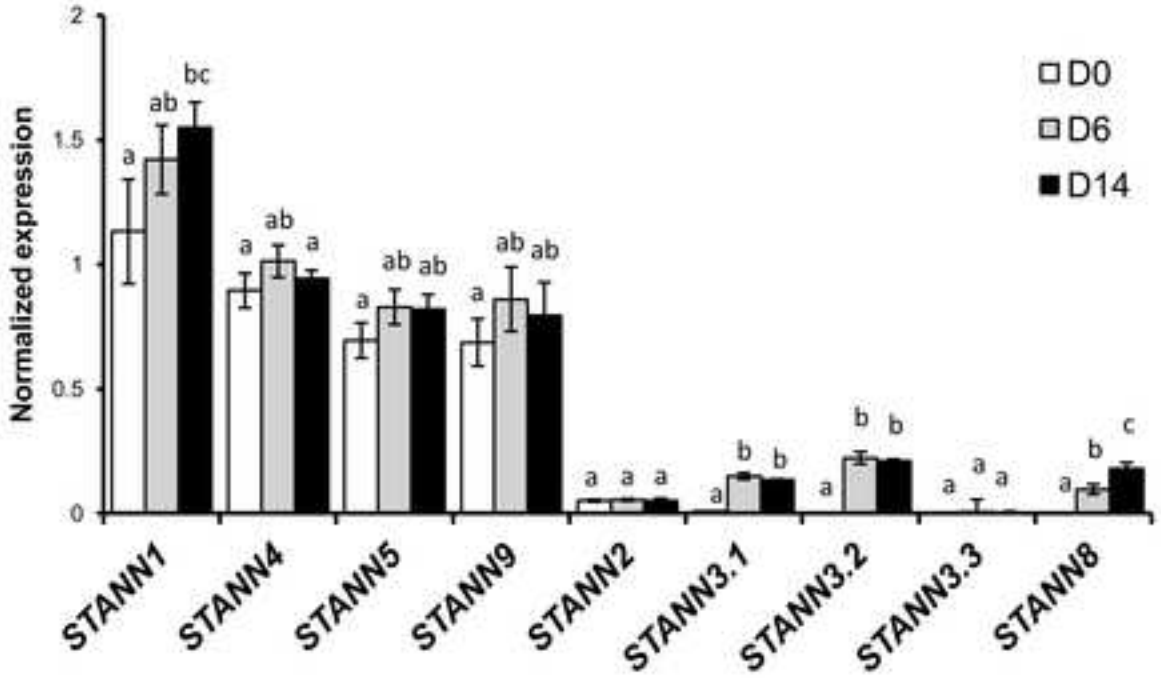

Fig. 3
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Figure 3. Drought tolerant phenotype of transgenic potato plants

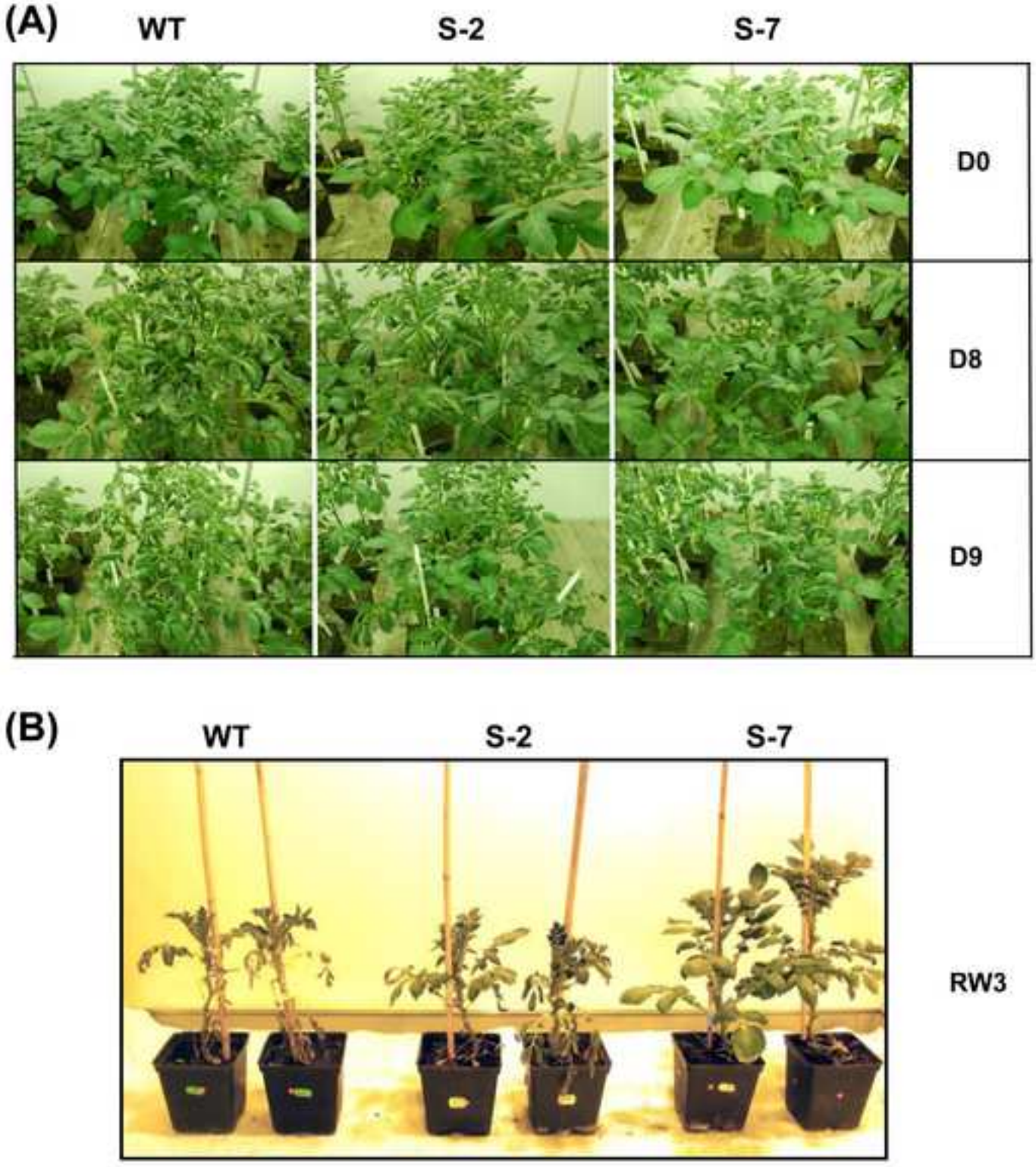

Fig. 4
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Figure 4. Examination of leaf water status.

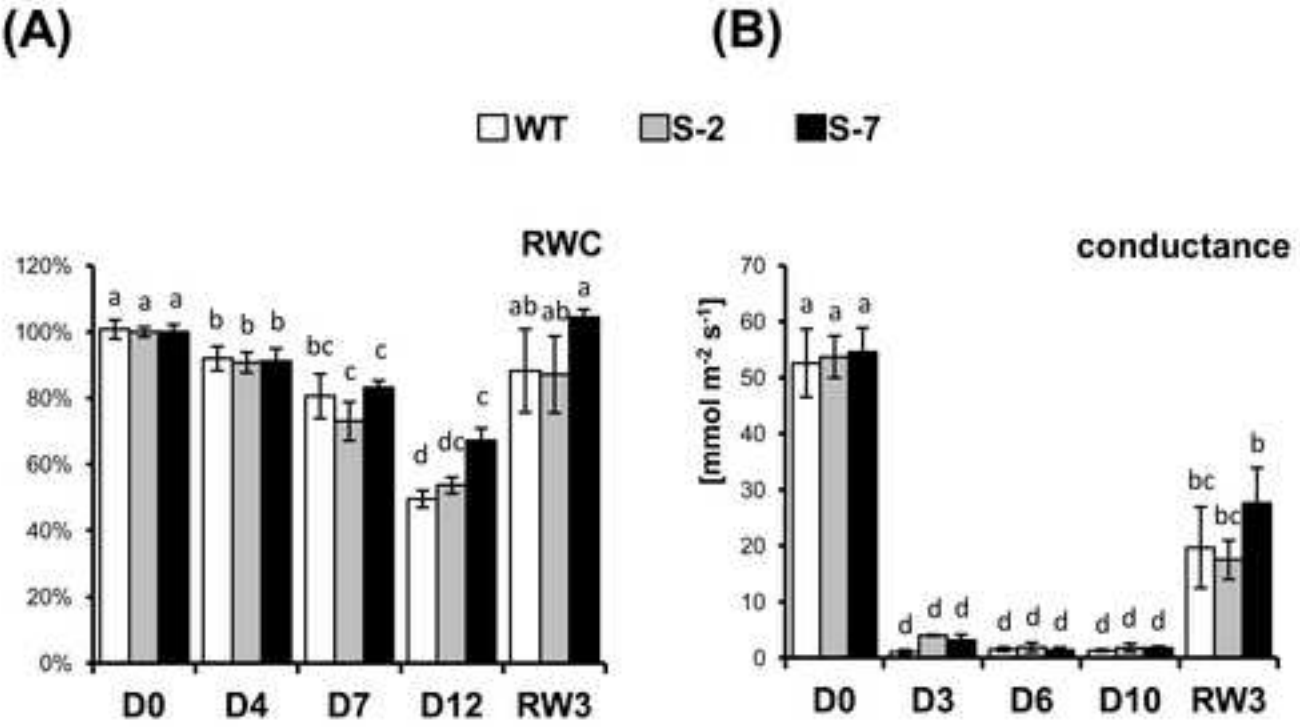

Fig. 5
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Figure 5. Net photosynthesis and photosynthetic performance of PSII during drought.

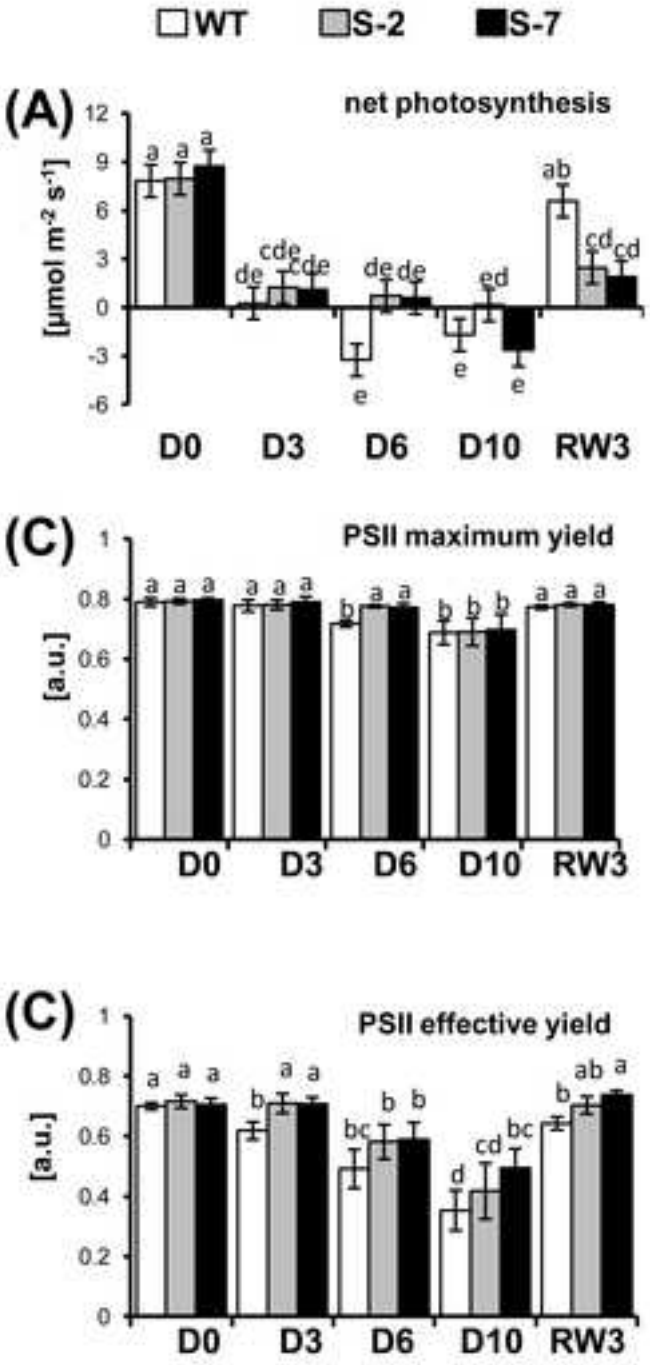

Fig. 6
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Figure 6. Photosynthetic pigments content during drought.

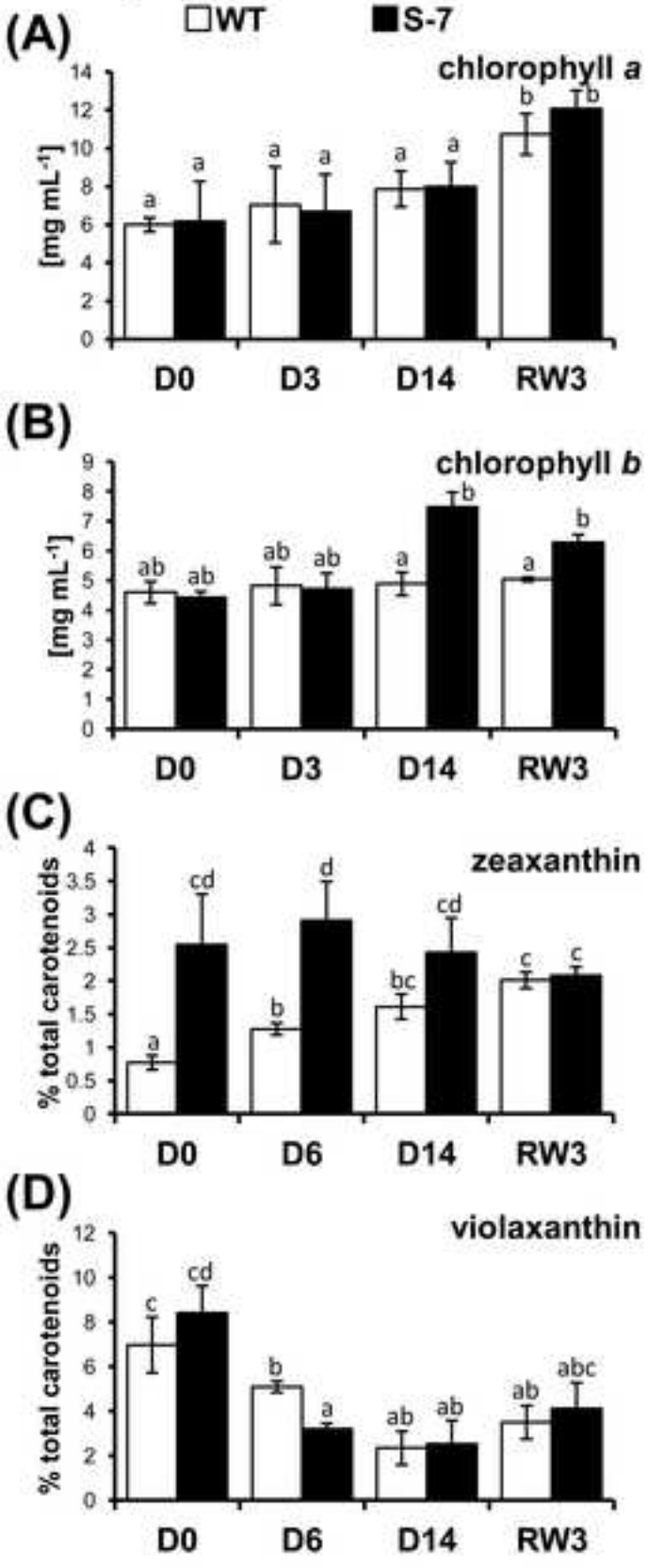

Fig. 7
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Figure 7. NPQ assayed in leaf of control potato

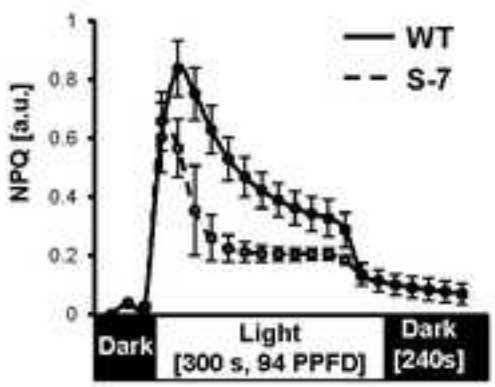

Fig. 8
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Figure 8. Accumulation of stress-related hormones during drought.

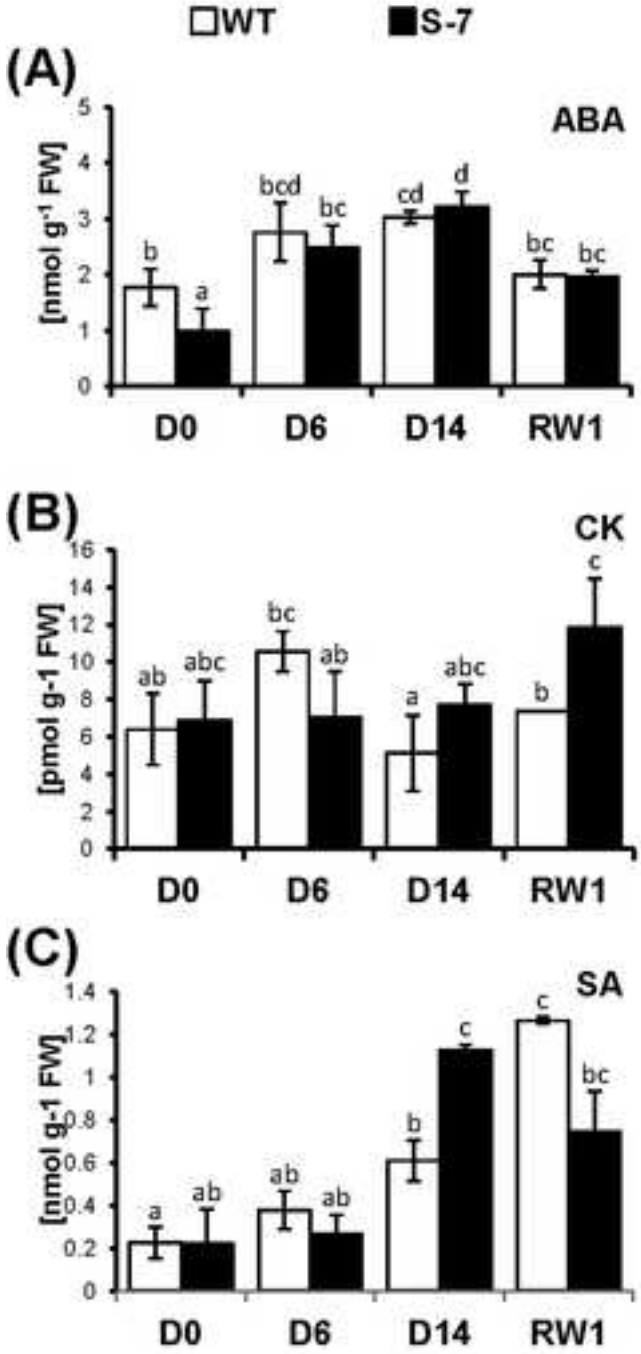

Fig. 9
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Figure 9. Accumulation of ROS (hydrogen peroxide and superoxide anion) and lipid peroxidation.

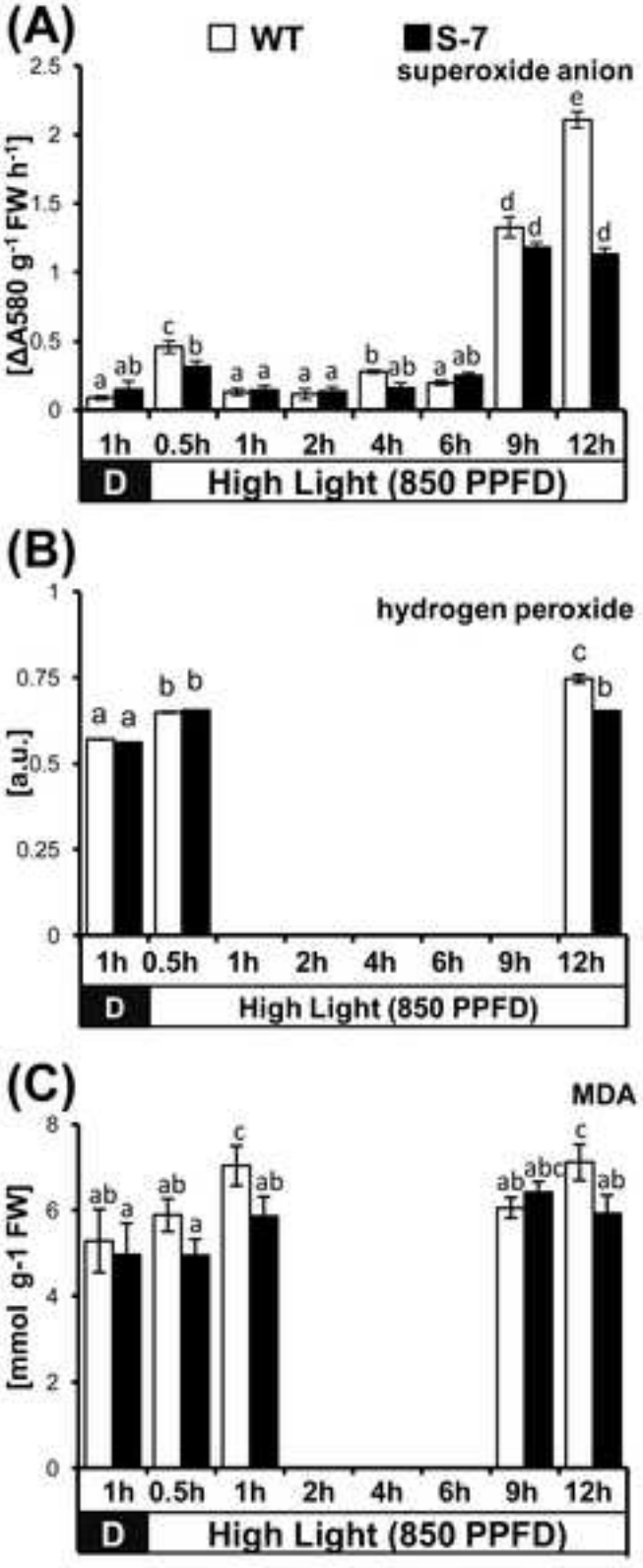

Fig. 10
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Figure 10. STANN1 attenuates photo-oxidative stress.

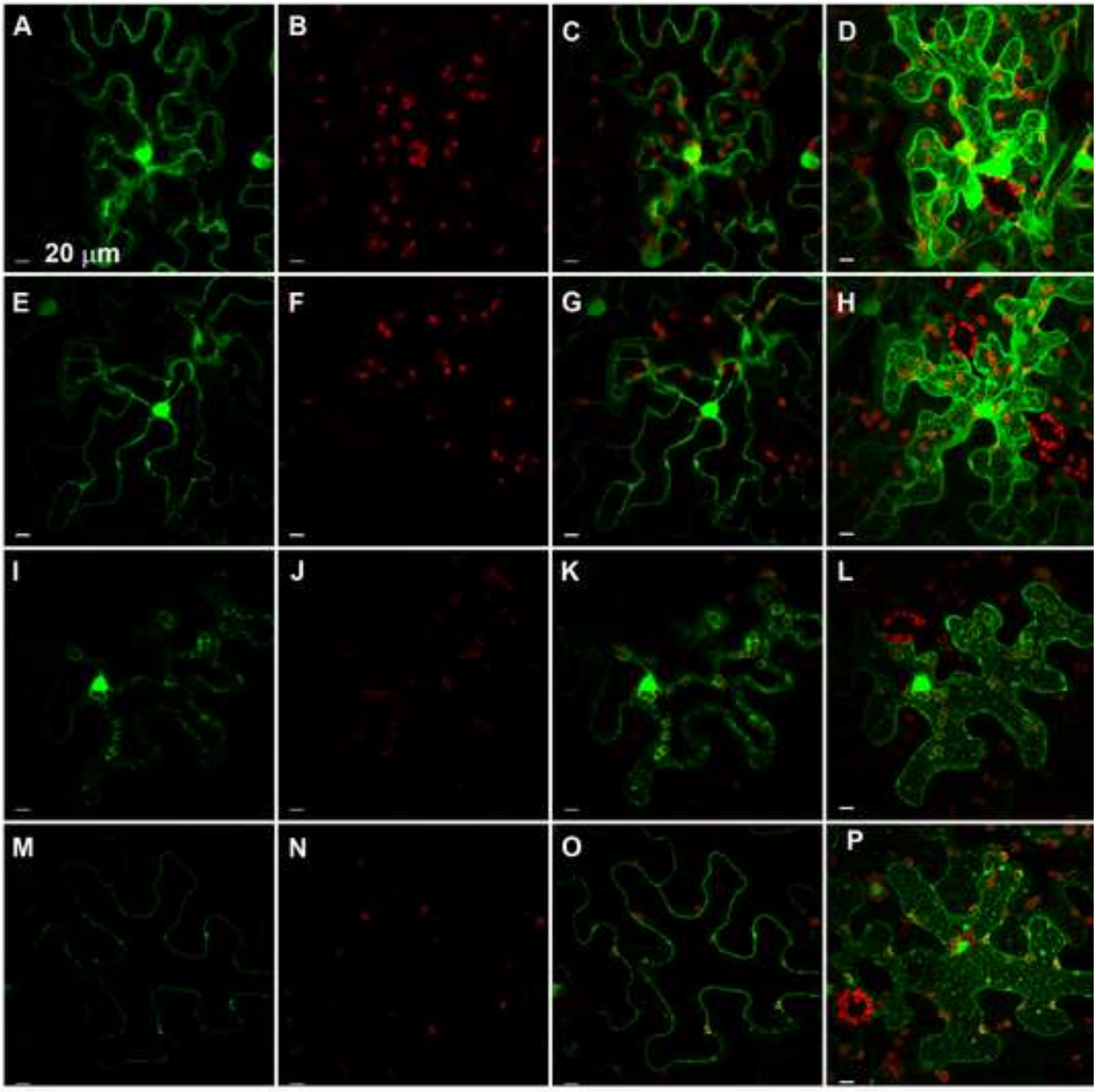

Fig. 11
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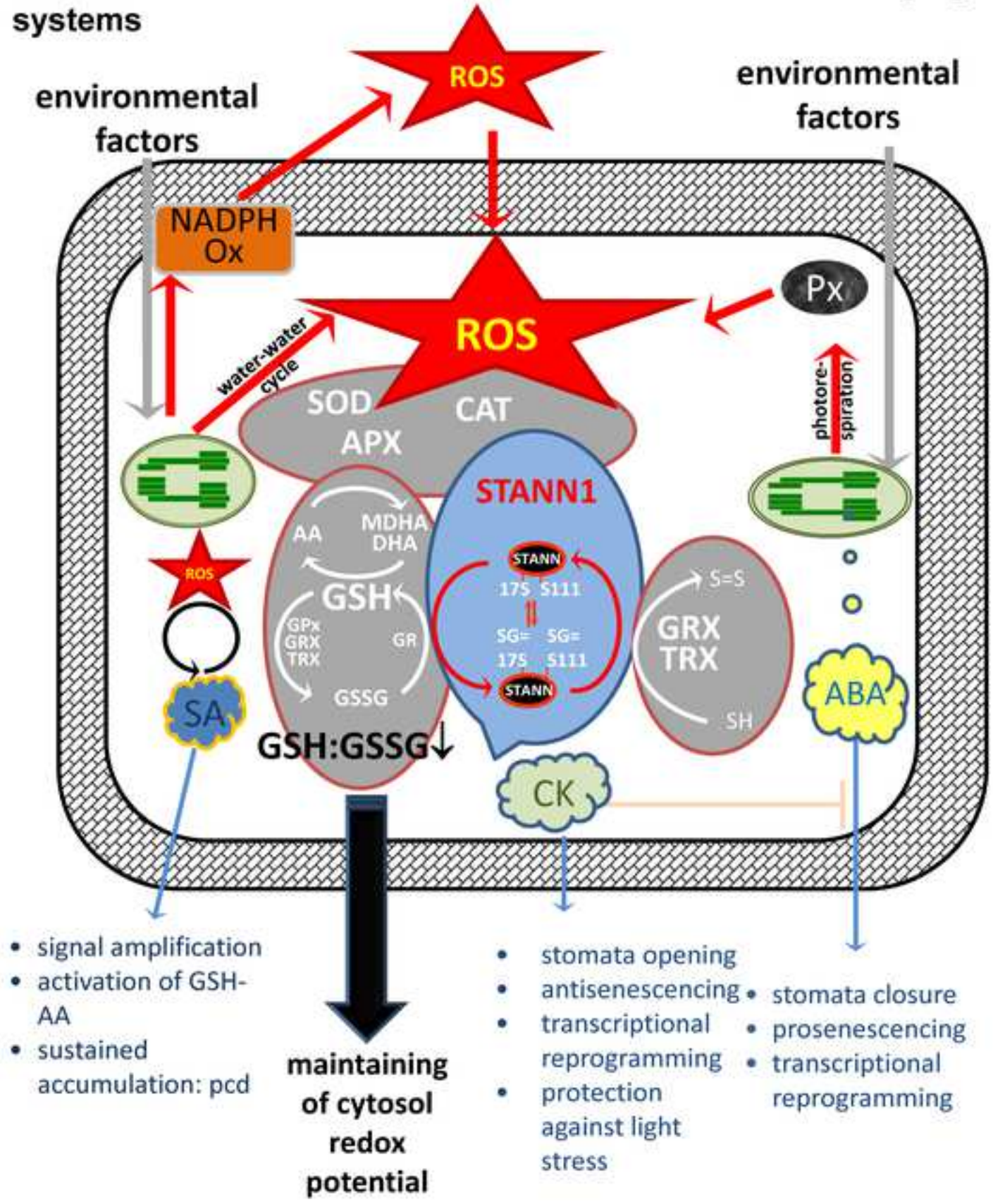
Figure 11. A simplify scheme depicting the interactions between STANN1 and innate mechanisms of ROS scavenging systems



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Potato Annexin STANN1 Promotes Drought Tolerance and Mitigates Light Stress in Transgenic *Solanum tuberosum* L. Plants

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Abbreviations:

ABA	abscisic acid
APX	ascorbate peroxidase
ATANN1	annexin 1 from *Arabidopsis thaliana* L.
ASC	ascorbate
Car	carotenoids
Chl*a*, Chl*b*	chlorophyll *a*, chlorophyll *b*
CK	cytokinins
Fv/Fm	the maximum quantum efficiency of photosynthesis
MeV	methyl viologen, paraquat
NPQ	non-photochemical quenching
PCD	programmed cell death
PETC	photosynthetic electron transport chain
PPFD	photosynthetic photon flux density [µmol m^{-2} s^{-1}]
PSI, PSII	photosystem I, photosystem II
PSBS	22-kD photosystem II subunit
SOD	superoxide dysmutase
STANN1	annexin 1 from *Solanum tuberosum* L.
SA	salicylic acid
Viol	violaxanthine
ViolDE	violaxanthine deepoxidase
XCar	xanthophylls
Y(II)	effective quantum yield of PSII
Zea	zeaxanthine

Abstract

Annexins are a family of calcium- and membrane-binding proteins that are important for plant tolerance to adverse environmental conditions. Annexins function to counteract oxidative stress, maintain cell redox homeostasis, and enhance drought tolerance. In the present study, an endogenous annexin, STANN1, was overexpressed to determine whether crop yields could be improved in potato (*Solanum tuberosum* L.) during drought. Nine potential potato annexins were identified and their expression characterized in response to drought treatment. *STANN1* mRNA was constitutively expressed at a high level and drought treatment strongly increased transcription levels. Therefore, *STANN1* was selected for overexpression analysis. Under drought conditions, transgenic potato plants ectopically expressing STANN1 were more tolerant to water deficit in the root zone, preserved more water in green tissues, maintained chloroplast functions, and had higher accumulation of chlorophyll *b* and xanthophylls (especially zeaxanthine) than wild type (WT). Drought-induced reductions in the maximum efficiency and the electron transport rate of photosystem II (PSII), as well as the quantum yield of photosynthesis, ~~and the electron transport rate~~ were less pronounced in transgenic plants overexpressing STANN1 than in the ~~wild type~~WT. This conferred more efficient non-photochemical energy dissipation in the outer antennae of PSII and probably more efficient protection of reaction centers against photooxidative damage in transgenic plants under drought conditions. Consequently, these plants were able to maintain effective photosynthesis during drought, which resulted in greater productivity than WT~~wild-type~~ plants despite water scarcity. Although the mechanisms underlying this stress protection are not yet clear, annexin-

mediated photoprotection is probably linked to protection against light-induced oxidative stress.

Introduction

Plants have developed passive and active strategies to survive environmental stresses such as drought, salinity, chilling, heat shock, heavy metals, UV radiation, ozone, mechanical stress, nutrient deficiency, hypoxia, and biotic stress [1]. Several stress-response genes have already been targets for bioengineering studies to improve plant stress tolerance [2]. However, ectopic expression of stress-inducible genes often results in developmental aberrations (e.g., stunted growth and irregular leaves) or reduced crop yields under non-stress conditions due to non-specific induction of programmed cell death (PCD) and/or premature senescence [3]. Current knowledge of stress-responsive pathways is based primarily on results obtained by imposing each stress individually, whereas plants in natural settings are generally challenged with multiple concurrent stresses, and the resultant signaling pathways may be superimposed and/or induce/antagonize each another [4-6]. New approaches to bioengineering stress tolerance in crop plants are needed to achieve sustainable improvements in crop biomass production [2].

Recent work shows that changes in redox poise can regulate plant cell function [7-~~10~~9] by acting as cellular signals [~~11]. Numerous studies have investigated redox-mediated stress responses [12-16]. Our understanding of the mechanisms that control production and metabolism of reactive oxygen species (ROS) in plants is incomplete. ROS are generated in several cellular compartments.~~10]. In light, the predominant location of ROS production is in chloroplasts [~~17, 18], where ROS are continuously formed as photosynthetic by-products under non-stress conditions.~~11]. The two main ROS sources there are the light-driven photosynthetic electron transport chains (PETCs) of photosystem I (PSI) and photosystem II (PSII). Environmental stress can trigger an

imbalance in redox homeostasis and affect chloroplast metabolism [~~17, 18]. The~~ 12]. Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the photosynthetic electron transfer chain (PETC) and utilization of oxygen as an alternative acceptor for excess electrons [13]. Changes in chloroplast ~~antioxidant system has been described~~redox poise activate secondary ROS-producing sources, such as ~~"loosely tailored" to maintain an endogenous ROS pool [20], which enables plants to quickly respond to fluctuating light levels. Consequently, significantly improving ROS scavenging enhances protection against sustained stress, but also desensitizes plant light responses and impairs environmental fitness. This result suggests that a delicate balance has to be maintained between ROS production and the activity of antioxidant systems. Chloroplasts also are involved in biosynthesis of hormones, carotenoids (Car), amino acids, and lipids. Therefore, the protection of chloroplast structure and function from environmental stresses is crucial for plant stress resistance and recovery.~~membrane NADPH oxidase complex [respiratory burst oxidase homologs (RBOHs)] [14] or photorespiration [15].

~~The two main ROS sources in chloroplasts are the light-driven photosynthetic electron transport chains (PETCs) of photosystem I (PSI) and photosystem II (PSII). Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the PETCs [21]. Under these conditions, oxygen can be utilized instead of $NADP^+$ as an alternative acceptor for excess electrons [22]. Several mechanisms have evolved to dissipate excess excitation energy, including nonphotochemical quenching (NPQ), which dissipates excess electrons as heat in PSII [23] and via the water-water cycle in PSI [21]. The water-water cycle produces hydrogen peroxide (H_2O_2), which is the most stable small ROS that can passively and actively cross membranes [24, 25]. In isolated intact~~

spinach chloroplasts and *Arabidopsis thaliana* (Arabidopsis) protoplasts, H_2O_2 produced under illumination can avoid neutralization by chloroplast antioxidant systems, and escape the organelle in a light-intensity-dependent manner [26-28].

Changes in chloroplast redox poise activate secondary ROS-producing sources in other cellular compartments, such as membrane NADPH oxidases [respiratory burst oxidase homologs (RBOHs)]. Abscisic acid (ABA) induces *RBOH* genes expression in Arabidopsis leaves and guard cells [29-30], *Hordeum vulgare* aleurone layer [31], and *Zea mays* seedlings [32]. NADPH oxidase-mediated ROS accumulation has been reported in ozone-treated Arabidopsis leaf [33, 34] and salt-treated Arabidopsis root tips [35].

Prolonged drought stress results in CO_2 deficiency due to stomatal closing and activates photorespiration. This rescue reaction dissipates excess reducing equivalents and energy, but generates H_2O_2 during salvage of the toxic byproduct 2-phosphoglycolate in peroxisomes. Under drought conditions, photorespiration is estimated to generate 70% of the H_2O_2 produced [36]. One of the major roles of photorespiration was proposed to be readjustment of redox homeostasis under abiotic stress conditions [37]. Cytokinin (CK)-mediated stimulation of photorespiration in transgenic tobacco plants resulted in substantially enhanced drought resistance [38].

Different buffering systems in discrete subcellular compartments are involved in maintaining cellular redox homeostasis and redox signaling. The primary pathway of H_2O_2 scavenging is the glutathione-ascorbate cycle (GSH-As cycle), which operates in chloroplasts, cytosol, mitochondria, and peroxisomes [21, 39]. Each cellular compartment contains a specific set of H_2O_2-scavenging enzymes, such as peroxidases, catalases (CAT), dismutases, glutaredoxins (GRX), and thioredoxins (TRX) [40, 41]. In

~~Arabidopsis and tobacco plants enhancing the antioxidant capacity of chloroplast and cytosol had a beneficial effect on photosynthesis and stress tolerance [42], whereas removal of any single antioxidant component reduced photosynthesis and stress tolerance [43-47]. There is evidence for cross-talk between antioxidant systems from different compartments [40, 48].~~

Annexins are a multigene, evolutionarily conserved family of calcium- and phospholipid-binding proteins [~~49]. They are~~16] that is present in all eukaryotes~~ and are characterized by~~. They have a highly conserved tertiary structure~~. The annexin superfamily is~~ defined by the presence in the molecule of four (or eight) approximately 70 amino acid ~~endonexin~~ motifs, ~~which contain~~each consisting of five α-helices~~ that are repeated four (or eight) times in the protein~~. The contribution of annexins to plant cell adaptation to adverse environmental conditions is well documented [~~49-53~~16-20]. However, an understanding of the primary physiological functions of plant annexins remains elusive. Initially, based on their prototypical characteristics, annexins were thought to participate primarily in membrane-related events, such as cellular transport, membrane-cytoskeleton interactions, or endo-/exocytosis [16; 20]. Later, it became clear that their cellular functions go far beyond this and include regulation of cellular redox poise and modulation of calcium transients upon stress.

Annexin 1 was identified in a genome-wide search of *Arabidopsis thaliana* (Arabidopsis) sequences capable of rescuing *Escherichia coli* ΔoxyR growth on high H_2O_2 concentrations [~~54].~~ 21]. Further experiments showed that Arabidopsis annexin 1 (ATANN1) is an element of the ROS signaling network in Arabidopsis. Deletion of functional ATANN1 reduced expression of glutathione-*S*-transferase Tau 1 (GSTU1) in seedlings after H_2O_2 treatment [22]. It is well accepted that upon salinity stress, ROS

trigger increases in Ca^{2+}, and ATANN1 was proposed to mediate calcium conductance activated by NADPH oxidases in root epidermal cells [22]. These data suggest that ATANN1 can function at a cross-road of calcium and ROS signaling [23; 24]. It is still an open question if other proteins from this family can function in a similar way.

Subsequent analyses confirmed that not only ATANN1, but also *Brassica juncea* annexins BJANN1 and BJANN3, and *Nelumbo nucifera* annexin NNANN1 ameliorated oxidative stress in homologous or heterologous cells and improved stress tolerance ~~[55-61].~~of tobacco, cotton, and Arabidopsis [23; 25-30]. In some cases, overexpression of annexins resulted in multi-stress tolerance. Transgenic tobacco plants expressing BJANN1 ~~were more tolerant of different abiotic stresses and~~ exhibited enhanced resistance to different abiotic stresses and infection with *Phytophthora parasitica* var. *nicotianae*, the latter possibly due to constitutively increased expression of several pathogenesis-related proteins [~~55~~25]. NNANN1 overexpression in Arabidopsis conferred enhanced tolerance to heat and oxidative stress [~~58]. Deletion of functional ATANN1 reduced expression of glutathione-S-transferase Tau 1 (GSTU1) in Arabidopsis seedlings after H_2O_2 treatment [62]. Under salinity conditions, ATANN1 mediates ROS-dependent Ca^{2+} signaling in roots at low (1.5 mM) external Ca^{2+} concentrations [63].~~27]. These studies generally used seedlings or leaf discs subjected to short-term stress treatments (in hours). There is a lack of information regarding annexin function in cell physiology, hormonal homeostasis, and metabolism during long-term exposure to environmental stress.

Potato is one the most important vegetable crops. Its global annual production in 2010 exceeded 300 million tons (FAOStat). Potato plants are highly efficient in terms of water usage (http://www.fao.org/potato-2008/en/potato/water.html), and produce more

food per water unit than any other crop [~~64~~31]. Therefore, potato could be a promising alternative to cereal crops. Modern potato cultivars are susceptible to drought, which is defined as a shortage of water in the root zone [~~65~~32]. Water deficit affects nearly all stages of potato development, and negatively impacts tuber numbers and quality (crop yield) [~~66, 67~~33; 34]. Only a few attempts to engineer potato drought tolerance have been reported (reviewed in [~~68~~35]). These studies had limited success because most transgenic plants did not exhibit good performance and productivity under non-stress and stress conditions. Potato annexin has not been considered for bioengineering applications; however, new proteomics research showed that STANN1 could be a candidate gene to improve stress tolerance. STANN1 was differentially expressed in potato tubers in response to wounding [~~69, 70~~36; 37], bruising (personal observation; [~~71~~38]), osmotic stress and salinity [~~72~~39], and was differentially expressed in potato aerial parts in response to osmotic stress and salinity [~~73]. In this study, we overexpress~~40]. We overexpressed potato annexin STANN1 and ~~observe~~observed the effects on plant biochemistry and physiology during drought ~~tolerance~~.

First, we investigated if increased expression of ATANN1 affected potato drought tolerance. We used the *S. tuberosum* genome to identify all potato annexins, and analyzed potential involvement in drought responses using semi-quantitative RT-PCT. Then, we characterized photosynthetic performance in transgenic plants overexpressing ATANN1 during prolonged water deficit around the root zone. One of the plant strategies to cope with environmental stresses is premature induction of the senescence program [41]; therefore, we analyzed the influence of STANN1 on long-lasting changes in hormonal homeostasis during drought. We also investigated possible annexin functions in modulating redox signaling, and assessed changes in drought stress

responses. Our working hypothesis was that annexin modulated plant stress responses by increasing the cytosolic antioxidant buffering capacity in transgenic plants. Studies on Arabidopsis ecotypes indicate that *ATANN1* mRNA levels differ in ecotypes adapted to very different local climatic condition (TAIR and our non-published data). In potato tubers, STANN1 levels did not differ in proteomes from different genetic backgrounds [~~74~~42]. Further experiments are necessary to elucidate if drought-tolerant potato landraces and cultivars could be generated by enhancing the level of annexin expression.

Materials and Methods

Generation of transgenic plants, transformation and growth conditions

S. tuberosum cultivar Sante (WT), medium-tolerant to drought, was used for transformation experiment (http://www.europotato.org).The *STANN1* cDNA sequence without the stop codon (957 bp; Acc. No. PGSC0003DMG400017714) was fused at the 3′ end to a 6×His-tag sequence and inserted into the XbaI restriction site of pROK2 [~~75~~43] between cauliflower mosaic virus 35S promoter and nopaline synthase (Nos) terminator sequences (Fig. S1A in Supporting Information file S1 Figures). This construct was used for *Agrobacterium tumefaciens*-mediated transformation of WT potato plants according to a previously published method [~~76~~44]. Regenerated transgenic plants were transplanted into separate glass tubes filled with 10 mL of Murashige & Skoog solid medium supplemented with 50 μg/mL kanamycin. The presence of the transgene cassette was verified with genomic PCR (data not shown). Expression of recombinant STANN1_6×His protein was confirmed by purification from leaves of WT and F1 transgenic plants (lines S-2, S-3, S-7, S-83, S91, S-97, and S-123) by Ni-NTA chromatography and detection with anti-HisTag primary antibody (Sigma-Aldrich). Recombinant ATANN1_6×His protein produced by bacterial overexpression was used as a positive control (WT protein extract) (Fig. S1B in Supporting Information file S1Figures).

Potato WT plants (*S. tuberosum* cv Sante) or transgenic lines in the "Sante" background (S-2 and S-7) were used for further experiments. Plants were cultivated in a growth chamber (or an air-conditioned greenhouse when indicated) under standard conditions (21±2°C; 16 h/8

h day/night; light intensity 110 to 130 PPFD (photosynthetic photon flux densities); 60–80% relative humidity.

Water stress

S. tuberosum plantlets sprouted from tubers were grown in plastic pots filled with 1 kg of sterilized soil (mixture of peat and sand, pH 5.5; prepared by the Plant Breeding and Acclimatization Institute) for 160–170 days. The field capacity (FC) was determined gravimetrically (g of water per g of soil). Pots were weighed every 2–3 days and the volume of water necessary to maintain the indicated FC was calculated individually for each plant. For well-watered control plants, FC was maintained at 65% (−0.8 MPa) for the whole experiment. Experimental drought was imposed after 8–10 weeks of growth (tuber initiation) (Fig. S2A in Supporting Information file S1Figures). Irrigation was decreased over 10 days to gradually reduce the FC to ~25% FC (−2.0 MPa) and was then maintained at this level until the end of the water deficit period. Irrigation was subsequently resumed with full soil saturation (rewatering). To estimate the impact of drought on potato productivity, plants were cultivated for an additional 11–12 weeks after rewatering (FC 65%) until physiological maturity. An exemplary schedule of FC changes is shown in Fig. S2B (supporting Information file S1Figures). Samples were collected at the beginning of the water deficit period (D0), and (depending on experiment) at different days of drought, i.e. 3rd (D3), 4th (D4), 6th (D6), 10th (D10), and 14th (D14), and at the first (RW1) and third (RW3) days after rewatering.

Identification of potato annexins

Annexins were identified in silico by searching for the endonexin domain (PFAM definition, PF00191, 66 aa) in six translation frames of the heterozygous diploid potato breeding line, *S.*

tuberosum L. group Tuberosum RH89-039-16 genome using the HMMSearch program from the HMMER3 package. According to PFAM, >93% of proteins from this family contained at least three consecutive repeats of the endonexin domain. By searching with a single repeat, the probability of missing a complete protein due to below-threshold partial hits or incorrectly defined intron-exon boundaries was minimized. Only hits with an E-value ≤0.001 were considered. To verify the presence and sequence of the predicted annexins in WT potato, genome primer sets were designed that corresponded to the 5′ (F) and 3′ (R) ends of the predicted open reading frames (ORFs, Table S1 in Supporting Information file S2Tables). Expression of putative annexin genes was verified using RT-PCR. Briefly, total RNA was isolated from WT leaves and reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania) with poly$(T)_{12-18}$ primer. Annexins were amplified from cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). PCR products were cloned with pJET Cloning Kit (Thermo Scientific) and their compliance with the predicted sequences was verified.

Semi-quantitative expression of annexins and stress-regulated genes

Gene expression was profiled over 14 days of drought in WT potatoes grown as described above. Samples were taken from the first fully-developed composite leaf at the top of the plant. For each time point, single leaf discs from four independent plants were collected, flash-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated with Trizol (Invitrogen, Scottland). Reverse transcription was performed as described above. Taq DNA Polymerase (Thermo Scientific) was used to amplify specific sequences from cDNA. Genes for semi-quantitative analysis were selected from PGSC_DM_v3.4_pep_fasta, which

contains a database of potato virtual translation products predicted according to similarity to annotated Arabidopsis genes. Specific primer sets for expression analysis were designed using PrimerSelect, Laser Gene10.0 DNASTAR (USA) (Table S2 in Supporting Information file S2Tables). The obtained sq-RT-PCR products were subjected by agarose gel electrophoresis, stained with ethidium bromide, and quantitated by densitometry using MultiGaugeV3.0 (Fuji) software. Expression was normalized with respect to the expression of potato elongation factor 1 alpha mRNA (EF1a; PGSC0003DMT400050664; [~~77~~45]). Each single experiment included four biological replicates, which were quantitated in three technical replicates. Experiments were repeated three times for each primer set and template.

Relative water content

Relative water content (RWC) was determined as described previously [~~78~~46] with the slight modification. For full saturation [equivalent to turgor weight (TW)] leaves were incubated in distilled water for 4 ~~hours~~h instead of overnight. Experiments were performed three times on at least five biological replicates for each genotype.

Extraction and determination of plant hormones

Leaf samples of ~0.5 g (without the main vein) from 8–10-week plants subjected to drought (as described above) were collected, immediately frozen in liquid nitrogen, and kept at −80°C until use. Samples were taken from the first fully-developed composite leaf at the top of the plant at the indicated time points. Sampling was performed ~~four hours~~4 h after the start of daily illumination. Three independent biological replicates were examined. Purification and analysis were performed as described previously [~~79, 80~~47, 48]. Briefly, leaf samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v) and the following

~~labelled~~labeled internal standards (10 pmol per sample) were added: $^{2}H_6$-ABA, $^{2}H_5$-*trans*Z, $^{2}H_5$-*trans*ZR, $^{2}H_5$-*trans*Z7G, $^{2}H_5$-*trans*Z9G, $^{2}H_5$-*trans*ZOG, $^{2}H_5$-*trans*ZROG, $^{2}H_5$-*trans*ZRMP, $^{2}H_3$-DHZ, $^{2}H_3$-DHZR, $^{2}H_3$-DHZ9G, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, and $^{2}H_6$-iPRMP (Olchemim, Czech Republic). Extracts were purified using a SPE-C18 column (SepPak-C18, Waters), and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction [containing abscisic acid (ABA) and its metabolites] was eluted with methanol and the second fraction (containing CK metabolites)] was eluted with 0.35 M NH_4OH in 70% methanol. Both fractions were separated by HPLC (Ultimate 3000, Dionex) and the hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and net photosynthesis were analyzed with a Portable Handheld Photosynthesis System CID 340 device (CID Bio-Science, Camas, WA, USA) according to the manufacturer's instructions. The maximum quantum efficiency of photosynthesis (Fv/Fm) and the effective quantum yield of PSII [Y(II)] were determined with CID 340 (CID Inc., USA) with a CI-510CF Chl fluorescence module and a CI-310LA light attachment (CID Bio-Science) providing actinic light. Measurements were performed 5 h after turning on the light, if not indicated otherwise, on the upper five fully-expanded unwrinkled leaves. Five plants were analyzed per time point. For maximal fluorescence (Fm) determination, plants were dark-adapted for 30 ~~minutes~~min (so all PSII reaction centers were closed) and then stimulated with saturating pulses of light (0.8 ~~seconds~~sec, 3,000 PPFD). The minimal fluorescence (Fo) with all PSII reaction centers opened was measured with modulated light of 0.25 PPFD.

Fv was calculated from the equation Fv= Fm-Fo. Y(II) was calculated using the equation Y(II) = (Fms-Fs)/Fms. The maximal fluorescence under light (Fms) was determined by allowing plants to adapt to light for 20 ~~minutes~~min and measuring the steady-state of chlorophyll (Chl) fluorescence (Fs). Next, a saturating pulse (0.8 ~~seconds~~sec, 3,000 PPFD) was applied and Fms was determined.

Gross non-photochemical quenching (NPQ) was estimated with a Dual Pulse Amplitude Modulation device, PAM-100 (Walz, Germany). For a single time point, six composite leaves from three to five control plants were analyzed. NPQ was calculated as (Fm − Fms)/Fms, where Fm represents the fluorescence of a dark-adapted sample and Fms represents a fluorescence of the illuminated sample. Plants were dark-adapted for ~20 ~~minutes~~min and kinetics were measured after repeated light pulses of 94 PPFD for 300 ~~seconds~~sec. Leaves were subsequently relaxed in darkness for 240 ~~seconds~~sec and fluorescence while continuously measuring and recording fluorescence.

Non-polar lipids extraction and carotenoids~~/chlorophyll~~ (Car)/Chl determination

Plant material was collected from 8–10-week-old plants exposed to drought. Samples were collected 4 ~~hours~~h after switching on the light at D0, D6, D14, and RW3. One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully~~-~~-expanded composite leaves, and total six discs harvested from two plants were combined as a single sample. Non-polar lipids were extracted at 4°C. Plant material after shredding in cooled mortar was transferred to a 15 mL Pyrex tube. After the addition of 3 mL acetone-methanol (8:2 v/v), the sample was perfused with argon and mixed vigorously by vortexing for 2 min. For the second and third extractions, hexane (9 mL) was added

and the sample was again perfused with argon before capping and shaking in a reciprocating shaker (PROMAX 2020, Heidolph, Germany) for 30 min in the dark. After shaking, the sample was incubated without agitation for 5 min to allow phase separation. The upper hexane phase was collected by aspiration and transferred to a 100 mL Erlenmeyer flask, perfused with argon, capped and stored in the dark in 4°C. In the second extraction stage, 2 mL of propanol was used in addition to hexane, and perfusion, shaking and phase collection were repeated as before. After removal of hexane, the polar phase was centrifuged for 15 min at 4500 rpm. The supernatant was combined with the two hexane phases, perfused with argon, and filtered through a Milipore syringe filter unit Millex-CV13 Filter Unit (0.22 µm). The combined hexan phases were then transferred to room temperature, evaporated to dryness under argon, and dissolved in 1 mL methanol-propanol-hexane 6:1:3 (v/v/v). Dissolved samples were transferred to 2 mL glass vials, perfused with argon, capped, and stored at −80°C.

Non-polar lipids were analyzed by injecting 5 µL of sample extract onto an ACQUITY UPLC HSS T3 1.0×150 mm 1.8 µm column and eluted with a gradient of solvent A [water and methanol (1:9, v/v)] and solvent B [methanol:isopropanol: hexane (2:1:1, v/v/v)], with a total of 210 ~~minutes~~min to transition from solvent A to B. Separation was monitored in the 300–750 nm range with a photodiode array detector. A single chromatogram at 436 nm was extracted, exported in ASCII format, and used for peak area integration analysis with GRAMS/AI software (Thermo Electro Corp, Finland).

Chl*a* and Chl*b* contents were estimated by recording the absorbance of the aforementioned extract at 663, 652, and 645 nm (Cary 50 Bio UV/VIS spectrophotometer, Varian, Australia) as described previously [~~81~~49].

ROS levels and lipid peroxidation during high light stress

One leaf disc (11 mm diameter) was taken from the third, fourth, and fifth fully-expanded leaves of potato plants, and for a total of six discs three discs were harvested from each of two plants were combined as a single sample. Immediately after harvesting, samples were vacuum-infiltrated with methyl viologen (MeV) at the indicated concentrations and then incubated in the dark for 1 ~~hour~~h under normal irradiance (150 PPFD). Images were obtained after 30 ~~hours~~h of incubation.

A similar procedure was used for ROS quantification, with the exception that a single MeV concentration (50 μM) was used and samples were exposed to high irradiance (850 PPFD~~).~~), well in excess of the levels that saturate photosynthesis in Arabidopsis (high light stress, HL). Samples were collected at the indicated time points. Superoxide anion ($O_2^{\bullet -}$) content was determined using a colorimetric nitro blue tertrazolium (NBT) assay as described previously [~~82~~50]. Hydrogen peroxide (H_2O_2), was detected with diaminobenzidine tetrahydrochloride (DAB) and quantified by counting pixels on scanned images using ImageJ software [~~83~~51]. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA) [~~84~~52].

Transient expression of STANN1_GFP in *Nicotiana benthamiana*

The *STANN1* sequence (without the stop codon) was introduced between NcoI and BcuI restriction sites at the 5′-end of the monomeric GFP (mGFP) coding sequence in pCAMBIA1302. Intact *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 transformed with empty pCAMBIA1302 expressing mGFP or pCAMBIA1302 expressing STANN1_mGFP as described previously [~~85~~53]. After 3 days, 1 cm diameter leaf discs were

excised and incubated with 50 μM MeV for 1 h~~our~~h in darkness, and then incubated for 4 ~~hours~~h in high light (850 PPFD). Fluorescence was immediately observed using a Nikon Eclipse TE2000-E inverted C1 confocal laser scanning microscope equipped with a 40× Plan Fluore oil immersion objective (numerical aperture, 1.30). mGFP and chloroplast autofluorescence were excited with a solid-state Coherent Sapphire 488 nm laser and detected using 515/30 band pass and 610 long pass emission filters, respectively. All samples were analyzed in triplicate. Three independent experiments were performed.

Statistical analyses

Data were analyzed using two-way ANOVA with Duncan's Multiple Range Test (DMRT) (for yield) and MANOVA regression models (for other experiments). Multiple comparisons between means were performed with a HSD Tukey test with a confidence limit of 95%.

Results

Identification of potato annexin genes

Genome-wide examination of the potato sequence database for annexins revealed the presence of 11 DNA segments encoding putative proteins displaying substantial similarity to previously characterized plant annexins. Two of these sequences were classified as pseudogenes due to several defects and a lack of continuity in any of the six ORFs. The remaining nine genes were located on chromosomes I, IV, V, and X and each encoded 5–6 exons (Fig. 1A). The positions and phases of introns in the putative potato annexin genes were consistent with those reported for rice annexins [~~86~~54] (Fig. 1B). The putative annexin sequences in the *S. tuberosum* genome were verified using genomic PCR (Fig. 1C), and the lengths of the amplified genomic products were as expected (Table S1 in Supporting Information file S2Tables). The degree of nucleotide sequence identity between the putative potato annexins was 41–92%. Sequences identified by bioinformatics approaches were confirmed experimentally. Reverse transcription polymerase chain reaction indicated that all nine genes were expressed in different potato organs (data not shown).

Multiple alignment of the putative potato annexin amino acid sequences with Arabidopsis annexins revealed that all but one of the potato annexins had Arabidopsis homologs (data not shown). The newly-identified potato genes were named accordingly as *STANN1*, *STANN2*, *STANN3.1*, *STANN3.2*, *STANN3.3*, *STANN4*, *STANN5*, *STANN8*, and *STANN9* (data not shown). The potato annexins formed a functionally diverse protein family that was differentially expressed in different plant organs (data not shown). The most striking genomic feature of the potato annexin family was triplication

of the annexin 3 gene on chromosome 1 (Fig. 1D). In addition, in an arrangement resembling that in Arabidopsis, potato *STANN3.1* and *STANN4* were adjacently localized and divergently transcribed, possibly from a shared promoter. The Inparanoid database groups all annexins in the same in-paralog cluster; however, we suspect that the annexin 3 variants (*STANN3.1*, *STANN3.2*, and *STANN3.3*) are within-species out-paralogs. Two duplications (ancestral gene → *STANN3.1* and the ancestor of *STANN3.2*; then the ancestor of *STANN3.2* → *STANN3.2* and *STANN3.3*) appear to have occurred prior to potato and tomato speciation, as *S. lycopersicum* contains two orthologs of the annexin STANN4 and STANN3.1. In turn, *STANN4* and *STANN3.1–3.3* are out-paralogs, as *STANN4* is moderately related to all *STANN3* variants but shares high sequence similarity with other annexins from *S. lycopersicum* or Arabidopsis. Multiplication of DNA segments within this region of chromosome 1 during *Solanaceae* evolution apparently took place independently at least twice. In tomato chromosome 1, the entire dyad of *SLANN3*/S*LANN4* was duplicated [~~87~~55] and gave rise to a tetrad located within a short segment of DNA (21,145 bp) that was not interspersed with other genes. This region of chromosome 1 represents a "hot-spot" in the *Solanaceae* family where duplications of a single gene or gene cluster occurred.

Characteristic of potato annexin proteins

Newly~~.~~–identified potato annexins had similar predicted molecular masses of 34–37 kDa and diverse isoelectric points (5.21–9.02). The overall tertiary structures, which were defined by four endonexin domains containing calcium binding sites, were well preserved (Fig. S3 in Supporting Information file S1Figures, Table S3 in Supporting Information file S2Tables). However, the primary amino acid sequences diverged

significantly, with the lowest amino acid identity of 20.9% between STANN4 and STANN5. Groups with higher similarities were identified, such as STANN3.1, STANN3.2, and STANN3.3. Annexins 3.2 and 3.3 were the most closely related with amino acid identities of 90.5% and 70.1% with STANN3.1, respectively. STANN3.2 and 3.3 differed in length (302 and 317 aa, respectively) due to lack of the 14-3-3 like domain on the C-terminus of STANN3.2. Similarly, the N-terminal end of STANN3.2 and STANN3.3, but not STANN3.1, contained a putative myristoylation motif (MG). To date, a myristoylation-mediating membrane localization has been confirmed only for mammalian AnxA13b. With respect to plant annexins, a myristoylation motif was found in poplar annexin EEE95606.1, but the functionality of this motif was not experimentally verified. In summary, despite extensive similarities, there were substantial differences between members of the STANN3 subfamily. This suggested that family might be unique to *Solanaceae*, and that distinct cellular functions evolved for each of the annexins.

The potato annexins contained canonical type II calcium binding sites G-X-GTD-{30-40}D/E solely in the first and occasionally fourth endonexin domains (Fig. S3 in Supporting Information file S1Figures). STANN4 and STANN8 appear to have lost calcium responsiveness as a result of substantial mutations (substitutions and insertions) in these regions. The calcium binding site in the fourth endonexin repeat was probably the only one preserved in STANN5. Tryptophan residues within the first endonexin repeat (G-W-GT) were conserved in potato annexins 1, 2, 8, and 9, but were replaced with phenylalanine (STANN5) or lysine (STANN3.1-3.3 and STANN4) in other annexins (Fig. S3 in Supporting Information file S1Figures). This phenylalanine modification is not predicted to interfere with calcium binding because phenylalanine

and tryptophan residues are both hydrophobic and possess aromatic rings. By contrast, the lysine modification may impede membrane translocation of annexin because introducing a positive charge into the calcium coordination site has the potential to disrupt calcium binding. Other amino acids or motifs important for the plant annexin tertiary structure were preserved in the potato proteins, such as histidine 40 (except in STANN3.2 and STANN4), cysteine 111 (except in STANN4), and cysteine 239 (except in STANN1).

Potato annexin gene expression during drought

To generate drought-tolerant potato, the genes whose products confer drought tolerance have to be identified. Potato annexins are a multigene family; therefore, we characterized the expression of all annexins during drought. Only five annexin genes (*STANN1*, *STANN4*, *STANN5*, *STANN9*, and *STANN2*) were expressed in the leaves of well-watered control WT plants (Fig. 2). At the onset of drought (D0), *STANN1* mRNA was the most abundant transcript (relative to the *EF1a* mRNA). Over time, the level of *STANN1* mRNA increased whereas *STANN4*, *STANN5*, *STANN9*, and *STANN2* mRNA levels remained unchanged. The difference in the accumulation of *STANN1* mRNA from D0 to D14 was statistically significant (Fig. 2). Concurrently, additional annexins were expressed that were not detected under control conditions. The levels of *STANN3.1* and *STANN3.2* mRNA (relative to *EF1a*) increased on D6 and remained elevated until the end of the drought period. The level of *STANN8* mRNA increased continuously during the whole period of water deficit (Fig. 2). However, these induced annexins were expressed at levels at least ten-fold lower than that of annexin 1. This

strongly suggested that STANN1 was the key annexin involved in the plant cell response to drought.

Tolerance to soil water deficit

Drought is one of the most devastating environmental stresses in modern agriculture; it reduces global crop yields in developed and developing countries [~~88~~56]. Continued efforts are required to obtain new crop varieties that assure food security. Annexins were shown to be a promising target in model plants; thus we wanted to verify if they can be used to improve stress tolerance in crop plants. To investigate the effect of STANN1 on drought tolerance in potato transgenic plants overexpressing STANN1 were generated by introducing the STANN1coding sequence under control of the 35S promoter. Transgenic plants displayed normal morphology without any discernible abnormalities and/or growth aberrations under well-watered conditions in growth chamber and in the greenhouse. Leaf turgidity was similar between transgenic and WT plants, which indicated that the leaf water status of WT and transgenic lines was comparable (Fig. 3A, upper panel; Fig. S4A in Supporting Information file S1Figures). During soil water deficit, STANN1 overexpression conferred sustained turgor maintenance, whereas leaf wilting was clearly visible on D8 in WT plants (Fig. 3A, middle panel). In WT the effect of drought was more apparent by D9, and leaves began to shrivel, roll, and curl up. Younger leaves near the top the of the plant were most severely affected (Fig. 3A, lower panel). Leaves of the transgenic lines S-2 and S-7 maintained turgor and did not show signs of dehydration. Rewatering restored leaf turgor and normal growth resumed within 1 day for transgenic plants and 3 days for WT (Fig. 3B). After 2 weeks of drought, S-2 and S-7 leaves were less damaged then those of

WT. Experiments were repeated four times in succeeding years, under greenhouse and growth chamber conditions, and in all cases similar results were obtained (Fig. S4 in Supporting Information file S1Figures). The exact number of irreversibly damaged leaves varied between experiments depending on the drought severity (intensity and length). Damage was consistently significantly lower in transgenic lines than in WT. For example, survival rates after a 3-week drought were 12% and 82% for WT and S-7, respectively.

The ability to preserve turgor in leaves is closely related to drought tolerance. To further characterize drought responses in transgenic plants overexpressing STANN1, RWC changes under water deficit were analyzed. RWC was comparable in WT and transgenic plants. Drought reduced RWC in WT and transgenic plants. However, differences between lines became apparent with increasing drought severity and this became statistically significant at D12 (Fig. 4A). Rewatering after 2 weeks of drought treatment restored control RWC values in WT and transgenic plants. The effect of drought on stomatal conductance (a measure of water and carbon dioxide vapor through the leaf stomata) was apparent by the third day after reducing watering, but the difference between WT and transgenic lines was statistically insignificant (Fig. 4B). Conductance remained low during the whole period of water deficit and only partially returned to control levels on the third day after the resumption of watering.

The real goal for any genetic engineering efforts in crop plants is to improve crop yield. We examined the productivity of transgenic plants under control conditions and under drought. STANN1 overexpression improved plant yield both in terms of the total tuber mass (Fig. S5A in Supporting Information file S1Figures) and consistency of the tuber size (Fig. S5B–C in Supporting Information file S1Figures). The net productivity

of well-irrigated WT and transgenic S-7 and S-2 lines was almost identical but tuber quality (size and uniformity) was enhanced in the transgenic lines. A 14-day drought decreased the tuber yield of the WT plants by half, whereas yield loss for S-2 and S-7 lines under comparable conditions was statistically less significant. Tuber quality in the transgenic lines was less impaired after drought compared with WT. On the basis of these results, we concluded that increasing STANN1 levels is a promising strategy to improve drought tolerance in potato.

Plant photosynthetic activity during drought

We showed that elevation of ATANN1 levels enhanced drought tolerance in potato, but the mechanism of this process was unknown. There is some indication that this could be due to annexin-mediated modulation of redox homeostasis. During drought, ROS accumulation in chloroplasts leads to oxidative damage of photosystems ~~[89, 90~~57; 58]. PSII catalyzes water oxidation and provides electrons for all further photosynthetic reactions; thus, its efficiency is crucial for the entire pathway. ~~The PSII complex is a highly vulnerable structure that is constantly photodamaged; to maintain activity, constant repair and reassembly of reaction centers with newly synthesized proteins is necessary [91]. PSII activity is involved in mediating plant adaptation to environmental conditions.~~ Drought impairs photosynthetic capacity and reduces leaf netto carbon uptake due to increased photorespiration activity (another sink for the absorbed energy) [~~92~~59]. To directly estimate the effect of STANN1 on drought-induced PSII damage, the photosynthetic performance of PSII in transgenic plants overexpressing STANN1 was characterized under drought conditions. Several physiological parameters related to plant vigor were analyzed to assess the effect of STANN1 overexpression. These

included, net photosynthesis (Pnet, associated with plant vitality and biomass production) (Fig. 5A), maximum efficiency of PSII in the dark-adapted state (a measure of the organization and vitality of PSII) (Fig. 5B), and effective quantum yield of PSII in illuminated samples (Fig. 5C). Under control conditions, STANN1 overexpression did not influence any of these parameters. By contrast, essentially all photosynthetic functions were disturbed during drought, and changes in the two overexpression lines were consistent.

~~Wild-type~~ WT Pnet declined to zero by D3 (Fig. 5A). Subsequently, it dropped to negative values by D3 and D10. In the two transgenic plant lines, Pnet remained positive until D10. After rewatering, Pnet increased in all three lines (Fig. 5A). Under control conditions, Fv/Fm values (Fig. 5B) were similar in the all three plant lines (~0.79), and was in the same range as in most investigated plant species. Drought negatively affected Fv/Fm in our experiments; this was observed by D3 in WT, but become apparent in transgenic plants arent on D6. In all three lines, Fv/Fm recovered to baseline within 3 days of rewatering. Measurements were performed on upper non-wrinkled leaves, indicating that apical shoot meristems were not irreversibly damaged by dehydration. In WT and transgenic plants Y(II) (Fig. 5C) declined steadily from the onset of drought, but the reduction appeared on D6 in transgenic plants, and the effect was significantly reduced compared to WT. Y(II) fully recovered in S-2 and S-7; however, even on the third day after soil resaturation, the physiological efficiency of PSII was not restored in WT. This suggested that photorespiration was activated later in STANN1 overexpressing plants then in WT. Thus, PETC was protected for a longer time against irreversible damage and diminished photorespiration-induced H_2O_2

accumulation in cytosol. These results show that PSII impairment in transgenic plants was fully reversible

Photosynthetic pigments content in transgenic plants

Drought activates premature senescence in plants [~~93~~60] and stimulates catabolism of photosynthetic pigments [~~94~~61], particularly ~~chlorophyll (~~Chl~~)~~ and ~~carotenoids (~~Car~~).~~. We determined the photosynthetic pigment contents under drought conditions to better understand the effect of STANN1 on photosynthetic machinery.

Chla and Chlb accumulation

In WT and transgenic lines under well-watered conditions, the total Chl content (11.2±0.01 and 10.6±2.29 mg mL^{-1}, respectively) and the ratio of Chl*a* to Chl*b* were similar. In WT accumulation of Chl*a* (Fig. 6A) and Chl*b* (Fig. 6B) did not change during drought and after rewatering, the level of Chl*a* increased to 180% of the control value at D0. During water deficit in S-7 line, the Chl*a* level was stable; however, Chl*b* levels increased and reached 168% at D14 compared to D0. Consequently, the Chl*a*/*b* ratio rose to 2.0. After rewatering, Chl*a* levels doubled and Chl*b* levels remained stable.

Xanthophyll (XCar)accumulation

The relative ~~xanthophyll (~~XCar~~) cycle is essential for harmless dissipation of excess excitation energy in PSII as heat (NPQ). The relative XCar~~ abundance in the total Car pool changes during the day depending on the incident light [~~95~~62]. To exclude diurnal fluctuations, samples were collected at the same time (approximately 4 ~~hours~~h after the start of daily illumination). Under non-stress conditions, STANN1 overexpression did not significantly affect the total Car level, but the XCar content increased [zeaxanthin

(Zea), 188%; violaxanthin (Viol), 144%] compared with that in WT plants (Fig. 6C–D). This result indicates that the ~~xanthophyll~~XCar cycle activity was higher in transgenic plants than in WT plants under the same light conditions. In WT plants, Zea content increased progressively during drought and reached a similar level to that in transgenic S-7 plants only after rewatering [0.35±0.01 pmol/g fresh weight (FW)]. In transgenic S-7 plants, the Zea level remained largely stable and fluctuated in the range of 0.31–0.34 pmol/g FW. Viol declined significantly during drought in both plant lines. The most significant reduction was observed during the first 6 days of drought, and was more pronounced in S-7 than in WT (57% and 10.5% reduction, respectively). At subsequent time points, the differences between lines disappeared and Viol remained at a stable level after rewatering (0.45±0.01 pmol/g FW in WT and 0.44±0.06 g^{-1} FW in S-7).

~~Nonphotochemical quenching~~NPQ activity

The PSII complex is a highly vulnerable structure that undergoes constant photodamage even under optimal conditions and mediates plant adaptation to the dynamic light environment. NPQ is an effective short-term mechanism that provides protection for PSII against excessive irradiation and allows excess excitation energy to be harmlessly dissipated as heat [63]. For efficient NPQ, XCar cycle effectiveness and the level of PsbS protein are essential. The observed differences in XCar accumulation prompted us to analyze gross NPQ performance in attached leaves of control, well-watered WT, and transgenic plants (Fig. 7). As expected, NPQ of S-7 and WT differed. Maximal NPQ occurred in S-7 plants after the start of daily illumination, and NPQ amplitude was ~25% higher in S-7 than in WT. The steady-state NPQ level was elevated and saturation was delayed in S-7 compared with those of WT. The *PSBS* (another key NPQ factor)

mRNA level during drought was higher in S-7 than in WT plants (Fig. S6A in Supporting Information file S1Figures). These results indicate that the NPQ capacity in transgenic line S-7 was greater than that of WT, which likely conferred better protection of PSII against photooxidative damage. ~~Electrons~~While excess absorbed electrons were redirected to H_2O_2 in WT plants, they were more efficiently dissipated as heat in transgenic plants ~~and~~, which prevented subsequent ROS accumulation ~~was lower than in WT~~.

Annexin overexpression affects hormonal homeostasis in plants subjected to drought

The drought phenotype of transgenic potato plants overexpressing STANN1resembled that of plants overproducing cytokinins (CK~~.~~). Compelling evidence indicates that the redox signaling network integrates with phytohormone-activated pathways [~~96~~64]. ROS are positioned upstream and downstream of at least some hormone-signaling pathways [~~14].~~65]. We therefore stress-hormone levels [pro-senescing: ABA and salicylic acid (SA); anti-senescing: CK] in leaves of WT and S-7 plants subjected to drought. Under well-watered conditions, the level of biologically active ABA in transgenic plants was significantly lower than in WT (Fig. 8A). However, this difference was insignificant by D6 after the initiation of drought This suggested that biosynthesis of ABA in transgenic plants during the first week of drought was more active than that in WT, which is consistent with a more pronounced reduction of Viol (ABA precursor) levels in transgenic plants (Fig. 6D). During the second week of water deficit, only a slight increase in ABA level was observed and maximum levels on D14 were similar WT and S-7 (3.21±264.01 and 3.02±101.59 nmol g^{-1} FW,

respectively) (Fig. 8A). As expected, ABA levels declined to control values on resumption of watering.

Under control conditions, annexin overexpression had no significant effect on CK levels (Fig. 8B; Table S4 in Supporting Information file S1Figures). The contents of active and total CK were similar and amounted to 6.35 and 6.90 pmol g^{-1} FW, and 506.34 and 542.08 pmol g^{-1} FW, in WT and S-7 plants, respectively. Drought stress was associated with down-regulation of *trans*-zeatin (tZ), the most physiologically active CK involved in the stimulation of cell division. During early drought stages (RWC ~85%, only minor difference from control conditions), the level of active CK in WT increased, especially compared to the less active isopentenyladenosine (iPR) levels. Active CK declined under severe drought conditions, with the exception of *cis*-zeatin (cZ) and its riboside (cZR), both of which were CK species associated with stress responses. After rewatering, active CK content strongly increased, especially that of *trans*-zeatin (tZ), whereas cZ and cZR levels substantially declined. High levels of active CK (including high levels of cZ) were maintained in S-7 even under severe drought conditions. These levels were substantially higher than in parental plants. After rewatering, active CK elevation was much more pronounced in S-7 than in WT. The level of storage compounds (CK O-glucosides) was generally low. By contrast, levels of deactivation products (CK N-glucosides) substantially increased during drought, probably as a result of the enhanced deactivation of CK (data not shown).

SA accumulation was reported in response to different abiotic stresses [~~97~~66]. STANN1 overexpression had no effect on SA levels under well-watered conditions. SA accumulation in WT and S-7 did not change significantly under moderate drought (D6). During the second week of water limitation, the SA level increased in both lines, and

SA accumulation in S-7 was approximately twice that in WT (Fig. 8C). The SA level declined in S-7 during recovery, but remained slightly higher than that observed at D0. By contrast, SA continued to increase in WT and exceeded the level observed in S-7. These data indicate that ROS-modulating systems are activated more rapidly and to a higher extent in transgenic plants overexpressing STANN1 than in WT plants.

In summary, genetic modification ~~did not~~ influence neither ABA synthesis ~~and~~~~ABA~~no ABA-dependent responses. The elevation in CK metabolism upon rewatering was consistent with phenotypic observations. SA levels in S-7 increased rapidly during drought and peaked by D14 but declined rapidly after rewatering. This suggested that SA-mediated activation of antioxidant systems during drought was faster in STANN1 overexpressing plants. In WT plants, delayed SA-mediated effects such as induction of PCD might be induced.

STANN1 mitigates drought-mediated oxidative stress in cytosol and chloroplasts

Although the experimental plants were grown under constant temperature conditions, heat stress response (HSR) was induced in WT and transgenic plants during drought. In WT plants, water deficit increased the accumulation of chloroplast-specific *HSP100* and cytosol-targeted *HSP40* mRNAs (compared to the EF1a normalization control), which peaked during the second week of drought. In transgenic plants, only *HSP100* expression was induced under water deficit (Fig. S6C–D). This result suggests that STANN1 overexpression mitigates cytosolic oxidative stress.

STANN1 mitigates photooxidative stress induced by MeV

Enhanced stress tolerance frequently reduces plant responsiveness to light [~~98].~~67]. The chloroplast antioxidant system is "loosely tailored" to maintain an endogenous ROS pool under control conditions [12], which enables plants to quickly respond to fluctuating light levels. Consequently, significantly improving ROS scavenging enhances protection against sustained stress, but also desensitizes plant light responses and impairs environmental fitness. To verify if annexin-mediated drought tolerance influenced light ~~stress~~ responses, we analyzed the effect of the photosensitizer MeV on transgenic plants overexpressing STANN1. MeV induces oxidative stress, which enables studies of oxidative tolerance and stress cross-tolerance in plants [~~99~~68]. MeV induces an oxidative burst by accepting electrons from PSI and transferring them to molecular oxygen, which results in massive H_2O_2 accumulation in light and generates oxidative stress in chloroplasts.

Leaf disc senescence assay

Leaf discs from WT, S-2 and S-7 plants were exposed to normal light (150 PPFD) in the presence of 10 and 50 μM MeV. The damage caused by MeV was visualized as the degree of leaf tissue bleaching. In the absence of MeV, exposure to light for up to 30 ~~hours~~h had no significant effect on leaf discs. By contrast, exposure to light during MeV treatment induced leaf tissue bleaching, which increased according to MeV concentration (Fig. S7 in Supporting Information file S1Figures). Transgenic plants S-2 and S-7 had higher tolerance to MeV, and exhibited lower levels of leaf disc bleaching in light.

Quantification of ROS and lipid peroxidation

To further analyze STANN1-mediated protection against light stress, leaf discs from WT and S-7 plants were subjected to the combination of relative excess light (850 PPDF) and 50 μM MeV. The levels of superoxide anion, hydrogen peroxide, and malonyldialdehyde (MDA) were quantified at the indicated time points (Fig. 9).

Exposure of WT to excess light and high MeV concentration induced biphasic accumulation of superoxide anions, with an initial peak at 30 ~~minutes~~min after induction and a second, more substantial and long-lasting, peak beginning at 9 ~~hours~~h after induction. In S-7, an initial increase in superoxide anion level was observed, which was significantly lower than that in WT~~..~~. The maximum level of O_2^- was the same in WT and S-7, but the kinetics of the second peak differed (Fig. 9A). In WT, the level of superoxide increased steadily from 6 to12 h after induction. In S-7 superoxide anion accumulation occurred during 6~~–~~9 ~~hours~~h after induction, reaching a similar maximal level as in WT at this time point, and the superoxide level then remained unchanged until 12 h after induction.

In WT, light-induced changes in H_2O_2 level were biphasic, with a second higher and sustained peak (Fig. 9B). The first peak occurred within 30 ~~minutes~~min and the second peak occurred by 12 ~~hours~~h after induction. In S-7, the first peak had a similar magnitude to that in WT. After several hours, no further accumulation of H_2O_2 was observed in S-7, and overall levels were significantly lower than in WT.

Lipid peroxidation, measured as an MDA equivalent, was apparent in WT only after 30 ~~minutes~~min and 12 ~~hours~~h. No statistically significant changes in the lipid peroxidation state were observed under high light stress in S-7 (Fig. 9C).

Annexin 1 attenuates cell death and protects chloroplast structure against oxidative stress

In our experiments, the annexin STANN1 attenuated both phases of chloroplast-derived oxidative stress. In transgenic plants overexpressing STANN1, the expression of nucleus-encoded PSII proteins (Fig. S6A–B) and HSPs was modified correspondingly (Fig. S6C–D). A transient mGFP expression assay was performed to confirm that tolerance to photooxidative stress was due to elevated STANN1 levels. In this experiment, STANN1 was produced as an in-frame C-terminal fusion with mGFP. *N. benthamiana* leaf discs were transformed with STANN1_mGFP (experiment) or mGFP (control) constructs. Leaf discs were then subjected to high light or to the combination of high light and MeV as described above. Leaves with similar fluorescent protein expression levels were used for analysis. Exposure to high light alone had no effect on cell structure, regardless of the construct used (mGFP-alone, Fig. 10A–D; STANN1_mGFP, Fig. 10E–H). High light plus MeV induced cytosol condensation and chloroplast damage (as determined by a decline in chloroplast autofluorescence) in mGFP-expressing cells (Fig. 10I–L). Annexin 1 overexpression attenuated both of these effects, and the cell morphology resembled that of control samples (Fig. 10M–P). Chloroplast fluorescence intensity was quantified and, there was no significant difference in mGFP fluorescence between plants transiently expressing mGFP and STANN1_mGFP. The difference in chloroplast autofluorescence (red) between the mGFP and STANN1_mGFP expressing leaves was statistically significant. This strongly suggests that the chloroplast structure was maintained in the presence of STANN1 protein.

Discussion

This study clearly ~~demonstrate~~demonstrates that elevation of endogenous STANN1 expression can be successfully employed to improve potato tolerance to water deficit. Under optimal conditions, genetic modification had no negative effects on plant phenotype, growth, or productivity. Reduction of the photosynthetic rate in response to water deficit is usually attributed to ROS-induced damage of lipids, pigments, and proteins in the photosynthetic apparatus. Overexpressed STANN1 relieved the negative effects of drought stress, such as degradation of photosynthetic pigments, reduction of photosynthetic activity, and loss of productivity. In transgenic plants, NPQ was induced more rapidly and had higher capacity in STANN1-overexpressing plants, which contributed to increased tolerance to photooxidative stress. Exposure to MeV reduced ROS accumulation and membrane lipid damage, so STANN1-overexpressing plants were not desensitized to light. Consequently, we assume that maintenance of photosynthesis during water deficit was due to protection against drought-induced oxidative stress and/or modification of redox/hormonal signaling in STANN1-overexpressing plants. We propose that manipulation of annexin expression is a valuable new approach for crop improvement that focuses on delay and/or attenuation of leaf senescence and maintenance of physiological processes when plants are exposed to challenging environmental conditions.

Annexin selection for transgenic experiment

Potato annexins ~~form a multigene family that encodes proteins with~~have similar tertiary structures but display different levels of primary amino acid ~~similarities.~~sequence similarity. Despite some extensive structural similarities, the individual annexins

displayed unique expression patterns in the different plant organs (data not shown) and in response to drought. This suggests that the specialization of individual family members towards unique roles in growth/development ~~and~~or adaptation to environmental conditions. ~~Recently~~ Indeed, recently it was shown that functional knock-out of annexin 5 (At1g68090) in Arabidopsis was male-sterile due to the abortion of pollen grains before mature pollen stage; however, on the basis of primary amino acid sequence, no specialized functions could be predicted for annexin 5. Detailed investigations revealed that ATANN5 ~~was expressed primarily during microsporogenesis. The observed pollen lethality was due to impairment in pollen development [100, 101]. This suggests that the specialization of individual annexin family members for unique roles in growth/development may be reflected in specific expression patterns. This also may be observed for adaptation to environmental conditions. It will be necessary to test if ectopic expression of any other Arabidopsis annexin under the ATANN5 promoter restores pollen development.~~

~~This suggests that the specialization of individual annexin family members for unique roles in growth/development may be reflected in specific expression patterns. This also may be observed for adaptation to environmental conditions.~~is the most abundantly expressed annexin during microsporogenesis [69; 70]. It will be necessary to test if ectopic expression of any other Arabidopsis annexin under the ATANN5 promoter restores pollen development.

Expression of four potato annexins was induced during drought. However, STANN1 expression was several-fold higher than other annexins. This strongly suggested that STANN1 was involved primarily in stress responses ~~rather than~~and argued against complementation within the annexin family, at least at the transcriptional level.

STANN1 is expressed in all plant organs (data not shown); therefore, the risk of inducing developmental aberrations due to ectopic *STANN1* expression during development is minimal. Hence, we considered STANN1 as a good candidate to improve drought tolerance in potato and possibly other crops.

STANN1 ~~affects NPQ and~~ mitigates chloroplast-induced oxidative stress in cytosol

~~Chloroplasts~~In light, chloroplasts are one of the major stress-induced ROS sources in plant cells~~.~~ [11]. Abiotic stresses reduce CO_2 assimilation, which results in over-reduction of the PETC [71]. Under these conditions, oxygen can be utilized instead of $NADP^+$ as an alternative acceptor for excess electrons [13]. Disruption of chloroplast redox poise permeates throughout the cell and activates secondary ROS sources in other compartments. In mesophyll cells of *Eupatorium adenophorum*, tenuazonic acid (TeA) inhibits electron flow along PSI and PSII and induces H_2O_2 accumulation in chloroplasts within 1 ~~hour. By 4 hours, H_2O_2 spread to the cell walls facing intercellular spaces [102].~~ h. By 4 h, H_2O_2 spread to the cell walls facing intercellular spaces [72]. The most prominent secondary ROS source is the membrane NADPH oxidase complex. ABA induces RBOH gene expression in Arabidopsis leaves and guard cells [14; 73], the *Hordeum vulgar*e aleurone layer [74], and *Zea mays* seedlings [75]. Furthermore, NADPH oxidase-mediated ROS accumulation has been reported in ozone-treated Arabidopsis leaves [76; 77] and salt-treated Arabidopsis root tips [78].

We provided evidence that photooxidative stress in potato leaves induced a biphasic oxidative burst, with the first transient peak after 1 ~~hour~~h and the second more significant peak occurring by 12 ~~hours~~h. In STANN1-overexpressing plants, both

phases of ROS accumulation were reduced. Biphasic ROS accumulation with a similar kinetics was reported in response to ozone and salt stress treatments [~~33-35~~76-78]. In Arabidopsis and tobacco, the first transient ROS peak occurring after O_3 treatment originated in the chloroplast, whereas the second required NADPH oxidase activity and undisturbed functioning of PETC [~~34~~77].

~~We assume that the first peak of ROS accumulation during photooxidative stress in potato is due primarily to chloroplast-generated ROS, whereas the second resulted from activation of secondary ROS sources.~~ The question arises as to how ~~proteins such as~~ annexins that contain no specific chloroplasts signal sequences ~~that target them to chloroplasts~~ can modulate processes inside chloroplasts and attenuate the first peak. Annexins are found occasionally in chloroplast proteomes of some plants (reviewed in [~~49~~16]). For example, a mustard (*Sinapis alba* L.) annexin was identified as a component of a multisubunit chloroplast RNA polymerase A complex [~~103~~79]; however, these results were not confirmed in a subsequent study [~~104~~80]. Overall, these chloroplast localizations remain exceptions. We believe that annexin-mediated protection of chloroplasts and photosynthesis could be an indirect effect of increased redox homeostasis buffering in the cytosol. ~~In Arabidopsis, the cross-talk between different ROS-scavenging systems in distinct cellular compartments was reported. During light stress, cytosolic antioxidant capacity had an essential role in protecting chloroplasts. Deletion of cytosolic ascorbate peroxidase APX1 induced degradation of thylakoid and stromal/mitochondrial APXs, a cytochrome b6f complex subunit protein, and the small subunit protein of Rubisco [43]. This result is in agreement with observations from other studies [26, 105], which reported that ROS accumulation rapidly reduced chloroplast antioxidant capacity due to APX inhibition~~We proposed

that the first peak of ROS accumulation during photooxidative stress in potato is primarily due to chloroplast-generated ROS, whereas the second peak results from activation of secondary ROS sources, and STANN1 overexpression protects chloroplasts by improving ROS-scavenging systems in the cytosol.

In Arabidopsis, deletion of cytosolic ascorbate peroxidase 1 (APX1) resulted in collapse of chloroplast ROS-scavenging system; this induced degradation of thylakoid and stromal/mitochondrial APXs, a cytochrome b6f complex subunit protein, and the small subunit protein of Rubisco [81]. At the same time, chloroplast APX is one of the very first targets for ROS-mediated inhibition, and ROS accumulation rapidly reduced chloroplast antioxidant capacity [82; 83]. In Arabidopsis and tobacco plants, enhancing the antioxidant capacity of chloroplasts and cytosol has a beneficial effect on photosynthesis and stress tolerance [84], whereas removal of any single antioxidant component reduces photosynthesis and stress tolerance [81; 85-88]. Manipulation of chloroplast/cytosol antioxidant capacity was successfully used to modulate potato tolerance to adverse conditions. Transgenic potato lines engineered to express cytosolic or chloroplast Cu/Zn-superoxide dismutase (Cu/Zn-SOD) from tomato displayed enhanced tolerance to MeV [89]. Overexpression of cytosolic Cu/Zn-SOD from *Potentilla atrosanguinea* improved drought stress tolerance and enhanced net photosynthetic rates [90]. Co-expression of Cu/Zn-SOD and APX in chloroplasts enhanced potato tolerance to multiple abiotic stresses, including chilling, high temperature, photooxidative stress, and drought [91]. Accordingly, the lack of chloroplast thioredoxin CDSP32 resulted in greater susceptibility of potato plants to oxidative stress [92]. Taken together, these data show that elevating cytosolic antioxidant capacity is a promising way to enhance stress tolerance in potato. STANN1

overexpression improved drought tolerance and mitigated photooxidative stress, similarly to that observed for plants with overexpression of ROS-scavenging enzymes. The accumulation of mRNAs coding for cytosolic HSP40 and chloroplast HSP100 was entirely or partially reduced during drought, suggesting that the HSR in transgenic lines developed more slowly and to a lesser extent. This is in agreement with a previous report [46] for *Solanum andigeanum*, in which the expression level of respective *HSP* mRNAs during drought was higher in less tolerant lines than in more resistant landraces.

In summary, ~~chloroplasts~~we assume that annexin-mediated protection of chloroplasts from ROS-induced damage is of utmost importance for plant stress resistance and recovery. Chloroplasts host crucial biosynthesis pathways (e.g., of hormones, Car, amino acids, and lipids) and are the site of cross-talk between basic metabolic pathways and stress responses, which places them in a key position with respect to coordination of defense responses [~~110]. This suggests that protection of chloroplasts from ROS-induced damage is of utmost importance.~~93; 94].

Cross-talk between redox signaling and phytohormone-mediated pathways in transgenic plants overexpressing STANN1

Cross-talk between ABA, SA, CK, JA and other phytohormone pathways modulates plant development and stress adaptation. Our results showed that increased STANN1 expression modified drought-induced hormone accumulation. We assume that this is an indirect consequence of STANN1-mediated modulation of cellular redox homeostasis.

Accumulating data indicate that multi-faceted and multi-level feedback interactions orchestrate hormone- and ROS-mediated signaling networks. Alterations in the cellular redox state were sufficient to modify hormone accumulation and their downstream effects [~~96~~64]. ROS signaling is positioned upstream and downstream of hormone-signaling pathways [~~13, 14~~65; 95]. Redox cues integrate with the action of different phytohormones such as ABA and SA in the coordination of plant growth and stress tolerance [~~111, 112~~95; 96].

~~Annexin elevation reduced the ABA steady-state level, but drought-induced ABA accumulation had similar kinetics in transgenic and WT plants, and levels similar to those in WT were eventually achieved in STANN1-overexpressing plants. Stress-dependent ABA accumulation typically results from elevated ABA synthesis [113]. ABA biosynthesis is induced by protonation of the thylakoid lumen and accumulation of oxidized ascorbic acid as a result of a reduction in PETC efficiency. Oxidized AA cannot function as a cofactor for violaxanthin de-epoxidase, which deactivates the XCar cycle in favor of ABA biosynthesis [114]. In leaves of ascorbate-deficient *vte1* Arabidopsis plants, the ABA level increased by 60% [115]. In transgenic potato plants overexpressing STANN1, the ABA biosynthetic pathway was fully functional. ABA is a key factor in abiotic stress responses (induces stomatal closing and transcriptional reprograming); therefore, it is of utmost importance that ABA content and ABA-dependent stress signaling pathways are similarly activated in WT and transgenic plants. However, the reduced steady-state ABA level indicated that biosynthetic pathways in STANN1-overexpressing plants under control conditions were directed toward XCar activity at the expense of ABA synthesis.~~

The control SA levels in transgenic plants were not significantly different than those in WT plants, and were similar to previously reported values for potato cv Desiree [~~116~~97]. *S. tuberosum* has higher basal SA levels than Arabidopsis, maize, tobacco, or tomato [~~117~~98]. Increases in SA levels in potato are relatively moderate (e.g., two-fold) after infection with *Phytophthora infestans*, compared with a 20-fold increase in Arabidopsis [~~118~~99]. There is a lack of data on SA accumulation in potato leaves during drought, although it has been shown that SA functions as a regulatory signal mediating drought stress responses in several plant species [~~119, 120~~100; 101]. In our experiments, SA increases during water deficit in both plant lines were similar (6-fold in WT and 5-fold in S-7 over basal level), which was in perfect agreement with observations in *Phillyrea angustifolia* [~~121~~102]. However, the SA peak was observed in WT plants only after rewatering, whereas in transgenic potato it occurred earlier, even during drought.

Recently, it became clear that SA is an important regulator of photosynthesis. In Arabidopsis, SA influences plant photosynthetic performance, and properly balanced SA levels are necessary for acclimation to changing light [~~122, 123~~103; 104]. The SA-mediated signaling pathway in Arabidopsis is involved in optimal photosynthetic activity under stress conditions by modulating redox homeostasis [~~124~~105]. SA enhances the cell antioxidant capacity during drought, although the mechanism of this process is unclear. Endogenous SA deficiency in potato results in ineffective induction of stress defense system and enhances stress sensitivity [~~116, 125, 126~~97; 106; 107]. During plant response to pathogen infection, SA inhibits the ROS-scavenging enzymes CAT and APX [~~127-129~~108-110] and stabilizes H_2O_2 levels. SA and H_2O_2 function as a positive-feedback amplifying loop; if not properly balanced, this loop exerts detrimental

effects for cell survival. In rice, reduced endogenous SA levels enhanced H_2O_2 accumulation and the appearance of spontaneous necrotic lesions during senescence and development of oxidative damage and in response to high light intensities [~~130~~111]. SA accumulation induces different responses depending on the timing and accumulation level; it induces stress-responsive defense systems such as antioxidant enzymes, or induces PCD in response to long-term elevations of SA levels. In WT plants, slow and prolonged SA accumulation despite the resumption watering may ultimately lead to PCD. Rapid SA accumulation in transgenic plants appears to indicate more efficient mobilization of SA-induced stress responses, and accounts for improved photosynthetic performance.

Recent work shows that CK has an important role in plant adaptation to environmental stresses such as drought, cold, osmotic stress, and light stress [~~131-134~~112-115]. In our experiments, CK species and their levels under control conditions were similar to those previously reported for potato cv Desiree [~~135~~116]. STANN1 overexpression did not influence CK profiles or steady-state levels, but CK levels were maintained during water deficit and rapidly increased after rewatering.

CK antagonizes many ABA-induced physiological responses to drought such as stomatal closure or leaf senescence [~~136~~117]. Maintaining CK biosynthesis during drought improves stress tolerance, confers protection against photooxidative stress, and mitigates reductions in photosynthesis [~~38, 83, 137-141~~118-123]. CK activity is anti-senescent and associated with maintenance of greater antioxidant activity. In creeping bentgrass, elevated CK levels due to senescence-driven expression of isoprenyl transferase (IPT), a key enzyme in CK biosynthesis pathways, conferred drought resistance, increased the levels and activity of scavenging enzymes such as APX and

CAT1, and reduced MDA accumulation [~~142~~124]. Similarly, elevated CK levels in tobacco plant leaves and chloroplasts conferred higher physiological parameters than those in controls [~~143~~125], and increased APX and dehydroascorbate reductase (DHAR) activity, which prevented over-oxidation of the chloroplastic ascorbate (ASC) pool. CK regulates stress responses on several levels, such as inducing stress-inducible gene expression [~~144-145~~126; 127], including peroxidases, GRX, and glutathione S-transferases~~.~~ (GSTs). Plants with reduced CK levels had lower ROS-scavenging capacity, exhibited more severe photodamage after high~~-~~ light treatment, and had reduced neoxanthin and Zea levels under control conditions, which declined further during photooxidative stress~~.~~ [128]. Similar effects were observed in scavenging enzyme activities, and a strong reduction in APX and SOD activities were observed under control conditions and in response to light stress [~~146~~128]. We assume that sustained biosynthesis of CK during drought in transgenic potato plants overexpressing STANN1 remediates oxidative stress and improves photosynthetic performance.

STANN1 overexpression affects ABA accumulation and NPQ

ABA is a key factor in abiotic stress responses (induces stomatal closing and transcriptional reprograming); therefore, it is of utmost importance that ABA content and ABA-dependent stress signaling pathways in WT and transgenic plants are similarly activated. The ABA biosynthetic pathway is a side-branch of the Car biosynthetic pathway, with Viol being a direct precursor [129]. Viol is synthesized from Zea by zeaxanthin epoxidase, which is constitutively active in darkness and sub-saturating light; hence, under such conditions, the level of Viol far exceeds the level of its precursor. Instead, under saturating light (when the proton gradient produced is too

high to be entirely consumed for CO_2 assimilation), the level of Zea increases as a consequence of violaxanthin deepoxidase (ViolDE) activation, which requires reduced ASC as an electron donor. Hence, the actual level of Zea is determined mainly by the processivity of ViolDE [130]. Elevation of the annexin level resulted in reduced the ABA steady-state level and concomitantly increased the relative content of photoprotective Zea and Viol, which suggests that the ABA synthetic pathway was disabled. However, drought-induced ABA accumulation had similar kinetics, and similar maximal ABA levels were eventually achieved in transgenic and WT plants.

Both in control conditions and during drought, the levels of Viol and Zea were higher in STANN1-overexpressing plants than in WT plants. Partitioning of Viol into competing biosynthetic pathways (reconversion to Zea or ABA biosynthesis) depends on the chloroplast ASC status. The accessibility of reduced ASC promotes ViolDE activity resulting in Zea accumulation, whereas depletion of reduced ASC activates ABA biosynthesis. In leaves of ASC-deficient *vtc1* Arabidopsis plants, the ABA level is increased by 60% [131]. The Arabidopsis mutant *npq1* with no functional ViolDE does not accumulate Zea in HL. This is accompanied by increased photodamage of photosynthetic apparatus (reduction in CO_2 assimilation and elevated lipid peroxidation) and strongly inhibited NPQ [132].

Overall, the plant capacity to dissipate excess light energy in a non-photochemical manner is also affected by redox poise. In leaves of two Arabidopsis ASC-deficient lines that over-accumulate ABA, *vtc1* and *vtc2-2*, NPQ at HL is decreased [133; 134]. Regeneration of the ASC pool is maintained mainly by DHAR [135] and suppression of DHAR in Arabidopsis results in lower induction of NPQ, while increased DHAR expression enlarges the size of the XCar pool [135]. For activity, DHAR requires

glutathione as an electron donor, and in Arabidopsis *pad2-1* mutants, a shortage of glutathione also impairs NPQ and compromises adaptation to severe drought stress [136]. Similarly, an increased level of another lipid-soluble antioxidant, α-tocopherol, restores the control level of NPQ in the Arabidopsis *npq1* mutant lacking Zea [137]. Over-accumulation of α-tocopherol in the *Chlamydomonas reinhardtii* double mutant *npq1 lor1* (lacking both Zea and lutein) restores tolerance to HL and tolerance to oxidative stress [138]. These results suggest that NPQ is dependent on redox homeostasis, probably due to the effect on the xanthophyll cycle and Zea accumulation. Hence, an enhanced buffering capacity in the cytosol upon overexpression of STANN1 could result in improved NPQ.

STANN1 affects redox homeostasis

Different hypotheses have been proposed to explain the molecular basis of annexin-mediated alleviation of oxidative stress, including innate peroxidase activity [~~54, 147, 148~~21; 139; 140], calcium-induced stabilization of peroxidases activity [~~149~~141], and modulation of calcium influx [~~62, 150~~142; 143]. Based on the results of our experiments, we assume that annexin-mediated reduction in oxidative stress in transgenic potato overexpressing STANN1 results from the annexin effect on thiol-disulfide homeostasis.

Downstream transmission of several environmental cues for H_2O_2 accumulation is sensed and mediated by several ROS-neutralizing systems, which are low-molecular-weight antioxidant buffers such as ~~ascorbate (~~ASC~~)~~ and glutathione (GSH), and oxidoreductases such as GRX, TRX, and scavenging enzymes [~~39~~144]. The most prominent ROS-scavenging and redox-signal perception system is GSH accumulation

and GSH oxidation to disulfide (GSSG) during ~~ascorbate~~ASC regeneration in the glutathione-~~ascorbate~~ASC cycle [~~151, 152~~145; 146]. This type of redox imbalance is transduced downstream by reversible formation of a mixed disulfide between GSH and a target protein (*S*-glutathionylation). An increased GSH:GSSG ratio was observed in plants exposed to chilling, heat stress, heavy metals, xenobiotics, drought, ozone, pathogen [147-153~~-159~~], and during oxidative stress resulting from deficiency in the H_2O_2-scavenging photorespiratory enzymes CAT or APX [~~36, 47, 160-164~~88; 155-158]. In maize and rice, the ability to maintain higher GSH:GSSG ratios was associated with greater stress tolerance [~~164~~158]. GSSG accumulation is a key determinant of cell death and growth arrest [~~165, 166~~159; 160].

Immunolocalization studies revealed that stress-triggered GSSG accumulation occurred in discrete subcellular compartments. Localization studies in Arabidopsis detected little or no accumulation in mitochondria, slight but significant accumulation in the cytosol, and prominent accumulation in vacuole and chloroplasts [~~162~~157]. GSSG sequestration in metabolically inert vacuole is thought to initiate catabolism, whereas accumulation in chloroplasts could have functional consequences for photosynthetic efficiency. Increased GSSG level is sufficient to trigger protein *S*-glutathionylation [~~167~~160], which is thought to regulate enzymatic protein activity [~~168~~161]. A large number of unidentified targets of this posttranslational modification represent chloroplast proteins (e.g., RuBisCO or glucose-6-phosphate dehydrogenase) [~~167~~161]. However, it is not clear if GSSG accumulation in chloroplast results from import or *in situ* synthesis. Isolated wheat chloroplasts can take up GSSG from the medium [~~36~~154]. Re-engineering of compartment-specific glutathione synthesis pathways suggested that cytosol-to-chloroplast GSSG transport also occurs *in vivo* [~~169~~162]. Specific GSSG

transporters have been identified in tonoplast but not in the chloroplast envelope [~~170~~163].

The presence of redox-sensitive cysteines has been shown for mammalian ANXA2 [~~171~~164] and ANXA1 [~~172~~165]. They are located in the extended C- or N -terminal end (Cys324 aa for ANXA1, and Cys8 and Cys334 for ANXA2), which confer structural diversity to proteins from this family. It cannot be easily generalized if other annexins contain cysteines susceptible to oxidation. ANXA2 was proposed to directly neutralize H_2O_2 with accompanying oxidation of only Cys8 [~~173-175~~166; 167]; subsequently, it would be reduced via the NADPH-dependent thioredoxin system (NTS) being thus an ultimate acceptor of electrons from NADPH. Oxidative damage in annexin A2-depleted cells enhanced oxidation of the ANXA2-binding proteins actin and transcription factor JunD. This suggests that ANXA2 can function directly as a protein reductase [~~174~~168].

The presence of reactive cysteines was confirmed in Arabidopsis annexin ATANN1. Implicated amino acids are localized within the endonexin domains and are highly evolutionarily conserved [~~53~~20] (Fig. S3 in Supporting Information file S1Figures). ATANN1 Cys underwent *in planta S*-nitrosylation within 20 ~~minutes~~min after NO treatment [~~176~~169] and *S*-glutathionylation within 30 ~~minutes~~min after ABA induction [~~56~~23]. MeV treatment resulted in oxidation of both cysteines residues of ATANN1 (Cys111 and Cys 239), although the exact type of modification (mixed disulfide bonds with GSH, or intramolecular disulfide bond) has not been defined [~~177~~170]. The closest ATANN1 homolog in *Brassica rapa*, BRANN1, appears to form a complex with peroxidase in floral buds [~~178~~171].

STANN1 contains two cysteine residues, Cys17 and Cys111. Among potato annexins, the former is unique for STANN1 and STANN3.2, whereas the latter is homologous to Arabidopsis Cys111. This arrangement resembles that of ANXA1, with reactive Cys8 in the N-terminal amino

acid. Elevated STANN1 levels mitigated photooxidative stress and diminished ROS accumulation, which suggested that STANN1 enhanced the capacity of cytosolic antioxidant buffer. Therefore, it appears that the evolutionarily conserved cysteine homologous to ATANN1 Cys239 is not necessary for such activity. Plant annexins can prevent ROS over-accumulation in a similar way to that previously shown for ANXA2: by direct ROS neutralization and further regeneration by NADPH-dependent thioredoxin/glutaredoxin system. Therefore, annexin would function as an ultimate acceptor of excess electrons leaking from over-reduced PETC. Alternatively, STANN1 may be used as an acceptor for ROS diminishing thus GSSG formation (Fig. 11). Annexins are abundant cytosolic proteins (accounting for up to 2% of the total soluble proteins). They possess redox-sensitive cysteines and could participate significantly in the cellular protein thiol pool. In transgenic plants overexpressing STANN1, annexin levels are higher and the antioxidant protective effect is increased. Reduced GSSG accumulation prevents a decline in the GSH:GSSG ratio and over-oxidation of the cellular environment. The latter mechanism could explain the broad-specificity of annexin-mediated protection, which is functional in bacteria and photosynthetic and non-photosynthetic eukaryotic cells. Glutathione is one of the most abundant non-protein thiols; it is present in cyanobacteria and proteobacteria, and in all mitochondria- or chloroplast-containing eukaryotes [~~179, 180~~172; 173]. The mechanism of GSH-mediated regulation and maintenance of cellular redox status is similar in all living organisms. Reversible oxidative thiol modifications modulate the function of proteins involved in many different pathways, including gene transcription, translation, protein folding, metabolism, signal transduction, and apoptosis**.**

Conclusions

The results obtained in this study clearly indicate that annexin overexpression has potential application for developing drought-tolerant crops. Enhanced drought tolerance

in transgenic potato overexpressing STANN1 confers greater tolerance to high- light stresses, stomatal closure, and diminished CO_2 supply. ROS accumulation was attenuated, which improved chloroplast function; genetically modified plants maintained efficient PSII under stress conditions. Maintenance of a high photosynthetic yield under sub-optimal conditions had a beneficial effect on crop yields and biomass production. Annexins are a promising target for manipulation of plant tolerance to environmental conditions.

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Figure legends.Figure 1. Annexin genes in potato genome.

(A) Localization of annexin genes on potato chromosomes. The Roman numerals at the top denote the chromosome, digits in brackets indicate chromosome size.

(B) Intron-exon organization of potato annexin genes.

(C) Genomic PCR confirming the presence of predicted annexin genes in WT potato. Specific primers anneal to the 5'- and 3'- ends of coding sequence of certain annexin gene, hence the length of the resulting PCR product is a sum of the respective coding sequence with introns.

(D) Schematic arrangement of *STANN3.1*, *STANN3.2*, *STANN3.3* and *STANN4* on chromosome I.

Figure 2. Profiling of annexin expression in WT potato leaves during drought.

Potato WT plants grew in the walk-in growth chamber under controlled conditions. After 8-10 weeks irrigation was gradually reduced to decrease the field capacity (FC) to 25% (which took approximately 10 days) and then maintained at this level till 14th day. Samples were collected from the first fully developed composite leaf from the top at indicated time points (D0 – beginning of drought, D6 – sixth day of drought, and D14 – fourteenth day of drought). RNA was isolated with Trizol and sq-RT-PCR was performed with primer sets specific for certain annexins. The level of expression was normalized against *EF1a* mRNA. Results are means ±SE ($n \leq 4$). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 3. Drought tolerant phenotype of transgenic plants.

Potato WT plants and transgenic lines (S-2, S-7) was subjected to drought as described above in Fig. 2. (A) Drought stress phenotype of WT (left column), S-2 (middle column) and S-7 (right column) plants. Photographs were taken on the beginning (D0), on eighth (D-8) and ninth (D-9) day of drought. Experiments were repeated twice in greenhouse and twice in growth chamber and gave similar results.

(B) Regeneration of potato plants after prolonged drought. The procedure of drought imposition was the same as described above but the FC was maintained at 25% until the twenty first day of drought (D21). On D22 plants were rewatered and after draining of gravitationally bound water FC was kept up at 65%. Photograph was taken on the third day after rewatering. Left side - two WT plants; middle – two S-2 plants, and right – two S-7 plants. Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.

Figure 4. Examination of leaf water status.

Potato WT plants (white bars) and transgenic lines: S-2 (gray bars) and S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. (A) Relative water content (RWC) analysis. Samples from the first fully developed undamaged leaf from the top of plant were collected at D0, D4, D7, D12 and 3 days after rewatering (RW3) and relative water content (RWC) was determined. Results are means ±SE (n=3). (B) Stomatal conductance were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-

310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results.

Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated 3 times and gave comparable results.

Figure 5. Netto photosynthesis and photosynthetic performance of PSII in potato plants during drought.

Potato WT (white bars) and transgenic lines: S-2 (grey bars) and S-7 (black bars) were subjected to drought as described in Fig. 2. (A) Netto photosynthesis, (B) maximum quantum yield of photosystem II (Fv/Fm) and (C) effective quantum yield of photosystem II,Y(II) were measured in fully expanded, attached leaves at D0, D3, D6, D10 and RW3. After D10 the leaf surface was wrinkled to such an extent that further analysis was impossible. Measurements were done with a CI-510CF Chl fluorescence module, actinic light was provided by a CI-310LA light attachment. Results are means ±SE (n=10). Experiment was performed three times and gave comparable results. Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values which are not significantly different at $p<0.05$ and belong to the same homogenic group.

Figure 6. Photosynthetic pigment content during drought.

WT (white bars) and transgenic line S-7 (black bars) were exposed to drought as described in Fig. 2. Samples were collected at the same time during the day at D0, D6, D14 and RW3 from third, fourth and fifth fully expanded leaves from top at 4 hours after turning the light. The level (A) chlorophyll *a*; (B) chlorophyll *b*; (C) zeaxanthine; and (D) violaxanthine were

determined Non-polar lipids were separated on an ACQUITY UPLC system (Waters) and peaks were integrated at 436 nm. The level of xanthophylls is expressed as percent of the total carotenoids. The level of chlorophyll is expressed as mg mL^{-1}. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 7. NPQ assayed in leaf of well-watered potato plants.

Potato WT (dashed line) and transgenic S-7 (solid line) grew in the walk-in growth chamber under controlled conditions and were watered to maintained FC at 65%. Performance of gross non-photochemical quenching (NPQ) were assayed on the first fully developed composite leaf from the top of plant at 4 hours after turning the light with Dual PAM-100. For measurement plants were adapted to dark for 20 minutes and then stimulated with repeated light pulses of actinic light (94 PPFD) for 5 minutes and once again subjected to dark for 6 minutes. Each point represents the mean ±SD (*n*=3-4). Experiment was repeated three times and gave comparable results.

Figure 8. Accumulation of stress-related hormones during drought.

WT (white bars) and transgenic line S-7 (black bars) were subjected to 14-day drought as described in Fig. 2. The level of (A) absicisic acid ABA; (B) sum of active cytokinins, CK; (C) salicylic acid, SA were determined at D0, D6, D14 and RW1. Samples (0.5g of fresh leaf tissue without the midrib) were collected from the first fully developed, undamaged leaf from the top of plant at 4 hours after turning the light. Labeled internal standards were added to the leaf samples before homogenization. Hormones were then extracted, purified using a SPE-C18 column and separated on a reverse phase-cation exchange SPE column. Hormones were quantified using a hybrid triple quadruple/linear ion trap mass spectrometer. The level of

ABA and SA is expressed as nmol g^{-1} of fresh weight; the levels of cytokinins – as pmol g^{-1} of fresh weight. Results are means ±SE (n=3). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$).

Figure 9. Accumulation of ROS (hydrogen peroxide and superoxide anion) and lipid peroxidation.

Potato WT (white bars) and transgenic line S-7 (black bars) grew in walk-in growth chamber under controlled conditions. Leaf discs were expunged from the third, fourth and fifth upper fully expanded leaves and immediately vacuum infiltrated with methyl viologen (50 μM). After 1 hour incubation in dark discs were exposed to high light irradiance (850 PPFD) for indicated times (0.5 – 24 hours). Superoxide anion was determined colorimetrically with nitro blue tetrazolium chloride 9NBT). Hydrogen peroxide was stained in tissue with diaminobenzidine tetrahydrochloride (DAB) and quantified using the ImageJ. Lipid peroxidation was estimated spectrophotometrically with thiobarbituric acid (TBA). Results are means ±SE (n=5). Homogenic groups are determined by Tukey HSD (Honestly Significant Differences) test. The same letters designate values belong to the same homogenic group ($p<0.05$). Experiment was repeated twice.

Figure 10. STANN1 attenuated MeV-induced photooxidative stress.

Confocal laser scanning image of the leaf epidermis of tobacco plant transiently expressing GFP (A-D and I-L) or STANN1_GFP (E-H and M-P). 3 days after infiltration leaf discs were excised and subjected to high light (850 PPFD) (A-H) or the combine treatment of high light (850 PPFD) and 50 μM MeV (G-L). The fluorescence was monitored with Nikon TE-2000E EZ-C1 exc. 488 nm and emission 515/30 and

605/75 for GFP and chloroplast, respectively. First column represent single focal plane, second – chloroplast autofluorescence acquired with the same excitation parameter for each construction to visualized the difference between responses to the same treatment, third – overlay of green and red fluorescence channels with GFP enhanced to visualized cells; right column – stack obtained with Volume Render program EZ-C1 combined with chloroplasts. Scale bar is 20 μm. Experiment was performed 3 times.

Figure 11. A simplify scheme depicting the interactions between cellular redox state and participation in ROS scavenging mechanisms.

Oxidative stress is an unavoidable consequence of environmental stresses. ROS accumulation begins in chloroplasts and then it spreads throughout the whole cell. Activation of a secondary ROS sources e.g. NADPH oxidase complex or photorespiration resulted in substantial H_2O_2 accumulation in cytosol. To avoid deleterious effects of ROS several compartment-specific mechanisms evolved, including accumulation of low-molecular-weight antioxidants (glutathione, ascorbate), scavenging enzymes (CAT, APX, SOD) and protein thiols (PRX, GRX and TRX) that undergoes a reversible cycles the thiol-disulphide exchange.

The redox-sensitive proteins sense, transduce, and translate ROS signals into appropriate cellular responses. Thus, precise regulation of size and redox status of the thiol pool is of essential importance for induction of appropriate responses. In plant cells glutathione is present in different compartments in milimolar concentrations and in quiescents it maintained largely in reduced state due to activity of glutathione reductases (GR) at expense of NADPH. Stress-induced ROS accumulation stimulates oxidation of glutathione (GSSG) and in the same time *de novo* synthesis of GSH. Disturbances in

GSH/GSSG ratio might non-specifically influence several downstream pathways, e.g. by induction of thiol-disulfide exchange on target proteins. Cellular redox potential depends primarily on the total concentration of the total glutathione and the extend of its oxidation. GSSG accumulation did not disturb the redox potential if it is compensated by increasing the total glutathione concentration. However, if size of total pool remains unchanged when the GSH:GSSG ratio increased the cell redox potential in the cytosol become more positive.

We propose that the improved stress tolerance of annexin STANN1-overexpressing potato plants results from amelioration of oxidative shift of the cytosolic glutathione redox potential. Elevation of STANN1 level had a pleiotropic effect on plant metabolism and physiology what suggested that not one specific but several downstream signaling pathways were touched. Disruption of the glutathione redox potential is sufficient to induce such effect; e.g., in transgenic tobacco with constitutive upregulation of glutathione content MAPK and SA signaling pathways were modified. Annexin posses oxidation-sensitive cysteines and can act as a reductant influencing thus the redox potential. During stress in transgenic plants the capacity of cytosol redox buffer was more reducing compared to WT what prevents oxidation of downstream targets and modulate timing as well as magnitude of stress response. It had a beneficial effect on cell survival, photosynthesis and delay senescence. Similar effects were observed in tobacco and Arabidopsis plants and over-expressing particular elements of antioxidant systems.

Supporting information legends:

File S1Figures.

Figure S1. Construction of transgenic plants.

A) Structure of the T-DNA region from pROK2 carrying STANN1_His6x that was used for *Agrobacterium* -mediated transformation. LB – left border; RB – right border; NPTII – neomycin phosphotransferase II, CaMV – cauliflower mosaic virus 35S promoter; NOS – nopaline synthase terminator;

(B) Expression of STANN1_His6x protein in F1 transgenic potato lines. Proteins were isolated from leaves of WT and F1 transgenic lines S-2, S-3, S-7, S-83, S-91, S-97 and S-123 grown *in vitro*. His-tagged proteins were purified with Ni-NTA agarose, subjected to SDS_PAGE and blotting followed by detection with anti-His primary Ab. The band detected in WT represents *Arabidopsis* annexin ATANN1_His6x (molecular weight *ca* 36 kD) produced in *Escherichia coli* that was added before purification to the ground protein to STANN1_His6x easily dimerized hence the two bands were detected, the lower with molecular weight corresponding to monomer and the upper corresponding to dimer.

Figure S2. Characteristics of experimental drought.

(A) Potato WT and S-7 plants after 8 week of growth at the phase of experimental drought implementation. Transgenesis has no impact on tuber development. Formation of stolon hooks and stolon swelling as well as first tubers are visible.

(B) Field capacity (FC) was normalized at the beginning of experiment and maintained at constant level (app. 65%); for control (well-irrigated plants) FC was maintained at this level throughout the whole experiment. For experimental drought FC was gradually lowered to 20% and kept at this level un till the end of drought. Rewatering was applied by full water saturation of the soil and after gravity draining of excess water FC was kept at the 65% untill the end of experiment.

Figure S3. Multiple alignment of amino acid sequences of putative annexins from potato and selected annexins from human, Arabidopsis and cotton.

The alignment was done with Cobalt (Constrain-based Multiple Alignment Tool). Gene Bank Acc Nos of employed sequences are as follows: human AnxA5 (NP_001145.1), Gossypium hirsutum GHANN1 (1N00), Arabidopsis thaliana ATANN1 (2Q4C) and for potato annexins: STANN1 PGSC0003DMG4000177114, STANN2, STANN3.1 PGSC0003DMG4000221817, STANN3.2 PGSC0003DMG401019427, STANN3.3 PGSC0003DMG402019427, STANN4 PGSC0003DMG400019446, STANN5 PGSC0003DMG400007966, STANN8 PGSC0003DMG400007482 and STANN9 PGSC0003DMG40001879.
The boundaries of endonexin repeats were marked on the basis of crystal structures obtained for GHANN1 (Hofmann et al., 2003) and ATANN1 (Levin et al., 2007) and are, respectively:
- 1st endonexin domain: 14-80 and 13-81;
- 2nd endonexin domain: 83-153 and 84-154;
- 3rd endonexin domain: 164-239 and 165-241
- 4th endonexin domain: 241-309 and 244-3111 respectively for cotton and Arabidopsis annexin.
Conserved histidine 40 is in red; methionine and cysteines from C3 cluster are in blue and underlined.
Calcium binding motifs G-X-GTD-{38-40}-D/E are marked by black boxes; potential N-terminal acylation motif is in bold; potential actin-binding domains IRI are in bold and italic;
C-terminal peptide similar to 14-3-3 proteins is marked by pale-green rectangle.
Amino acid residues of high conservation are shown in red, medium - in blue.

Figure S4. Drought tolerant phenotype of transgenic S-7 potato plants.

Each image depicts two WT plants (left side) and two transgenic S-7 plants (right side) subjected to experimental drought. Drought was started on D0 and lasted 21 days. During that time watering was gradually reduced so as to lower the FC to 20%. After reaching that level it was maintained until 21 days after onset of experiment. The soil was than fully saturated with water (rewatering) and FC was maintained at 65% until the end of experiment.

D10 - irrigation withheld for 10 days, D14 - irrigation withheld for 14 days, D21 - irrigation withheld for 21 days, RW5 – rewatered for 5 days.
Experiments were repeated four times and similar results were obtained both in greenhouse and in growth chamber.
In WT symptoms of wilting clearly appeared after 10 days of drought; in S-7 they were apparent only after 2 weeks. On the 21st day WT were severely affected with damaged stems and dry leaves. At the same time in S-7 plants the upper leaves still maintained turgor. After rewatering only a few leaves in WT regenerated; instead, new shoots developed from below-ground parts after at least a week of regular irrigation. In contrast, the S-7 plants preserved their upper leaves and after rewatering returned to a normal healthy look within hours. The exact number of irreversibly damaged leaves varied between experiments, but it was always significantly lower than in WT.

Figure S5. Potato yield during drought.

(A) Irrigated Water Use Efficiency (IWUE) is a quotient of crop produced per unit per amount of water supplied (IWUE = Y / W [g/pot/mL of water])
(B) An exemplary tuber yield per plant. Potato plants WT, S-2, and S-7 were grown in a greenhouse. After 8-10 weeks of growth plants were subjected to drought stress by restricting irrigation to achieve 20% FC and kept at this level until 14th day. After that time plants were rewatered and cultivated in optimal conditions for additional 10 weeks until physiological maturity. Tubers were lifted immediately after withering of haulms. The weight of all fresh tubers from single plant was determined immediately after harvesting. Experiments were repeated twice and gave similar results.
(C) Quantification of tuber yield experiments. Results are shown as mean ±SD (n=10)

Figure S6. Expression of genes coding for PSII proteins and HSPs.

Relative quantification of *PSBS* (A), *LHCB4* (B) *HSP100* (C) and *HSP40* (D) mRNAs in leaves of WT (white bars) and transgenic S-7 (black bars) potato plants during three-week drought and after rewatering. The data represents the mean ±SE from at least four measurements. Homogenic groups are determined by Tukey HSD

(Honestly Significant Differences) test, the same letters designate days which are not significantly different at $P < 0.05$ and belong to the same homogenic group.

Figure S7. The effect of photooxidative stress on potato leaves.

Leaf discs (F ~ 1 cm) were excised from leaves of WT or transgenic plants S-2 and S-7 and immediately infiltrated with (A) 50 mM Tris-Cl, pH 7.5 (B)10 mm MeV or (C) 50 mM MeV in 50 mM Tris-Cl, pH 7.5. Subsequently, leaf discs were exposed to light of 150 PPFD for 30 h.

File S2Tables.

Table S1. Primer pairs used for identification of potato annexins.

Primer pairs corresponding to the predicted 5' (F) and 3' (R) ends of the particular annexin genes were designed on the basis of published potato genome sequence. Gene length refers to the total length of exons and introns. Individual primer pairs (F - forward, R - reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA).

Table S2. Primer pairs used for sq-RT-PCR.

Primers for semi-quantitative analysis of expression of annexins and other genes in potato. Individual primer pairs (F- forward, R- reverse) were designed with PrimerSelect, Laser Gene10.0 DNASTAR (USA) to span intron–exon boundaries to exclude interference from genomic DNA contamination. Amplified fragments were between 300 and 500 base pairs. The genes were selected from PGSC_DM_v3.4_pep_fasta containing database of potato virtual translation products on the basis of their homology to annotated Arabidopsis genes. Analyzed genes were as follows: annexins: STANN1-9; HSP100 (heat shock protein 100 kDa); HSP40 (heat shock protein 40kDa, DNAJ); PSBS (chlorophyll a/b- binding photosystem II 22kD subunit S); LHCB4 (light-harvesting complex binding protein 4). As a reference the housekeeping gene for Elongation Factor a1 (EF1a) was used.

Table S3. Characterization of putative potato annexin proteins.

chlo – chloroplast; cyto – cytoplasm; cyto_ER – cytoplasm/membrane of endoplasmatic reticulum; cysk – cytoskeleton; ER – endoplasmatic reticulum; extr – extracellular; mito – mitochondria; nucl – nucleus; plas – plastids; vacu – vacuole

Table S4. Cytokinins in leaves of WT and S-7 potato plants under drought.

S. tuberosum WT and transgenic S-7 plants were subjected to 2-week drought or well-watered. At time points indicated 0.5 g of tissue (without the main vein) was collected 4 hours after beginning of the day from fully expanded leaves. Hormone levels were analyzed by LC-MS as described in Materials and Methods (n=3). Data are shown as pmol g-1 FW.

Abbreviations: tZR, trans-zeatine riboside; tZ, trans-zeatin; iPR, isopentenyl adenosine riboside; iP, isopentenyl adenine; cZR, cis-zeatin riboside; cZ, cis-zeatin.