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Expression of the *Arabidopsis* high-affinity hexose transporter STP13 correlates with programmed cell death

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Abstract **We report the biochemical characterization in *Xenopus* oocytes of the *Arabidopsis thaliana* membrane protein, STP13, as a high affinity, hexose-specific H^+-symporter. Studies with kinase activators suggest that it is negatively regulated by phosphorylation. *STP13* promoter GFP reporter lines show GFP expression only in the vascular tissue in emerging petals under non-stressed conditions. Quantitative PCR and the p*STP13*-GFP plants show induction of *STP13* in programmed cell death (PCD) obtained by treatments with the fungal toxin fumonisin B1 and the pathogen *Pseudomonas syringae*. A role for STP13 in PCD is supported by microarray data from e.g. plants undergoing senescence and a strong correlation between *STP13* transcripts and the PCD phenotype in different accelerated cell death (*acd11*) mutants.**

Keywords: Sugar transport protein; Programmed cell death

1. Introduction

Sugars serve as essential sources of energy and carbon, but are also important signaling molecules modulating light and hormonal responses in plants [1,2]. Sugar transport and regulation of sugar homeostasis by transporters is essential for the plant life-cycle as exemplified by severe growth inhibition and sterility in knockout mutants of the *Arabidopsis thaliana* sucrose transporter *SUC2* [3]. The importance of regulated sugar localization is further demonstrated by heterologous expression of invertases, enzymes degrading sucrose into glucose and fructose, in different compartments in tobacco [4,5]. Apoplastic or vacuolar expression of invertase leads to stunted growth, development of spontaneous necrotic lesions, and activation of resistance responses, whereas cytoplasmic overexpression produces no visible phenotype.

In *Arabidopsis*, the disaccharide sucrose is transported by members of the sucrose carrier family (SUC) [6] and monosaccharides are transported by members of the sugar transport protein (STP) family that includes 14 predicted members and probably by several other of the more than 50 transporters, encoded in the *Arabidopsis* genome, that share significant homology to major facilitator superfamily (MFS) monosaccharide transporters [7,8]. STP1 [9], STP2 [10], STP3 [11], STP4 [12], STP6 [13], STP9 [14] and STP11 [15] have been characterized biochemically and exhibit broad substrate specificity towards both D-hexoses and D-pentoses with K_m in the range of 10–100 μM. Notably, STP6 is hexose-specific and is the only characterized STP that exhibits significant affinity towards fructose. STPs differ substantially in their developmental, environmental and spatial expression [16]. For example, STP2 has a specific role in resorption of glucose released from callose degradation upon pollen maturation [10].

Several observations suggest that sugar homeostasis and sugar transport play important roles in plant defense and PCD, and three *Arabidopsis* STPs have been suggested to play a role in plant defense based on their expression profile. *STP4* transcripts are induced upon infection by a range of pathogens [17,12], *STP1* mRNA levels increase upon treatment with the defense-related plant hormones salicylic acid and methyl jasmonate [18,19] and expression of the low affinity transporter *STP3* is induced by wounding [11]. Similarly, the *Arabidopsis* sucrose transporters *SUC2* and *SUC3* are induced upon infection with the beet cyst nematode *Heterodera schachtii* [20], and upon wounding [21], respectively. Putative monosaccharide transporters of the MFS have been associated with senescence, as evidenced by induction of *Arabidopsis SFP1* in aging leaves [22], and by induction of the hexose transporters *CST2* and *CST3* in *Chenopodium rubrum* suspension culture cells treated with plant hormones that regulate senescence [23]. In mammals, there is precedence for glucose starvation and the monitoring of glucose transport as an essential part of execution of PCD [24]. However, the precise link between sugar transport(ers) and PCD remains unclear.

We recently identified STP13 (At5g26340) as a glucose transporter in a functional genomics approach for screening plant transporter functions by expression cloning in *Xenopus* oocytes (Nour-Eldin, H.H., Nørholm, M.H.H. and Halkier, B.A., unpublished data). Here, we characterize STP13 biochemically and show a clear correlation between induction of the *STP13* and the appearance of PCD, which suggests a role of this transporter in PCD.

2. Materials and methods

2.1. PCR and in vitro transcription

STP13 cDNA was amplified with Pwo Polymerase (Roche) from the EST clone RAFL06-74-007 from Riken BRC [25,26] with the primers T7 and RAFL 4–6 3 (Table 1) that introduced a 5′ T7 promoter and a

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Table 1
Primers used in this work

Primer	Sequence
T7	AATTAACCCTCACTAAAGGGTTGTAATA CGACTCACTATAGGG
RAFL 4–6 3	TTTTTTTTTTTTTTTTTTTTTTTTTTGCTATG GCCCTTATGGCCGAGCTCT
actinF	GGTCGTACTACCGGTATTGTGCT
actinR	TGACAATTTCACGCTCTGCT
stp13F	TCGAAAGAGGTCGTCTCGAT
stp13R	ACATTGCTGGAAAATCTGTAAAGC
STP13PF	ATCGCGAGCTCGGAAATCGTTGTAACCCAAAC
STP13PR	CTAGTCTAGACCTGAATATCTCTTAGAAGC

3′ polyA tail. An aliquot containing 1 μg of PCR product was in vitro transcribed using the T7 mMessage mMachine kit (Ambion®) according to the manufacturer.

2.2. Oocyte preparation and uptake assays

Oocytes were prepared as described previously [27], and subjected to injection of 50 ng cRNA. The oocytes were incubated for 2–3 days at 17–18 °C, and then assayed for transporter uptake activity. Assays were performed in saline buffer (90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM MES) adjusted to pH 5 with TRIS. Oocytes were pre-incubated in assay buffer for 5 min to ensure intracellular steady state pH [28], and subsequently transferred to 500 μl buffer containing 0.037GBq [^{14}C]glucose (11.2 GBq/mmol; Amersham) in a final concentration of 15 μM glucose. After 30 min incubation, the assay was stopped by washing the oocytes four times in ice cold buffer. Oocytes were transferred to scintillation tubes containing 100 μl 10% SDS and disrupted by vortexing. 2.5 ml EcoScint™ scintillation fluid (National Diagnostics) was added, and radioactivity quantified in a scintillation counter. Each uptake consisted of at least seven oocytes. Kinetic parameters were determined using the Sigmaplot software (SYSTAT). In the kinase modulation studies, a mix of the kinase activators 8-bromo adenosine 3′–5′ cyclic monophosphate (500 μM, Sigma), forskolin (50 μM, Applichem), 3-isobutyl-1-methylxanthine (500 μM, Sigma) or the protein kinase A inhibitor 2-(*para*-bromocinnamoylamino)-ethyl-3-isoquinoline-sulfonamide (H89, 50 μM, Sigma) was included in the preincubation and uptake buffers.

2.3. Plant growth and real-time PCR analysis

Plants were grown in soil and benzothiodiazole treated as previously described [29]. Pools of at least 10 whole, 2–3 weeks old plants were used for each RNA isolation by use of standard protocols (RNAgents Total RNA, Promega). PCD was artificially induced in *Arabidopsis* rosette leaves by pressure infiltration of *Pseudomonas syringae* or 10 μM of the fungal toxin fumonisin B1 (FB1, Sigma) or by applying a 0.5 μl 10 μM FB1, 0.001% Silwett SL77 droplet. Reverse transcriptase PCR was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad) with a Bio-Rad iCycler and DyNAmo™ SYBR® Green qPCR Kit (Finnzymes) according to the manufacturer. After initial denaturation at 95 °C for 3 min, gene products were independently amplified by 45 cycles of: 95 °C, 15 s; 58 °C, 15 s; 72 °C, 15 s, 80 °C, 15 s. To avoid detection of potential primer dimers and genomic DNA, the primer3 software [30] was used to design primers for *STP13* and the endogenous control *Actin1* (Table 1) such that for both genes one primer was intron-spanning and detection was performed at the final 80 °C step. PCR quality was followed by melting curve analyses and agarose gel electrophoresis. Each sample was analyzed at least three times. Relative gene expression of *STP13* was normalized by comparison with actin (At2g37620) and calculated as previously described [31].

2.4. Construction of a STP13 promoter GFP fusion

A 2 kb fragment of the STP13 promoter was amplified from *Arabidopsis* (Col-0) genomic DNA, using primers *STP13*PF and *STP13*PR (Table 1), with *Pwo* polymerase (Roche). The fragment was subcloned into pBGFP0 [32] with the restriction enzymes *Sac*I and *Xba*I (New England Biolabs) and the construct transformed into Arabidopsis (Col-0).

3. Results

3.1. Biochemical characterization of STP13 in Xenopus oocytes

Screening of a cDNA library of *Arabidopsis* transporters in *Xenopus* oocytes with a ^{14}C-glucose uptake assay identified STP13 as a glucose transporter (Nour-Eldin, H.H., Nørholm, M.H.H. and Halkier, B.A., unpublished data). The gene had previously been annotated as an STP due to its homology to other well-characterized STP family members [16].

Biochemical characterization of *STP13* cDNA heterologously expressed in *Xenopus* oocytes showed that the recombinant protein mediated glucose uptake following Michaelis–Menten saturation kinetics with an apparent K_m value of 74 ± 14 μM towards D-glucose (Fig. 1A and B). Uptake experiments with ^{14}C-fructose and ^{13}H-ribose showed that STP13-injected oocytes facilitated the uptake of 14 pmol fructose in 30 min (Fig. 1A), whereas neither the water-injected control nor the STP13-injected oocytes facilitated ribose uptake (Fig. 1A). The substrate specificity of STP13 was further studied in competition assays using common sugars in 50-fold excess (Table 2). Glucose uptake was reduced by the hexoses D-galactose, D-mannose, D-fructose, but not significantly by

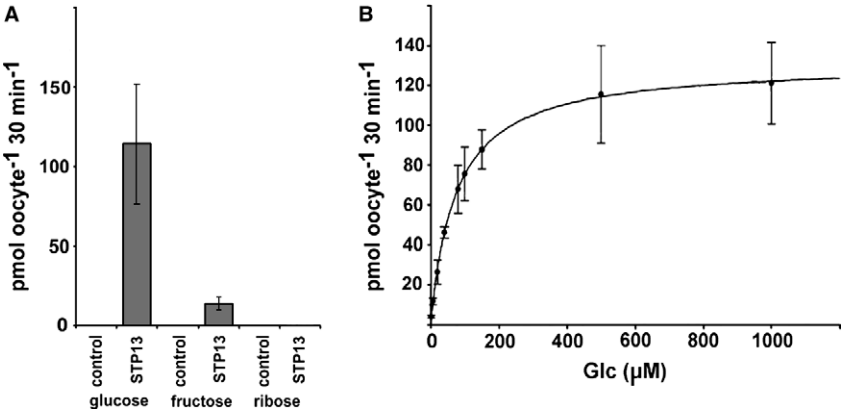

Fig. 1. Characterization of STP13 mediated glucose uptake in *Xenopus* oocytes. Oocytes were injected with 50 ng *STP13* cRNA and incubated with radiolabelled glucose, fructose or ribose. After 30 min, oocytes were washed and uptake measured. (A) STP13 mediated glucose-, fructose and ribose uptake in oocytes compared to water-injected controls. (B) Kinetic parameters of STP13-mediated glucose uptake were determined using Sigmaplot software.

Table 2
Analysis of STP13-mediated glucose uptake in *Xenopus* oocytes under different conditions

Uptake conditions	% Glucose uptake
750 μM D-glucose	10 ± 3.3
750 μM D-galactose	31 ± 6.9
750 μM D-mannose	37 ± 10.0
750 μM D-fructose	32 ± 7.1
750 μM D-sucrose	91 ± 17.5
750 μM D-ribose	78 ± 12.0
750 μM D-xylose	105 ± 24.1
750 μM 3-OMG	13 ± 5.6
750 μM L-glucose	96 ± 22.9
pH 5	100 ± 11.7
pH 7	6 ± 0.3
200 μM CCCP	22 ± 1.8
400 μM 2,4-DNP	33 ± 1.6
400 μM NaN_3	85 ± 19.5
Kinase activator mix	23 ± 3.3
H89	99 ± 8.7

Oocytes expressing STP13 were incubated with 15 μM ^{14}C-labelled glucose in a saline buffer at pH 5, except where uptake was tested at pH 7. Competing substrates were added in 50-fold excess (750 μM). Protonophores and sugars were added at the concentrations indicated. NaN_3 was added 30 min prior to assay start to ensure proper depletion of ATP. After 30 min, the oocytes were washed and the uptake of glucose measured. Data represents three independent experiments with standard deviations.

the pentoses D-xylose and D-ribose. In addition, the glucose analogue 3-*O*-methyl-glucose significantly reduced glucose uptake, whereas L-glucose or the disaccharide sucrose did not. Transport was abolished at pH 7 as compared to pH 5, and by the addition of the protonophores carbonylcyanide *p*-chlorophenyl hydrazone (CCCP) and 2,4-dinitrophenol (2,4-DNP) (Table 2). Preincubation for 30 min with sodium azide (NaN_3), an inhibitor of ATP synthesis, had no significant effect on glucose uptake. Together, these data show that STP13 is a high affinity H^+/D-hexose symporter.

Phosphoproteomics has identified STP13 as a phosphorylated membrane protein in vivo [33,34]. A potential regulatory role of phosphorylation on STP13 activity was studied using *Xenopus* oocytes expressing STP13 in the presence of protein kinase A activators or the protein kinase inhibitor H89 (Table 2). Glucose uptake by native STP13 was reduced 77% in the presence of kinase activators, whereas the H89 inhibitor had no significant effect. The data suggest that STP13 activity is negatively regulated by phosphorylation.

3.2. Microarray and real-time PCR analysis of the correlation of *STP13* expression with programmed cell death

To investigate the physiological role of STP13, global gene expression data at the NASC website [35] were examined. STP13 was found to be upregulated by various stress treatments, particular in tissues undergoing PCD. For example, significant up-regulation of STP13 was found in cell suspension cultures in which PCD had been induced [36] and in senescing leaves [37]. Other microarray data showed strong induction of *STP13* in the *Accelerated Cell Death 11* (*acd11*) mutant that germinate and develop cotyledons normally, but undergoes spontaneous PCD and constitutively expresses salicylic acid dependent defense related genes at the two- to six-leaf stage [29].

Real-time PCR was used to examine the effect of treatments with the pathogen *P. syringae* on *STP13* expression. STP13 specific primers (Table 1) were designed and tested with a standard BLAST search against the *Arabidopsis* genome sequence, to avoid detection of homologous transporter genes. *STP13* mRNA were isolated and quantified from plants treated with virulent and avirulent *P. syringae* strains four days after infil-

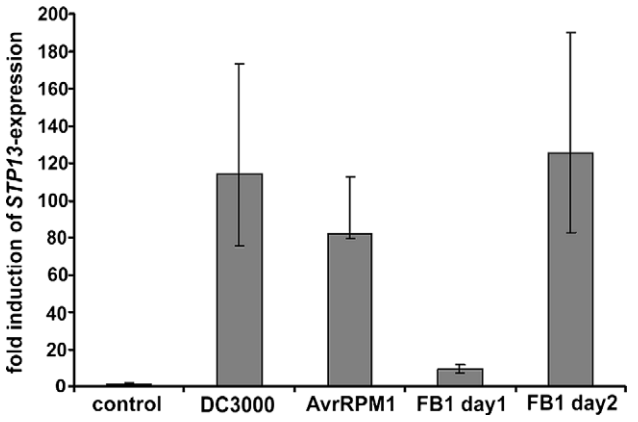

Fig. 2. Analysis of *STP13* mRNA levels in plants challenged with pathogens and the fungal toxin fumonisin B1 (FB1). *STP13* transcripts were quantified by real-time PCR using RNAs extracted from plants pressure infiltrated with virulent (DC3000) and avirulent (AvrRPM1) *Pseudomonas syringae* four days after the treatment, and from plants treated with FB1 one and two days after the treatment. The data are expression ratios relative to untreated plants.

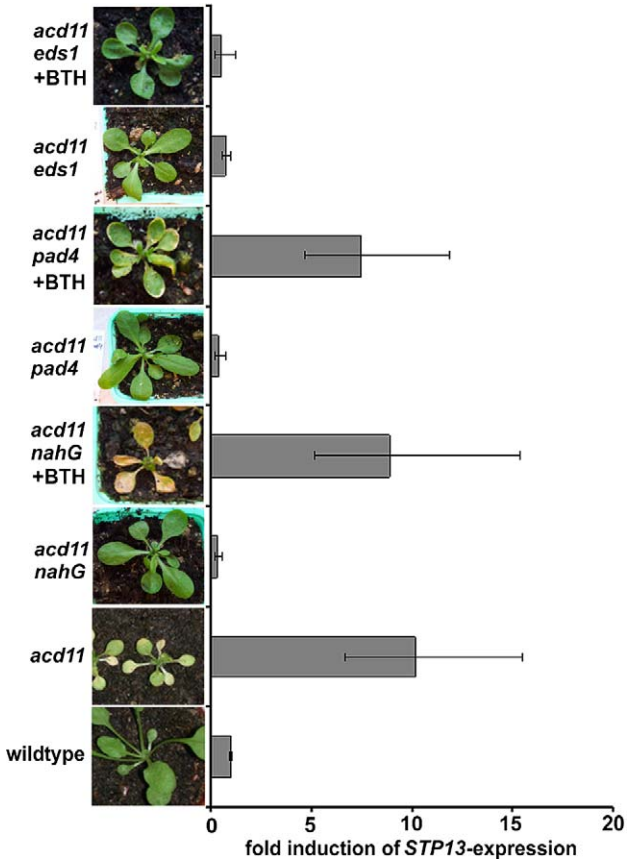

Fig. 3. Analysis of *STP13* mRNA levels in *acd11* genetic backgrounds. *STP13* transcripts were quantified by real-time PCR using RNAs extracted from wildtype and from different genetic backgrounds of the *acd11* mutant. The data are expression ratios relative to untreated wildtype plants.

tration, when serious cell death symptoms had occurred. Both the cell death inferred by the virulent and avirulent strain heavily induced *STP13* expression to around 100-fold (Fig. 2). Similarly, the effect of the PCD-inducing fungal toxin fumonisin B1 (FB1) [38] on *STP13* mRNA levels were examined by pressure infiltrating 10 μM FB1 in *Arabidopsis* rosette leaves. No visual death symptoms were observed one day after FB1 application (data not shown), but *STP13* expression was elevated approximately 10-fold (Fig. 2). On the second day after FB1 application death symptoms had clearly occurred (data not shown), and *STP13* levels were elevated to approximately 100-fold (Fig. 2). Hence, induction of PCD with both pathogens and a PCD-inducing toxin is followed by induction of *STP13* transcription.

We used the genetically well-characterized *acd11* mutant as a PCD model system to further study the correlation of *STP13* induction with PCD and defense responses. Quantitative real-time PCR showed that *STP13* is 10-fold upregulated in *acd11* (Fig. 3), which confirms the microarray data [29]. The double mutants *acd11/nahG*, *acd11/pad4-2* and *acd11/eds1-2*, which are repressed to varying degrees in their PCD phenotype, were used to correlate the severity of the *acd11* PCD-phenotype with *STP13* expression (Fig. 3). Introduction of *nahG*, encoding the bacterial enzyme salicylate hydroxylase [39], completely suppressed *STP13* expression, as it does the *acd11* PCD-phenotype [29], and subsequent application of the salicylate analog benzothiodiazole (BTH) fully restored the *acd11* phenotype and induced *STP13* expression. PAD4 and EDS1 are proteins with an unknown function that are necessary for induction of one type of PCD [40,41]. Mutations in PAD4 and EDS1 completely suppressed the PCD-phenotype of *acd11* as well as *STP13* expression. Compared to *acd11/nahG*, both the PCD-phenotype and *STP13* expression were only partially restored by BTH in *acd11/pad4-2*, whereas neither the PCD-phenotype nor *STP13* expression were restored by BTH in *acd11/eds1-2*. Thus, *STP13* expression tightly follows the severity of the PCD-phenotype in different *acd11* genetic backgrounds. BTH application did not induce *STP13* expression in control tissue from wildtype, *nahG*, *pad4-2* or *eds1-2* backgrounds (data not shown). In addition, introduction of the *ein2-1* and *jar1-1* mutations, that affect ethylene and jasmonate signaling [38], into the *acd11* background had no effect on *STP13* expression, but also have no effect on the *acd11* phenotype (data not shown).

3.3. *STP13 promoter GFP fusion analysis of STP13 expression in planta*

As an independent method to analyze for a correlation between *STP13* expression and PCD activation, we generated transgenic plants expressing an *STP13* promoter GFP reporter construct. As cell death is accompanied by the synthesis of a range of autofluorescent compounds, we included a nuclear localization signal in the construct to allow us to distinguish between autofluorescence and the GFP signal. In healthy *pSTP13:GFP* lines, nuclear localized GFP was only found in the vascular tissue of young emerging petals (Fig. 4A and B) and in senescing leaves (Fig. 4C). However, upon application of a droplet of the PCD-inducing FB1 [38], fluorescent nuclei

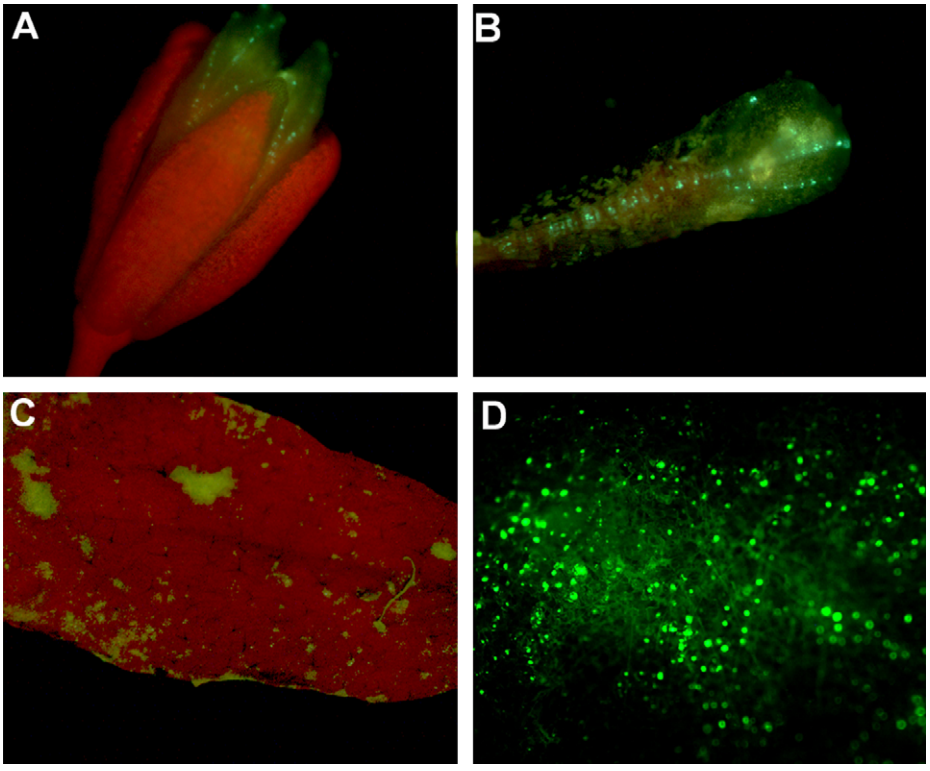

Fig. 4. Analysis of GFP expression in *Arabidopsis* transformed with a *pSTP13NLS:GFP* construct. (A) Fluorescence from GFP in nuclei from cells in the vascular tissue of emerging petals. (B) Dissection of a petal from the whole flower shown in A. (C) Fluorescence of GFP in a senescing leaf. (D) Induction of PCD with a 0.5 μl 10 μM droplet of the fungal toxin fumonisin B1 on one half of an *Arabidopsis* rosette leaf. The photo covers the area with the small 0.5 μl droplet and was taken 24 h after toxin application using an optic filter to omit red chlorophyll autofluorescence.

appeared within 24 h (Fig. 4D), which was 1–2 days before dead cells were observed by trypan blue staining (data not shown). The pattern of *pSTP13:GFP* expression suggests that STP13 is involved in PCD, senescence and possibly petal development.

3.4. Characterization of two stp13 t-DNA knockout lines

The physiological role of STP13 was further studied using two *stp13* mutant lines (Salk_045494 and Salk_021204) obtained from the SALK T-DNA collection [42]. The T-DNA insertions generated knockout mutants as evidenced by the absence of *STP13* transcripts using RT-PCR (data not shown). The morphology of *stp13-1* and *stp13-2* was closely followed from germination until senescence. No abnormalities could be observed when plants were grown under optimal conditions (data not shown). *Stp13-1* was crossed to *acd11* to investigate whether the absence of *STP13* influenced PCD in *acd11*. Trypan blue stainings showed that the double mutant *acd11/stp13* as well as the FB1-treated *stp13* single mutant did not differ significantly from *acd11* and a FB1-treated wildtype, respectively. This suggests that knockout of STP13 is not sufficient to produce clear phenotypes related to non-stressed growth or PCD activation, possibly as a consequence of functional redundance with other sugar transporters. Similarly, no difference in phenotypes were observed in the response of the *stp13* knockouts to pathogens such as *P. syringae* or the necrotroph *Alternaria brassicicola* (data not shown).

4. Discussion

We report the biochemical characterization of the *Arabidopsis* sugar transport protein STP13. In *Xenopus* oocytes, STP13 mediates glucose uptake that follows saturation kinetics with an apparent K_m value of 74 ± 14 μM. This establishes STP13 as a high affinity *Arabidopsis* glucose transporter with a K_m value similar to the other characterized members of the STP family. In addition, uptake of ^{14}C-fructose, but not ^{13}H-ribose, combined with competition studies using several hexoses and pentoses indicate that STP13 has broad substrate specificity towards all tested D-hexoses, which is agreement with the annotation of STP13 to the STP family.

Out of the 14 members in the *Arabidopsis* STP family, only STP1 has been characterized electrophysiologically to be a H^+/monosaccharide symporter [43]. STP13 is 59% identical and 75% similar to STP1 at the amino acid level, and STP13 glucose uptake activity is reduced at high pH and by common protonophores, but not by the ATP-synthesis inhibitor NaN_3. Combined with the substrate specificity analysis, this indicates that STP13 is a hexose-specific H^+-symporter. The ability of STP13 to recognize the ketose fructose is a rare property, only previously described for STP6 [13]. This is interesting since degradation of the highly abundant disaccharide sucrose releases both glucose and fructose, and suggests that STP6 and STP13 play particularly important roles in plant fructose transport.

Protein phosphorylation appears to be involved in regulation of complex networks in sugar signaling and transport [44,45]. For example, sucrose transport is inhibited by the protein phosphatase inhibitor okadaic acid in *Beta vulgaris* L. [45]. Recently, phosphoproteomics on the *Arabidopsis* plasma membrane has identified phosphorylation sites in the sucrose transporter SUC5, and in the monosaccharide transporters STP1 and STP13 [33,34]. The latter correlates with our data which showed that glucose uptake was inhibited by kinase activation in STP13-expressing oocytes, suggesting that STP13 is negatively regulated by phosphorylation. Further studies are necessary to clarify whether the inhibitory role of phosphorylation is direct or indirectly acts by lowering the amount of STP13 protein in the membrane.

Several lines of evidence suggest that STP13 plays a role in PCD. QPCR and *pSTP13:GFP* analysis of *STP13* expression in senescing plants, in the *acd11* mutant, and in plants treated with *P. syringae* and the PCD inducer FB1 correlate the expression of *STP13* to the appearance of PCD symptoms. Microarray data available from NASC [35] suggest that *STP13* is induced by several abiotic treatments such as cold, salt and osmotic stress. This does not contradict that STP13 plays a specific role in PCD, since abiotic stress is known to induce PCD in plants [46].

The role of glucose in PCD and plant defense is subject to a continuing debate. It has been suggested that plant transporters may reduce glucose concentrations in the apoplast to minimize pathogen growth [17]. We observed no obvious differences between wildtype plants and *stp13* knockouts in their response to different pathogens, which may, however, be due to redundant sugar transporters, such as e.g. STP1, STP3, STP4 or the putative monosaccharide transporter SFP1, that have all previously been implicated in plant defense and/or PCD. Alternatively, transporters have been suggested to provide carbohydrates to tissues with an elevated energy demand [12]. This energy may be needed to directly initiate PCD from reactive oxygen species, which are important for the spread of PCD in plants, and which require glucose metabolism [47]. In mammals, several studies favor a contrasting model that links glucose starvation to PCD. For example, glucose uptake is reduced in human T cells treated with apoptosis-inducing compounds [48,49], and inhibition of glucose transporter expression triggers apoptosis in murine blastocyst [50]. In plants, the latter model is supported by a study in which glucose transport was effectively inhibited in tobacco cells treated with the elicitor cryptogein that induces PCD in whole plants. [51]. This inhibition of glucose transporter activity involved phosphorylation, which is similar to our findings that protein kinase A activators effectively inhibit STP13 uptake activity.

In conclusion, our data show that expression of STP13 is correlated with PCD, which may suggest that sugar transport plays a role in PCD in plants. Such a link has been found in mammals, which suggests that sugar homeostasis plays an important role in PCD across kingdoms. Further investigations of the physiological role of STP13 in modulating extra- and intracellular glucose levels are necessary to understand the fundamental regulatory role of sugar transport in PCD.

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