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DROUGHT STRESS

Expression analysis of candidate genes as indicators for commencing drought stress in starch potatoes

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Abstract

Drought stress is a major problem for potato production and will be of grave importance due to climate change and the resulting temperature peaks along with drought periods in the vegetative growth phase of potato. Plants, as sessile organisms, adapt to their environment morphologically as well as biochemically. To cope better with abiotic stresses like drought, plants developed strategies like reactive oxygen species (ROS) detoxification and fast reacting stomatal closure, as well as signalling cascades leading to a quick response to stress. This study aimed at analysing eight genes of interest, derived from a former proteomic study, and determining their suitability for detection of commencing drought stress in early growth stages of potato. For this aim, six starch potato genotypes, which differed in stress response in previous studies, were examined for plant growth and physiological parameters in two experiments in an open greenhouse after seven and 14 days of stress. Besides lower shoot biomass after drought stress, which was already visible after seven days and became stronger after 14 days, weaker root growth was also detected after 14 days. The observed differences between the experiments can presumably be explained by temperature peaks and high radiation prior to and during the first experiment, which took place earlier in the year. The expression of the eight genes was studied in young leaves of four genotypes after 7 days of water withdrawal. Gene expression patterns were dependent on the studied genes. Three genes, *cell wall/vacuolar inhibitor of fructosidase (INH1)*, *peroxidase 51-like (POD)* and *subtilase family protein (SBT1.7)* showed consistent changes in gene expression after seven days of stress between all genotypes. The *INH1* gene was found to be upregulated in all genotypes in two independent experiments after drought stress. This correlates with the results at the protein level, where INH1 was also found to be higher abundant in two genotypes of potato (Wellpott et al., DGG-Proceedings 10, 2021). Therefore, this gene might be an appropriate candidate for the detection of commencing drought stress in potato.

KEYWORDS
drought stress, *invertase inhibitor 1*, open greenhouse, *Solanum tuberosum*, *subtilase*

Key points

- Drought stress was applied in all analyzed genotypes as indicated by growth reduction.
- Setup of stress experiments under open greenhouse conditions is of major importance regarding classification of tolerance levels.
- *Cell wall/vacuolar inhibitor of fructosidase (INH1)* represents a promising candidate for the detection of early drought stress in young potato plants.

1 | INTRODUCTION

Potato is one of the most important food crops together with rice, wheat and maize comprising around 5000 cultivars worldwide. Based on the high adaptability of the plant, potatoes are cultivated in many parts of the world (FAO, 2021). In addition to direct consumption of table potatoes and its use as fodder for animals, starch potatoes are of importance due to their high starch content for industrial purposes such as the production of paper, adhesives and thermoplastics (Röper, 2002; Vreugdenhil et al., 2014).

There are considerable differences in potato yields between the individual continents. In addition to technical and economic development in individual regions, this is due to climatic differences (FAO, 2021). Because of the foretold climate change, potato production worldwide is under severe pressure. Although being adaptable, the plant is rather sensitive to drought stress due to their shallow root system (van Loon, 1981). Drought influences plant growth in form of overall poor growth, reduced photosynthesis rate, reduced leaf area, smaller tubers and lower starch content (Gervais et al., 2021; Sprenger et al., 2015). Especially prolonged drought and heat periods are known to negatively affect the appearance and physiological properties of the tuber, which drastically reduces the overall quality and market value.

Drought stress is a major problem in potato production, and recent years have displayed more severe weather extremes, leading to an obligation in alteration of culture management, e.g. irrigation of cultures (Haverkort & Verhagen, 2008). More intense heavy rains occur, followed by dry periods, during which there is not enough water available for the plants in the soil (Intergovernmental panel on climate change [IPCC], 2022). The forecast of a higher frequency and severity of drought periods in spring and early summer, which correlates with the time of highest vegetative growth, will increase the need for more tolerant potato varieties to this abiotic stress.

One of the first reactions of plants to drought stress is a reduction in growth (Dahal et al., 2019). Reduced stem elongation can provide a reduction in canopy area and decreases the overall transpiration area to avoid further water loss. Plants also react to drought on a molecular level. Abscisic acid (ABA) is shown to be increased after drought stress and induces processes such as the regulation of stomatal closure and primary metabolism (Mustilli et al., 2002; Ruan et al., 2010; Yang et al., 2020). Further, plants respond to drought stress by activating signalling processes (Schaller et al., 2018) and generating ROS (Demidchik, 2015).

Previous transcriptomic studies investigating reactions to drought stress in potato either analyzed long-term drought stress (Aliche et al., 2022; Evers et al., 2010) or short-term drought stress under greenhouse conditions or in cell cultures (van Muijen et al., 2016). Complementing these previous reports, this study examined candidate genes after short-term drought stress in an open greenhouse and in an early vegetative growth phase.

The candidate genes were selected based on a previous proteomic study and were encoding proteins of differential abundance in more tolerant potato genotypes after drought stress compared to control plants in a rain-out-shelter trial (Wellpott et al., 2021). Based on this study, we selected eight genes of interest (GOIs), which might play a role and represent potential marker genes for drought stress or drought stress tolerance in potato. From these eight GOIs, Wellpott et al. (2021) found five associated proteins to be higher abundant in two rather tolerant to drought stress genotypes 'Eurostarch' and 'Tomba': ZBD (zinc-binding dehydrogenase family protein; enzymes), RPT5a (regulatory particle triple-A ATPase 5A; folding, sorting and degradation), 13-LOX (lipoxygenase; lipid metabolism), SHMT (serine transhydroxymethyltransferase; carbohydrate metabolism/amino acid metabolism) and INH1 (cell wall/vacuolar inhibitor of fructosidase; enzymes). Three of the eight proteins were found to be lower abundant on protein level after drought stress: Glyx (lactoylglutathione lyase/glyoxalase I family protein; signal transduction), POD (peroxidase 51-like; biosynthesis of other secondary metabolites) and SBT1.7 (subtilase family protein; folding, sorting and degradation) (Table 1).

The aim of this study was to analyse whether the regulation of these differentially abundant proteins also occurred at the transcriptional level. Therefore, we determined plant growth and physiological responses to drought stress of six starch potato genotypes in an open greenhouse after seven and 14 days of commencing drought stress. Because yield loss was reported to be greatest when drought occurred in the vegetative and tuber initiation phase (van Loon, 1981), drought was presented to the plants in this study four weeks after acclimatization. The responses of the eight GOIs were analyzed by quantitative reverse transcription (qRT)-PCR in four contrasting genotypes after seven days.

2 | MATERIALS AND METHODS

2.1 | Plant material and experimental setup

Six starch potato genotypes ('Eurobravo', 'Eurostarch', 'Kiebitz', 'Maxi', 'Ramses' and 'Tomba'), kindly provided by the respective breeders, were used in the drought stress experiments of this study. These

TABLE 1 Genes of interest (GOI) with name, function, KEGG pathway, and abundance of associated protein in Wellpott et al. (2021).

GOI	Protein name (Phureja DM1-3 v6.1)	KEGG pathway (second revision)	Abundance of protein 'Eurostarch' (fold change stress/control; Wellpott et al., 2021)	Abundance of protein 'Tomba' (fold change stress/control; Wellpott et al., 2021)
Glyx	Lactoylglutathione lyase/glyoxalase I family protein	Signal transduction	0.63	0.63
ZBD	Zinc-binding dehydrogenase family protein	Enzymes: oxidoreductases	1.72	1.85
RPT5a	Regulatory particle triple-A ATPase 5A	Folding, sorting and degradation	1.62	2.53
13-LOX	Lipoxygenase	Lipid metabolism	1.54	1.50
SHMT	Serine transhydroxymethyltransferase	Carbohydrate metabolism/ Amino acid metabolism	1.52	1.94
POD	Peroxidase 51-like	Biosynthesis of other secondary metabolites	0.64	0.63
SBT1.7	Subtilase family protein	Folding, sorting and degradation	0.43	0.36
INH1	Cell wall/vacuolar inhibitor of fructosidase	Enzymes	2.21	1.56

genotypes were selected based on their stress susceptibility index (SSI) according to Fischer and Maurer (1978) calculated for the tuber yield (Meise et al., 2019). 'Tomba' and 'Maxi' responded rather tolerant under drought stress based on tuber yield. 'Eurostarch' was between tolerant and sensitive, whereas 'Kiebitz' and 'Eurobravo' responded rather sensitive in the test set under drought stress (Meise et al., 2019). 'Ramses' was not tested in the study by Meise et al. (2019), however, was described as more tolerant compared to a test set (Schumacher et al., 2021). However, according to Sprenger et al. (2015), 'Ramses' and 'Tomba' reacted rather sensitive towards drought stress in field experiments, but tolerant when early drought reactions were investigated in pot trials. 'Eurobravo', 'Eurostarch' and 'Maxi' responded sensitive to early drought stress in the study of Sprenger et al. (2015).

Nodal cuttings were propagated in vitro on solid MS medium (Murashige & Skoog, 1962) containing 3% sucrose and 7.5 g L^{-1} Plant Agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). Cultivation took place at 18°C in a 16 h photoperiod with a PPFD-PAR of 35 µmol m^{-2} s^{-1}. Three-week-old plants were transferred to pot substrate (70% peat, 30% clay, limed to pH 5.5 to 6.5) and were acclimatized for three days by reducing air humidity to regular greenhouse conditions. Cuttings were taken for greater stem stability and after a rooting period of twelve days, they were planted in 2 L containers (ø 14 cm, height 18 cm) with 1700 g of a growing medium consisting of pot substrate: sand (1:1 [v/v]; substrate: Einheitserde T, Einheitserdewerke Werkverband e.V., Sinntal-Altengronau; and sand: size 0–2 mm, washed, declared as sand, Lehmann, Burgdorf). All pots were fertilized three times over two weeks with a 1‰ solution of Ferty 3 Mega fertilizer (N–P–K: 18–12–18 + 1.2 MgO, total volume per plant: ~300 mL). The experiments took place in 2021 in an open greenhouse (glass roof, open sides) in Hanover, Germany (52°23′36.4″ N 9°42′14.3″ E) from June 23 to July 16 (experiment 1) and from July 20 to August 12 (experiment 2). The total of 576 experimental plants and 96 boundary plants per experiment were arranged in 24 blocks in a block design. Each block contained one plant per genotype, treatment and evaluation day resulting in a total of 24 plants per block. Drought stress was applied for seven or 14 days. Stressed plants were not irrigated until a water holding capacity (WHC) of 15% was reached (~day 7). Control plants were irrigated to a WHC of 60% by daily weighing. These levels were maintained until evaluation (Figure 1). Six additional plants per variant served as recovery plants after seven and 14 days of water withdrawal, respectively. After stress application for seven or 14 days, they were rewatered for nine days to a WHC of 60%.

Throughout the whole experiment, the shoot length (from the soil surface to the shoot tip) was recorded, and SPAD values were measured with a chlorophyll meter SPAD-502 (Konica Minolta Sensing Europe B.V., Nieuwegein, the Netherlands) on the first fully developed leaf of each plant (Table S1). At each evaluation, eight (start of experiment, day 7, day 14) or six (recovery day 7 and recovery day 14) plants (=biological replicates) were harvested and the roots were thoroughly washed to remove the substrate to record the fresh mass. Shoots were separated from roots carefully and weighed. After 48 h at 70°C, the dry mass of shoots and roots was determined. Relative increase in dry mass for shoot and root dry mass data was calculated by dividing the difference between dry masses at day 7 and day 14, respectively, and dry masses at day 0 by the dry masses at day 0.

For gene expression analysis, the third leaflets of the first fully grown leaf of five biological replicates were harvested from extra plants, immediately frozen in liquid N and stored at −80°C until further use. Additionally, the relative water content (RWC) in percent in leaves was calculated from the weight of the youngest fully developed leaf of a plant after harvest, after 24 h in water (100%) and after 48 h of drying (0%).

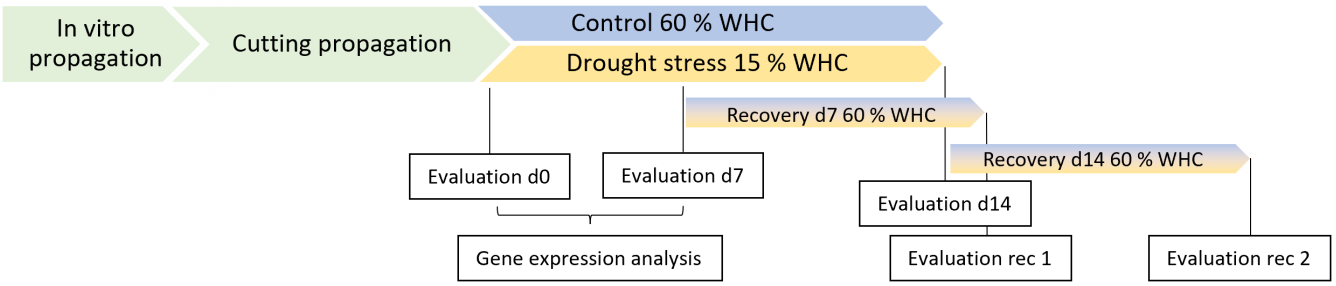

FIGURE 1 Timeline of drought stress experiments in an open greenhouse. Six starch potato genotypes were propagated in vitro, acclimatized, and once propagated via cuttings. Drought stress variants were watered daily to a WHC of 15%, control plants received water to 60% WHC. Evaluations took place on d0: start of the experiment, d7: seven days under drought stress, d14: 14 days under drought stress, rec 1: nine days of recovery (60% WHC) after seven days of drought stress, rec 2: nine days of recovery (60% WHC) after 14 days of drought stress. Samples for gene expression analysis were taken at d0 and d7.

2.2 | RNA isolation and cDNA synthesis

Frozen leaf samples of five biological replicates of the four genotypes 'Eurobravo', 'Eurostarch', 'Maxi' and 'Tomba' from control conditions and after seven days of drought stress (commencing-drought) were separately homogenized in a mixer mill at 27 Hz for 2.5 min (MM400, Retsch, Haan, DE). RNA was extracted from 100 mg of homogenized plant material by using the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany). Instructions of the manufacturer were followed and the DCT lysis buffer was used. Genomic DNA was removed with DNase I according to the manual (Thermo Scientific, Waltham, MA, USA), and the integrity of RNA was determined in a 1% agarose gel. For cDNA synthesis, the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) was used following the instructions of the manufacturer using the oligo-dT primer and 1 µg RNA as a template. The cDNA was diluted 1:10 and stored at −20°C until further use.

2.3 | Primer selection

Eight candidate genes were selected based on identified differentially abundant proteins in starch potato leaves under drought stress (Wellpott et al., 2021). For their selection, a focus was set on proteins that were differentially abundant in rather tolerant genotypes 'Eurostarch' and 'Tomba'. Primers were designed meeting the criteria of 18–24 bp length, GC content 40%–60%, amplification product 80–250 bp and a melting temperature T_M 60°C (Table S2). Primers were tested for specificity with Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) aligning it to the *Solanum tuberosum* subsp. *tuberosum* genome (NCBI: txid4113). Sequence information for all GOIs was provided by Spud DB (http://spuddb.uga.edu) using the genomic sequence of *Solanum tuberosum* group Phureja DM1-3 v6.1. All primers were tested in a standard PCR with cDNA of genotype 'Eurostarch' as a template and an annealing temperature $T_A = 60$°C and checked on a 1.5% agarose gel. The PCR products were sequenced by Sanger sequencing (Sanger et al., 1977). A list of all used primers is provided in Table S2. Sequencing results can be found in the LUH data repository under the following link: https://doi.org/10.25835/td4w2pg9. Alignments were performed via MAFFT v7 (Katoh & Standley, 2013) using Benchling (benchling.com).

2.4 | RT-qPCR

The real-time quantitative RT-PCR was performed using the Applied Biosystems QuantStudio 6 Flex System (Thermo Fisher Scientific, Waltham, MA, USA). All primers were tested with a pool of all cDNA samples for their efficiency. Primer efficiencies calculated in the software QuantStudio™ Real-Time PCR Software v1.3 are listed in Table S2. Only primers with single peaks in the melt curve analysis were selected for further analysis. Genes *EF1a* (elongation factor α), *APRT* (adeninphosphoribosyltranferase) and *Cyclo* (cyclophilin) were used as reference genes (Nicot et al., 2005). They were tested for stability in RStudio (2022.07.1 Build 554) based on R version 4.1.3 (R Core Team, 2022) using the NormFinder algorithm (Andersen et al., 2004). Because of a stability value >0.25, *EF1a* was excluded from calculations of the normalized gene expression. Each sample was measured in three technical replicates. Five biological replicates were analyzed for each genotype ('Eurobravo', 'Eurostarch', 'Maxi', 'Tomba') at the start of the experiment (T0) and after seven days under control conditions (T7C) and drought stress (T7S). In total, diluted cDNA of 120 samples was mixed with Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) diluted 1:4 (v/v) for analysis with every primer pair (final concentration in reaction: 0.2 µM). Following PCR conditions were used: one cycle at 95°C for 60 s, 40 cycles at 95°C for 15 s and one cycle at 60°C for 60 s. Subsequently, melting curve analysis (60°C to 95°C with an increment of 0.5°C/15 s) was conducted to determine specificity of amplification. Data was further processed with QuantStudio™ Real-Time PCR Software v1.3. Data are shown as normalized gene expression (Pfaffl, 2001).

2.5 | Statistical analysis

Graphics and statistical analysis for growth data as well as for gene expression data were performed in R version 4.1.3 (R Core Team, 2022) using RStudio v. 2022.07.1 Build 554 (RStudio Team, 2022). Figures were produced using the packages 'ggplot2' (Wickham, 2016), 'cowplot' (Wilke, 2020), 'ggpubr' (Kassambara, 2020), 'ggsci' (Xiao, 2018) and 'RcolorBrewer' (Neuwirth, 2014). The data were tested for normal distribution with the Shapiro–Wilk test, an analysis of variance (ANOVA) was calculated to assess main treatment and genotype effects and interactions, and means were compared pairwise by Tukey tests at $p < .05$. To minimize unwanted site effects, a randomized complete block design with 24 blocks was used. When normal distribution was not given, the data was either log-transformed or further analyzed by a Kruskal–Wallis test with Bonferroni adjustment. Packages used for statistical analyses were 'emmeans' (Lenth, 2022), 'multcomp' (Hothorn et al., 2008) and 'agricolae' (de Mendiburu, 2021).

3 | RESULTS

3.1 | Growth parameters under drought stress after seven and 14 days

Noticeable differences between treatments in the morphology and growth of all genotypes before development of flower buds were observed in two experiments over time. Plants after 7 days of water withdrawal showed lower height, darker leaves that began to wilt and overall poorer growth than control plants. These observations were even more pronounced after 14 days of stress (Figure 2). There were significant differences in the biomass data between the two experiments. Plants of experiment 1 showed lower dry mass than plants of experiment 2 in control and stress conditions. This might be due to temperature differences in the week before the start of the drought treatment as well as in the first seven days of stress between the experiments and higher sum of global radiation throughout the first experiment (Tables S3, S4 and Figure S1). In experiment 1, which took place in June 2021, temperature peaks were detected on days −5/−4 (31.2/31.4°C daily mean temperature measured in the canopy). On these days in experiment 2, which took place in July 2021, the daily mean temperature was considerably lower (26.5/22.8°C). Another peak in experiment 1 was observed on day 4 (32.2°C) of the experiment, whereas in experiment 2 the temperature was rather moderate (24.8°C).

Since the genotypes 'Kiebitz' (experiment 1 0.22 g/experiment 2 0.53 g), 'Ramses' (0.23 g/0.38 g) and 'Tomba' (0.29 g/0.58 g) entered the experiments with lower shoot dry mass compared to the other genotypes ('Eurobravo': 0.5 g/1.11 g, 'Eurostarch': 0.53 g/0.93 g, 'Maxi': 0.43 g/0.58 g), the growth data are shown as relative increase in dry mass, to account for these differences (Figures 3 and 4). Absolute mass data are provided in Table S5 and an analysis of variance (ANOVA) of the increase in dry mass can be found at https://doi.org/10.25835/td4w2pg9 (file: Statistics.docx).

After 7 days of water withdrawal, the plants of all genotypes showed a lower increase in shoot dry mass under drought stress than under control conditions. For genotype 'Maxi', this difference was significant in both experiments (reduction of 54.6% and 43.2% in experiments 1 and 2, respectively), as well as for 'Eurobravo' (53.2%) and 'Eurostarch' (54.5%) in experiment 2 (Figure S2). In experiment 1, 'Eurobravo' gained significantly more shoot mass than all other genotypes (1.0 ± 0.14 g). Moreover at this timepoint, the relative increase in dry mass was not significantly different between treatments (Figure 3a,b). 'Ramses' showed the highest and 'Kiebitz' the lowest relative increase in dry mass in both experiments after seven days. High variation and no significant differences in relative increase in dry mass in roots between control and drought-stressed plants were recorded after seven days for all genotypes (Figure 3c,d).

After 14 days of drought stress, a significantly reduced relative increase in dry mass of shoots was noticed for all genotypes in experiment 2 when comparing stress to control variants (74.8% 'Eurobravo', 72.9% 'Eurostarch', 66.4% 'Kiebitz', 79.6% 'Maxi', 67.6% 'Ramses' and 72.8% 'Tomba', see Figure 4a,b and Figure S2). In experiment 1, this was only observed for 'Eurobravo' (76.1%) and 'Maxi' (76.6%). In experiment 1, the relative increase in dry mass of shoots did not differ between genotypes in the control treatment. In the stress treatment, 'Ramses' showed the highest relative increase in dry mass, while 'Eurostarch' and 'Maxi' showed the lowest relative increase in dry mass of shoots. In experiment 2, genotypic differences were not as pronounced. 'Kiebitz', 'Ramses' and 'Tomba' expressed higher relative increase in dry mass than 'Eurostarch' and 'Maxi' with the lowest relative increase in dry mass of shoots. For the increase of root mass, no significant differences between control and drought stress variants were recorded in experiment 1 (Figure 4c,d). In experiment 2, however, for 'Maxi' (59.9%) and 'Tomba' (55.0%), the relative increase in dry mass of roots of drought stressed plants was significantly lower than that of control plants.

3.2 | *INH1*, *POD* and *SBT1.7* displayed consistent changes of gene expression in all genotypes after seven days of drought stress

The normalized expression of the candidate genes was analyzed in leaf material at the start of the two experiments (day 0) and after seven days under drought stress (day 7) to determine the early stress response of the analyzed potato genotypes (Table 2).

Expression of *Glyx* (*lactoylglutathione lyase/glyoxalase I family protein*) did not show significant changes after seven days between control and stress (Tables 2 and S6). *13-LOX* (*lipoxygenase*), *RPT5a* (*regulatory particle triple-A ATPase 5A*), *SBT1.7* (*subtilase family protein*) and *SHMT* (*serine transhydroxymethyltransferase*) differed in their regulation of expression between experiments 1 and 2. While no changes in gene expression was detected in experiment 1 for *13-LOX* and *SHMT*, this changed in experiment 2 as the expression in

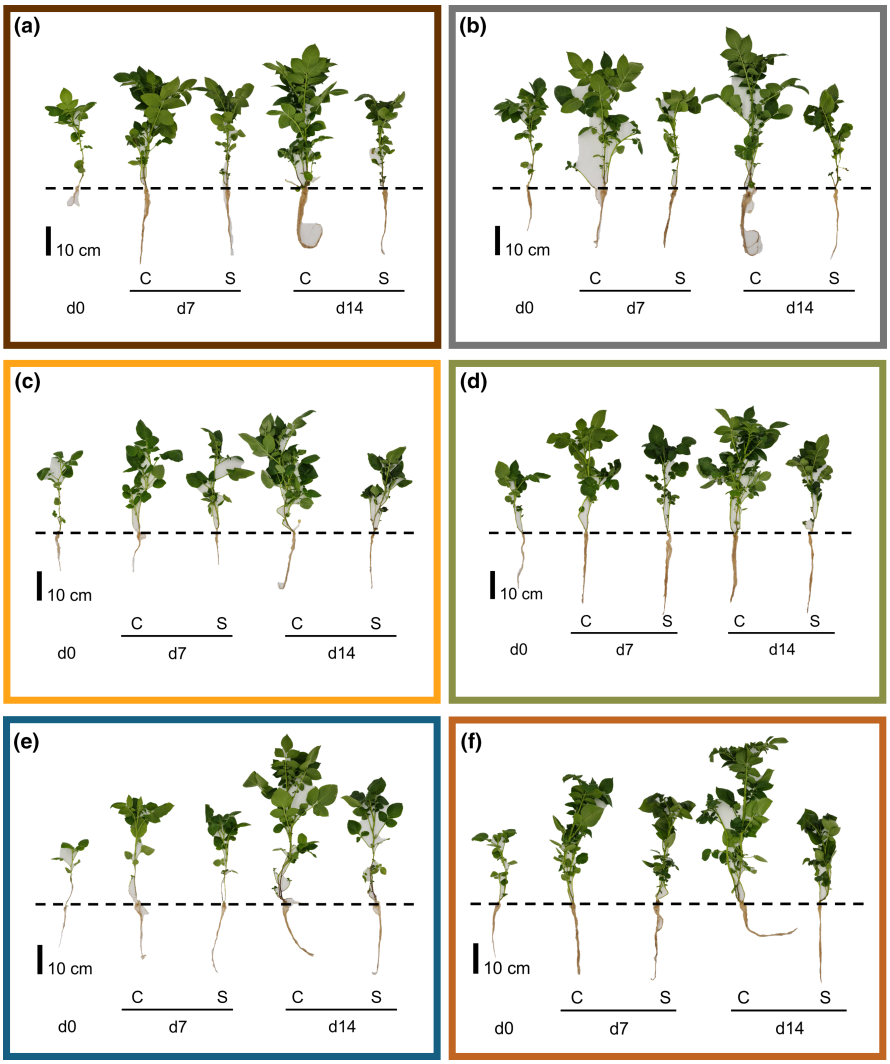

FIGURE 2 Plants at the start of the experiment (d0) and after seven (d7) or 14 days (d14) at either control (C) or drought stress (S) conditions. (a) 'Eurobravo', (b) 'Eurostarch', (c) 'Kiebitz', (d) 'Maxi', (e) 'Ramses', (f) 'Tomba. C: control plants (60% WHC), S: stressed plants (15% WHC).

'Eurobravo', 'Eurostarch' and 'Maxi' decreased for *13-LOX* and decreased in 'Maxi' and 'Tomba' for *SHMT* (Tables 2 and S6). While in experiment 1, the gene expression was reduced under stress for all genotypes except 'Tomba' for *RPT5a*, no alteration was detected in experiment 2. Expression analysis for *ZBD* displayed no alteration in level, except for 'Tomba' in experiment 1 where it was significantly upregulated. Furthermore, a reduction in expression was detected for *POD* after 7 days of water withdrawal for all genotypes, except 'Tomba' in experiment 1, where there was no visible change (Figure 5a,b). Highest expression levels of *POD* were observed in 'Maxi' and 'Tomba' in experiment 1 and in 'Tomba' in experiment 2. The lowest fold change (stress/control) showed 'Eurostarch' in experiment 2 (0.03). For the gene *SBT1.7*, a gene for a subtilase family protein, a significantly lower expression in stressed plants was detected in 'Eurobravo' in experiment 1, while a reduction to the same level took place in the stressed variants of all genotypes in experiment 2 (Figure 5c,d). After 7 days of water withdrawal, genotypes in experiments 1 and 2 displayed a higher expression of INH1 (*cell wall/vacuolar inhibitor of fructosidase*), except for genotype 'Tomba' in experiment 1 (Figure 5e,f). Fold changes (stress/control) reached from 3.77 ('Maxi') to 4.3 ('Eurostarch') in experiment 1 and were more pronounced in experiment 2 (from 6.31 in 'Maxi' to 15.51 in 'Eurobravo') (Table S6).

If the normalized gene expression at day 0 before starting the experiments was considered, all genotypes showed a higher expression level of *INH1* and *SBT1.7* in experiment 2 than in experiment 1 (Table S7). Furthermore, 'Tomba' displayed a higher gene expression of *Glyx*, *RPT5a*, *ZBD* and *SHMT* on day 0 in experiment 2 than in experiment 1. This was also the case for 'Maxi', except for *RPT5a*. 'Eurobravo' also showed higher gene expression of *ZBD* in experiment 2. Expression of *POD* and *13-LOX* was on a similar level in both experiments in the respective genotypes (Table S7).

4 | DISCUSSION

In the early growth phases of potato, drought has a huge impact on quality and quantity of the later yield. Therefore, in this study, early responses to drought stress in late vegetative or early tuber initiation phases of potato were analyzed.

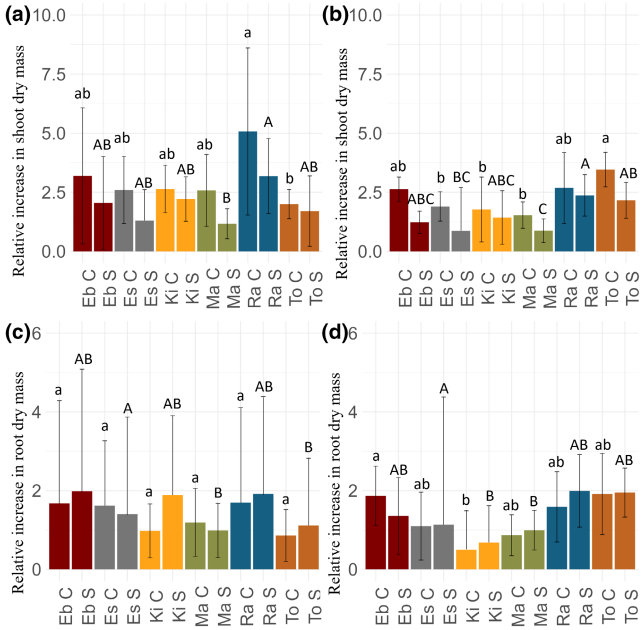

FIGURE 3 Relative increase in dry mass in shoot (a, b), and root (c, d) in gram after seven days of drought stress with standard deviation, $n = 8$. a/c: experiment 1, b/d: experiment 2. Eb: 'Eurobravo', Es: 'Eurostarch', Ki: 'Kiebitz', Ma: 'Maxi', Ra: 'Ramses', To: 'Tomba'. C: control, S: stress. Statistical analysis: Kruskal–Wallis test with Bonferroni correction. Significance codes: ***$p < .001$; **$p < .01$; *$p < .05$.

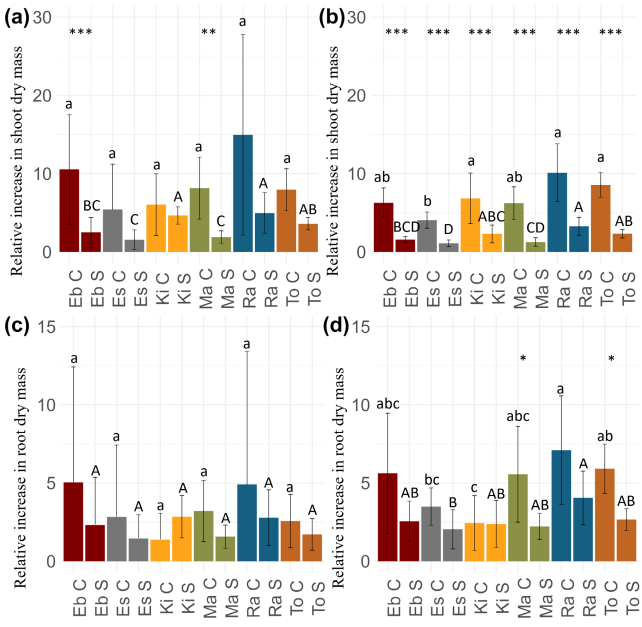

FIGURE 4 Relative increase in dry mass in shoot (a, b), and root (c, d) in gram after 14 days of drought stress with standard deviation, $n = 8$. a/c: experiment 1, b/d: experiment 2. Eb: 'Eurobravo', Es: 'Eurostarch', Ki: 'Kiebitz', Ma: 'Maxi', Ra: 'Ramses', To: 'Tomba'. C: control, S: stress. Statistical analysis: Kruskal–Wallis test with Bonferroni correction. Significance codes: ***$p < .001$; **$p < .01$; *$p < .05$.

4.1 | Drought decreases overall plant growth after 7 and 14 days of stress

After 7 days (commencing stress), a reduction in plant height, reduced increase in shoot dry mass, darker leaves and wilting was determined. Also, the RWC was significantly lower in stressed plants in genotypes 'Eurostarch', 'Maxi' and 'Tomba' after seven days in experiment 2 (Figure S3). After 14 days (intensified stress), these changes became more pronounced. This can be seen in the data of the recovery plants. All rewatered plants of all genotypes recovered from drought stress and resumed growth (Table S5, 'Rec 1' and 'Rec 2'). The RWC dropped from day 7 to day 14. In experiment 1 'Eurobravo', 'Eurostarch', 'Ramses' and 'Tomba' showed a significantly lower content in stressed plants compared to the control. In experiment 2, only 'Eurostarch' and 'Kiebitz' showed a significantly lower RWC in stressed plants. A reduction in shoot growth under abiotic stress is well described and is among the first visible signs of plant responses to stress (Dahal et al., 2019). Cells enter a status of growth arrest until stress relieve, therefore reducing the leaf area and minimizing water loss through the leaf area (Takahashi et al., 2019).

No significant effect on root growth could be detected after 7 days of drought stress for both experiments (Figure 3c,d). After 14 days of drought stress, still no alteration in root growth was observed in experiment 1 (Figure 4c,d). However, for experiment 2, a significant reduction in root dry mass was observed for 'Maxi' and 'Tomba'. This is in agreement with previous results by Boguszewska-Mańkowska et al. (2020) and Lahlou and Ledent (2005), who reported that root growth reduction took place under drought stress in a genotype-specific manner. More tolerant genotypes were shown to have constant root biomass under stress compared to control plants. Based on our data, this was observed for all genotypes in experiment 1 and for 'Eurobravo', 'Eurostarch', 'Kiebitz' and 'Ramses' in experiment 2, indicating that surrounding conditions in experiment 2 might have been more favourable for genotype distinction.

The overall difference in growth between the experiments was striking. Three of six analyzed genotypes ('Eurobravo', 'Eurostarch' and 'Maxi') showed significantly higher shoot increment in control plants than in stressed plants after 14 days of drought stress in the first experiment. Important differences between the experiments, which may explain the differences, were the temperature peaks before the beginning of the drought stress phase and the higher radiation in experiment 1 (Table S4). Additional heat stress, or more generally double stress, leads to a series of reactions in the plant, which do not mirror the responses under single stress (Meise et al., 2018; Pandey et al., 2015). Mittler (2006) displayed potential correlation effects based on a metadata search of potential double stressors, and heat and drought stress were described as potential negatively correlated. In addition, the differences between genotypes that Meise et al. (2019) or Sprenger et al. (2015) could not be reproduced in the growth data with our setup. However, there are major differences between our experimental setup and those conducted so far. First, in the present study, plants were derived from in vitro cultivation without

TABLE 2 Mean values of normalized expression of eight genes of interest (GOIs) in leaf tissue of four potato genotypes at the start of the experiment (day 0) and after seven days (day 7) of cultivation under control conditions or drought stress (±SD).

Experiment	Day	Genotype	Variant	Glyx	ZBD	RPT5a	13-LOX	SHMT	POD	SBT1.7	INH1
1	0	Eurobravo	Start	0.082 ± 0.037 a	0.162 ± 0.026 a	0.361 ± 0.052 a	0.490 ± 0.135 a	10.656 ± 1.480 a	0.025 ± 0.007 a	0.100 ± 0.027 a	0.077 ± 0.017 a
		Eurostarch	Start	0.064 ± 0.032 a	0.150 ± 0.023 a	0.363 ± 0.069 a	0.483 ± 0.129 a	8.029 ± 3.208 a	0.019 ± 0.004 a	0.120 ± 0.022 a	0.099 ± 0.039 a
		Maxi	Start	0.061 ± 0.027 a	0.151 ± 0.029 a	0.248 ± 0.045 b	0.261 ± 0.068 b	4.042 ± 1.560 b	0.021 ± 0.005 a	0.074 ± 0.017 a	0.076 ± 0.028 a
		Tomba	Start	0.034 ± 0.012 a	0.169 ± 0.028 a	0.229 ± 0.070 b	0.416 ± 0.186 ab	3.348 ± 0.623 b	0.031 ± 0.018 a	0.094 ± 0.048 a	0.087 ± 0.044 a
	7	Eurobravo	Control	0.053 ± 0.033 a	0.156 ± 0.069 b	0.422 ± 0.084 a	0.400 ± 0.284 a	7.023 ± 4.801 a	0.038 ± 0.014 ab	0.315 ± 0.205 a	0.184 ± 0.063 a
		Eurostarch	Control	0.039 ± 0.015 a	0.141 ± 0.065 b	0.392 ± 0.087 a	0.272 ± 0.210 a	5.533 ± 3.895 a	0.031 ± 0.016 b	0.186 ± 0.126 a	0.253 ± 0.096 a
		Maxi	Control	0.049 ± 0.024 a	0.204 ± 0.042 ab	0.424 ± 0.063 a	0.161 ± 0.042 a	4.033 ± 1.495 a	0.095 ± 0.041 a	0.125 ± 0.042 a	0.340 ± 0.155 a
		Tomba	Control	0.079 ± 0.018 a	0.296 ± 0.050 a	0.336 ± 0.047 a	0.284 ± 0.167 a	7.576 ± 1.286 a	0.070 ± 0.016 a	0.187 ± 0.076 a	0.177 ± 0.028 a
		Eurobravo	Drought stress	0.035 ± 0.007 c	0.116 ± 0.021 c	0.240 ± 0.045 b	0.286 ± 0.206 a	4.665 ± 1.418 b	0.004 ± 0.003 b	0.104 ± 0.062 b	0.768 ± 0.444 ab
		Eurostarch	Drought stress	0.067 ± 0.021 b	0.145 ± 0.047 bc	0.236 ± 0.040 b	0.326 ± 0.154 a	7.444 ± 2.947 ab	0.004 ± 0.003 b	0.118 ± 0.053 ab	1.090 ± 0.193 ab
		Maxi	Drought stress	0.076 ± 0.025 b	0.242 ± 0.051 b	0.317 ± 0.036 ab	0.189 ± 0.139 a	6.398 ± 2.162 ab	0.028 ± 0.013 a	0.137 ± 0.046 ab	1.283 ± 0.329 a
		Tomba	Drought stress	0.141 ± 0.023 a	0.390 ± 0.081 a	0.374 ± 0.046 a	0.563 ± 0.273 a	9.966 ± 1.679 a	0.054 ± 0.016 a	0.233 ± 0.076 a	0.247 ± 0.029 b
2	0	Eurobravo	Start	0.115 ± 0.021 a	0.232 ± 0.056 bc	0.366 ± 0.053 a	0.579 ± 0.094 a	9.721 ± 1.276 a	0.020 ± 0.003 b	0.205 ± 0.052 a	0.270 ± 0.054 b
		Eurostarch	Start	0.073 ± 0.024 a	0.161 ± 0.012 c	0.309 ± 0.007 ab	0.474 ± 0.073 ab	9.130 ± 1.543 a	0.019 ± 0.005 b	0.177 ± 0.021 ab	0.303 ± 0.049 b
		Maxi	Start	0.105 ± 0.027 a	0.260 ± 0.021 b	0.271 ± 0.012 b	0.324 ± 0.067 b	8.575 ± 0.382 a	0.016 ± 0.005 b	0.135 ± 0.011 b	0.617 ± 0.114 a
		Tomba	Start	0.124 ± 0.045 a	0.359 ± 0.048 a	0.369 ± 0.039 a	0.301 ± 0.126 b	8.424 ± 1.013 a	0.033 ± 0.002 a	0.136 ± 0.010 b	0.316 ± 0.033 b
	7	Eurobravo	Control	0.082 ± 0.038 a	0.207 ± 0.023 b	0.322 ± 0.039 ab	0.494 ± 0.128 ab	10.211 ± 1.323 a	0.014 ± 0.005 b	0.373 ± 0.029 a	0.383 ± 0.063 a
		Eurostarch	Control	0.049 ± 0.006 a	0.211 ± 0.018 b	0.307 ± 0.014 b	1.076 ± 0.260 a	9.944 ± 1.220 a	0.019 ± 0.002 b	0.351 ± 0.069 a	0.303 ± 0.110 ab
		Maxi	Control	0.073 ± 0.048 a	0.279 ± 0.033 a	0.370 ± 0.067 ab	0.332 ± 0.127 b	14.354 ± 4.918 a	0.022 ± 0.007 b	0.233 ± 0.049 b	0.419 ± 0.123 a
		Tomba	Control	0.054 ± 0.012 a	0.335 ± 0.022 a	0.400 ± 0.026 a	0.329 ± 0.086 b	14.197 ± 2.387 a	0.054 ± 0.015 a	0.211 ± 0.042 b	0.196 ± 0.050 b
		Eurobravo	Drought stress	0.071 ± 0.159 a	0.214 ± 0.067 b	0.361 ± 0.139 a	0.274 ± 0.114 a	7.948 ± 2.877 a	0.001 ± 0.000 b	0.042 ± 0.019 a	3.122 ± 5.634 a
		Eurostarch	Drought stress	0.057 ± 0.008 ab	0.210 ± 0.022 b	0.344 ± 0.026 a	0.171 ± 0.029 a	9.851 ± 1.346 a	0.001 ± 0.000 c	0.028 ± 0.007 a	3.146 ± 0.525 a
		Maxi	Drought stress	0.046 ± 0.012 ab	0.309 ± 0.014 a	0.338 ± 0.024 a	0.120 ± 0.038 a	6.737 ± 2.800 a	0.001 ± 0.000 bc	0.029 ± 0.009 a	2.642 ± 0.334 a
		Tomba	Drought stress	0.042 ± 0.020 a	0.297 ± 0.038 a	0.358 ± 0.032 a	0.364 ± 0.255 a	5.725 ± 0.909 a	0.003 ± 0.001 a	0.045 ± 0.015 a	1.721 ± 0.162 b

Note: Letters a–c display significant differences in between a box of four genotypes in one variant and one gene of interest (Tukey test or Kruskal–Wallis test with Bonferroni correction, $n = 5$). Heat map colours reach from green (lowest value) over yellow and orange to red (highest value) and were calculated for every column separately. For *INH1*, *POD* and *SBT1.7* see also Figure 5.

Abbreviations: 13-LOX, lipoxygenase; Glyx, Lactoylglutathione lyase/glyoxalase I; INH1, cell wall/vacuolar inhibitor of fructosidase; POD, protein peroxidase 51-like; RPT5a, regulatory particle triple-A ATPase 5A; SBT1.7, subtilase family protein; SHMT, serine transhydroxymethyltransferase; ZBD, zinc-binding dehydrogenase family protein.

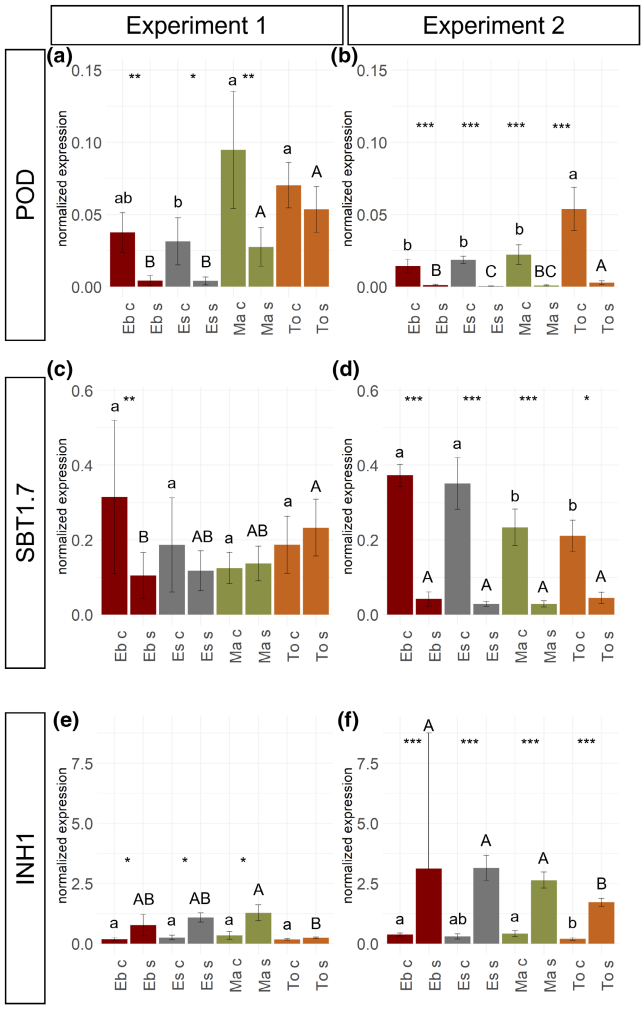

FIGURE 5 Normalized expression of the genes *protein peroxidase 51-like* (*POD*; a,b), *subtilase family protein* (*SBT1.7*; c,d), and *cell wall/vacuolar inhibitor of fructosidase* (*INH1*; e,f) after seven days under drought stress or control conditions in four potato genotypes with standard deviation, $n = 5$. a,c,e: experiment 1, b,d,f: experiment 2. Eb: 'Eurobravo', Es: 'Eurostarch', Ma: 'Maxi', To: 'Tomba'. c: control, s: stress. For *INH1* Eb s only positive SD is given. Statistical analysis: Kruskal–Wallis test with Bonferroni correction. Significance codes: ***$p < .001$; **$p < .01$; *$p < .05$.

storage starch from a seed tuber. Because the size of the seed tubers influences the performance of the plants, the comparability of plants from in vitro culture is limited (Köhl et al., 2021). Furthermore, the substrate is an essential factor for drought stress trials (Köhl et al., 2021). In our study, a large amount of sand was used in the substrate (50%), as this corresponds more closely to the soil properties in Lower Saxony (Goffart et al., 2022). Also, 2 L containers were chosen instead of larger pots because the plants were not cultivated to natural maturity as in other studies, where yield was analyzed. The open greenhouse is a rigid structure with an immovable roof. This contrasts with a rain-out shelter or closed greenhouse as were used in previous studies. This suggests that external circumstances such as pot/ container size, substrate and environment play an important role in plant response and tolerance groups can only be named within a setup.

This points to the importance of recording and considering physical growth conditions in stress experiments, especially under the semi-controlled settings of open greenhouse and field experiments.

4.2 | Stable expression of *Glyx* and *ZBD* under commencing drought stress, *RPT5a* expression differs between experiments

The candidate genes in this study were selected based on differentially abundant proteins identified in Wellpott et al. (2021) after drought stress. Significantly higher protein abundances under drought stress were shown for *RPT5a*, *ZBD*, *INH1*, *SHMT* and *13-LOX*, whereas lower abundances under drought stress were detected for *POD* and *SBT1.7*.

No alteration in gene expression was recorded for *Glyx*, a protein of the glyoxalase system, after seven days in stressed plants compared to control plants of each analyzed genotype. The protein detoxifies methylglyoxal (MG) in the first step of the glyoxalase system, which was proposed as a signalling molecule under abiotic stress (Hoque et al., 2016; Kaur et al., 2014). Likewise, expression of *ZBD* was not altered during commencing drought stress after seven days, the only exception being 'Tomba' in experiment 1, where *ZBD* expression was significantly increased. Zinc-finger proteins are a family of diverse proteins containing the zinc-finger motif. Comparing the obtained *ZBD* sequence in the SpudDB database showed that the most likely protein was an allyl alcohol dehydrogenase (Soltu.DM.03G015960) (Spud, 2022). Alcohol dehydrogenases (ADH) are encoded by a multigene family in plants and have been reported to play a critical role in plant growth, development and adaptation (Jörnvall et al., 2010; Strommer, 2011). As allyl alcohol dehydrogenases generate NADPH, which can be used as a coenzyme in photosynthesis, no alteration in gene expression might indicate a steady need for reducing agents.

RPT5a was shown to be downregulated in commencing drought stress after seven days in experiment 1, the exception again being 'Tomba' where no alteration in gene expression was detected. However, in experiment 2, differences were not detected for any genotype between control and stressed plants. RPT represent a large family of regulatory particles for ATPases that have a conserved AAA-motif. They are associated with the 26S proteasome and are essential for the unfolding of the substrates for degradation through mechanical shift (Bar-Nun & Glickman, 2012). The neighbours RPT5/6 within the RPT complex were reported to be essential for the binding of ubiquitin chains from marked proteins to the proteasome (Lam et al., 2002). The decrease in gene expression after seven days of drought stress compared to control plants in *RPT5a* might be explained by phases of high temperature before the sampling of leaves in experiment 1. High temperatures might have led to a sort of priming or stress memory effect and a subsequent drop in gene expression at the sampling date (Liu, Able, & Able, 2022).

4.3 | *LOX* activity is connected to light and temperature

Expression of *13-LOX* (*lipoxygenase*) was downregulated under drought stress in experiment 2 in 'Eurobravo', 'Eurostarch' and 'Maxi'. In contrast, in experiment 1, there was no alteration in expression after stress. The gene expression level of *13-LOX* in experiment 1 (in control and stress variants) was similar to the expression level after stress in experiment 2. Lipoxygenases could be correlated positively to ABA synthesis after drought stress and are linked to plant development and stress adaption (Deluc et al., 2009; Liavonchanka & Feussner, 2006). They can be divided into *9-LOX* and *13-LOX* based on their position of fatty acid oxygenation (Bae et al., 2016). *13-LOX* genes are expressed mainly in the above-ground plant organs, whereas *9-LOX* genes are produced mostly in roots and tubers. *13-LOX* genes play a role in the oxylipin biosynthesis through the lipoxygenase (LOX) cascade in the plant. Well-studied oxylipins are jasmonates, which activate transcription of genes involved in plant defence (Royo et al., 1996). *LOX* activity is also associated with tuberization in potato and their expression can be directly correlated to light range and temperature (Nam et al., 2005). The occurring temperature peaks in experiment 1 and the correlation between light, temperature and *LOX* expression indicate that *13-LOX* was downregulated by both stresses, heat/oxidative stress and drought and can presumably be linked to postponing of tuber formation.

4.4 | Results indicate a rapid stress response for *SHMT*

Stomatal closure causes downregulation of photosynthesis due to less available CO_2. This also leads to changes in gene expression of some genes involved in carbohydrate metabolism, such as *SHMT*. SHMT is a pyridoxal-5'-phosphate (PLP)-dependent enzyme which is linked to catalysing the conversion of glycine to serine and vice versa. *SHMT* activity results in one-carbon units, which are important for many cellular processes, including the synthesis of chlorophyll (Jabrin et al., 2003; Ruszkowski et al., 2018). In plants, mitochondrial SHMT enzymes provide these amino acids for chlorophyll biosynthesis and are linked to photorespiration (Douce et al., 2001; Liu, Pan, et al., 2022). Furthermore, ROS production is increased under stress, leading to damage to cellular components. One strategy of the plant to protect and adapt to oxidative stress is the detoxification of ROS (Demidchik, 2015) which also involves SHMT (Fang et al., 2020). *SHMT* expression was significantly decreased after seven days of commencing drought stress only in experiment 2 in 'Maxi' and 'Tomba'. In experiment 1, *SHMT* expression was increased in 'Eurostarch', 'Maxi' and 'Tomba', but those alterations were not significant. Hourton-Cabassa et al. (1998) also observed a downregulation of *SHMT* after drought stress in potato. Ambard-Bretteville et al. (2003) showed a drastic downregulation of *SHMT* after an upregulation 8 h after the onset of drought stress in potato. These outcomes and the fact that the enzyme was higher abundant in potato leaves after drought stress in Wellpott et al. (2021), indicate a rapid response of *SHMT* expression, which should be verified by analysing earlier time points after stress.

4.5 | Commencing drought stress reduces *POD* and *SBT1.7*, but induces *INH1* expression

Goals of the gene expression analyses were to find evidence whether regulation occurred at the transcriptional level for the selected proteins of interest and to identify possible markers for early drought stress in potato. The genes *POD*, *SBT1.7* and *INH1* showed very consistent regulation in all genotypes after commencing drought stress after seven days with *INH1* displaying the highest normalized expression levels.

A reduction of gene expression was detected for *POD* and *SBT1.7*. Reduction of gene expression was evident for *POD*, a peroxidase superfamily protein, in experiment 1 and 2. However, an exception was 'Tomba' in experiment 1, where no significant change in gene expression was detected. Peroxidases function in detoxification of hydrogen peroxide (H_2O_2), which is known to be related to cell wall modifications and is well known as a signalling molecule under oxidative stress (Boguszewska et al., 2010; Kopyra & Gwóźdź, 2003; Mittler, 2002). Most studies published report an increase in gene expression of peroxidases (which differ from the POD found in this study), or the activity of the enzymes produced after drought stress (for review see: Suzuki et al., 2012). Whether this could be also the case for our peroxidase early after stress remains unclear. Earlier time points might be more conclusive as for the gene expression of *POD*.

Expression of *SBT1.7* (also referred to as ARA12; Engineer et al., 2014), a calcium-dependent subtilase, was reduced in all genotypes for experiment 2 and in the genotype 'Tomba' in experiment 1. Subtilases comprise a diverse group of serine peptidases, most of which are targeted to the cell wall or were predicted to range in the extracellular space of potato plants (Norero et al., 2016; Schaller et al., 2018). They are known to function in cell growth and development through the regulation of the activity of extracellular signalling molecules as well as properties of the cell wall (Schaller et al., 2018). Reduced gene expression of *SBT1.7* might therefore display a reduced cell growth, as also indicated by the growth data of the plants after seven days of commencing drought stress. As protein abundance was also found to be reduced, this gene might comprise a target for further analysis upon drought stress to develop biomarkers (Wellpott et al., 2021).

INH1, an invertase inhibitor, was found to be significantly upregulated under commencing drought stress in both experiments, the only exception again being 'Tomba' in experiment 1. In potato, *INH1* was described to be highly expressed in leaves and flowers compared to *INH2*, which was more prominent in tubers and roots (Brummell et al., 2011). *INH1* was previously described upregulated by Aliche et al. (2022) after drought stress and by Yang et al. (2020) to give rise to drought tolerance when overexpressed

in sweet potato. However, they also found a trade-off with growth, as overexpression of *INH1* led to growth reduction in mutant lines. Therefore, cell wall and vacuolar invertase inhibitors are important regulators of plant growth. They are also known to be important regulators of sink-source strength and sugar-related signalling and were shown to be involved in stress responses, e.g. cold-induced sweetening of tubers in potato (Brummell et al., 2011; Castrillon-Arbelaez & Delano-Frier, 2011). *INH1* also plays a major role in drought stress-mediated stomatal closure to reduce water loss (Chen et al., 2016; Kulik et al., 2011; Matsuoka et al., 2021). ABA levels increase in plant cells under abiotic stress, activating SnRK2 family proteins and thus lead to stomatal closure, which is a common response of the plant to drought stress (Mustilli et al., 2002). Gene *INH1* (*cell wall/vacuolar inhibitor of fructosidase*) was shown to specifically inhibit many proteins from the SnRK2 family (Kulik et al., 2011; Matsuoka et al., 2021). Yang et al. (2020) demonstrated that the gene *INH1* (*cell wall/vacuolar inhibitor of fructosidase*) activates the ABA-regulated pathway and therefore ABA biosynthesis in sweet potato after drought stress, resulting in enhanced drought tolerance. Other than that, invertases hydrolyse sucrose into glucose and fructose and thus *INH1* plays a major role in regulating the primary metabolism and development of the plant (Ruan et al., 2010). An increase of *INH1* gene expression in potato leaves after seven days of commencing drought stress might therefore directly help plants to cope with starting water deficiency. Since *INH1* was found to be higher abundant after drought stress on protein level only in the more tolerant genotypes 'Eurostarch' and 'Tomba' (Wellpott et al., 2021), the protein could also be a candidate for the detection of commencing drought stress tolerance.

5 | CONCLUSION

In this study, we successfully applied drought stress in all analyzed genotypes. No trends concerning different levels of tolerance between the genotypes could be detected in the recorded growth data in contrast to results of previous evaluations which took place in different settings. This was likely due to the fact that experiments outside a climate chamber are subject to natural variations in physical growth conditions and in most previous studies, the plants were analyzed after natural maturity. There is no clear correlation between tuber yield and early shoot mass in potato. This indicates that the setup of stress experiments is of major importance regarding classification in tolerance levels of individual genotypes. We observed additional heat stress and higher radiation in the first experiment, which led to an alteration in response of the potato plants. This can be reinforced by variable gene expression data of *RPT5a*, *13-LOX* and *SBT1.7*. However, there is no evidence of priming in potato plants after drought stress (Köhl et al., 2023). Early drought stress experiments are therefore suitable to derive markers for drought stress tolerance. Out of the eight GOIs investigated in this study, *INH1* was found to comprise a strong candidate for detection of commencing drought stress in early stages of potato development.

AUTHOR CONTRIBUTIONS

Katharina Wellpott: Formal analysis; writing – review and editing; visualization; methodology; investigation; writing – original draft. **Jannis Straube:** Writing – review and editing; methodology. **Traud Winkelmann:** Project administration; supervision; writing – review and editing; funding acquisition; investigation. **Christin Bündig:** Project administration; supervision; writing – review and editing; writing – original draft; funding acquisition; investigation.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Research Data Repository of the Leibniz University Hannover at https://doi.org/10.25835/td4w2pg9.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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