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Cultivar-specific seedling vigor and expression of a putative oxalate oxidase germin-like protein in sugar beet (Beta vulgaris L.)

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Abstract For genetic screening and breeding purposes, an in vitro germination system that reflects relative field emergence potential was used to screen for germination-enhancing and stress-induced genes from germinating seedlings from two varieties of sugar beet. Three full-length germin-like protein (GLP) gene classes were recovered from stress-germinated seedlings of a superior emerging variety. GLP gene expression, oxalate oxidase protein activity, the H_2O_2 content of stressed seedlings, but not catalase activity, were induced by stress germination conditions (e.g. excess water, NaCl, mannitol, or oxalate) in a good emerging hybrid and were not induced in a poor emerging variety. Only one of the three germin-like protein genes (*BvGer*165) was differentially regulated, and was induced only in the good emerger. Hydrogen peroxide promoted germination and partially compensated solute-depressed germination percentages. Unlike other solute recovery by hydrogen peroxide regimes, recovery in oxalic acid plus H_2O_2 was cultivar-independent. A block in oxalate metabolism is postulated to contribute to lower germination under stress in the lower emerging variety. Selection for stress-induced germin expression, or for down-stream targets, presents the first direct target to enable breeding for improved field emergence of sugar beet.

Keywords Germination · Abiotic stress · Hydrogen peroxide · Seedling vigor · Molecular phenotyping

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Introduction

Field emergence of sugar beet (*Beta vulgaris*) is an annual concern for growers, and improving emergence potential has not been amenable to selection due to developmental and environmental influences on seed quality. On average, 60% of the planted seed contributes to the maximum stand count in Michigan (Anonymous 1997), with a range of emergence from 0 to 90% depending on environmental and disease pressure during stand establishment. Field emergence and stand establishment are largely determined by seedling vigor, which has been defined as the ability of physiologically viable seed to germinate and flourish in diverse environments (Kneebone 1976). For sugar beet, the first 8 weeks of growth are most critical (Durr and Boiffin 1995). Improving emergence potential is problematic because both the seed germination environment the and seed production environment profoundly influence seedling performance (Johnson and Burt 1990). Germination is the first phase of emergence, resulting from a culmination of events such as imbibition of water that initiates metabolic activity and leads to subsequent cell enlargement and division (Thomas 1993; Copeland and McDonald 1995).

A novel stress test appeared promising for assigning relative field emergence potential, demonstrating that germination of commercial quality sugar beet seed (e.g. >92% germ) can be discriminated in vitro to reveal higher and lower vigor seedlots. Germination in water showed the same relative ranking of varieties as their emergence in the field, and germination in hydrogen peroxide solution promoted germination to the extent observed with traditional germination methods used in commercial quality assessment (McGrath et al. 2000). In this report, we have begun the search for possible reasons of enhanced in vitro germination of sugar beet by hydrogen peroxide, since the effect is dramatic, and has potential in breeding for enhanced emergence.

We examined two commercially acceptable sugar beet hybrids known to differ in relative field emergence. We show that the good emerging commercial hybrid specif-

ically induced expression of a gene with high similarity to known germin-like protein (GLP) genes, and that this gene is not induced in the lesser emerging commercial hybrid. Expression of this particular germin-like protein appears to be a physiological marker for enhanced sugar beet germination and, by extension, field emergence. We propose that the specific GLP induced is an oxalate oxidase based on correlative evidence of increases in oxalate oxidase activity and internal hydrogen peroxide concentrations, and suggest that the oxalate oxidase metabolic product, hydrogen peroxide, markedly increases expression of stress-related genes during aqueous germination regimes and leads to enhanced seedling vigor.

Materials and methods

Seed germination

The same seedlots of two commercial sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. altissima Döll.) hybrids, USH20, a diploid (Coe and Hogaboam 1971), and ACH185, a triploid (American Crystal, Moorhead, Minn.), with commercially acceptable germination percentages (>92%) were used throughout these experiments. Standard germination assays were performed on moist pleated germination paper (TeKrony and Hardin 1966; Bornscheuer et al. 1993). Germination in solution was performed as described (McGrath et al. 2000). Briefly, four replicates of 25 seeds each were immersed in 15 ml of solution, and germination counts were done 96 h post-immersion, with an intermediate count and change of solution at 48 h. A seed was scored as germinated when the radical protruded from the seed ball. All values reported are germination percentages after the total 96-h incubation. Germination treatments in solution included 150 mM of NaCl, 200 mM of mannitol, 18 MOhm of water, 88 mM of H_2O_2, 120 mM of oxalic acid pH 4.0 and some combinations of these. Field emergence was assessed using six replicates of 100 seeds/10 m plot in each of 3 years at the Saginaw Valley Bean and Beet Research Farm (Saginaw, Mich.).

Differential display

RNA was isolated from 1,000 to 1,500 4-day old seedlings (Logeman et al. 1996). First-strand cDNAs were synthesized from 1 μg of total RNA (pre-treated with DNAse I) with oligo-dT primer (1 μM) and MMLV reverse transcriptase (1,000 units) at 42 °C for 1 h. Differential display (DD-PCR) was performed on USH20 total RNA samples using the Delta Differential Display kit (Clontech) following the manufacturer's instructions. Amplified products were fractionated in 6% denaturing polyacrylamide gel (Sambrook et al. 1989), dried, and autoradiographed for 24 h. Fragments with signal differences between control (filter paper germinated seedlings) and treatments were excised, re-amplified, cloned in pTAdv TA vector (Clontech) and sequenced. A germin-like DD-PCR fragment (amplified with the P4/P4 primer combination) was used to recover 14 full-length cDNA clones from a Lambda Zap cDNA library (Stratagene) constructed from USH20 4-day old solution-germinated seedlings, and each clone was sequenced in both directions. Encoded protein products were deduced from nucleotide sequences via GENSCAN (Burge and Karlin 1998). Nucleotide and amino-acid sequence homologies were determined with BlastN, BlastX and BlastP search algorithms (Altschul et al. 1997). Multiple sequence alignments were performed via ClustalW (Thompson et al. 1994).

RNA gel-blot analyses

Eight hundred nanograms of polyA+ mRNA were fractionated in 1.2% formaldehyde-agarose gels, blotted on nylon membranes (HybondN, Amersham) and hybridized with ^{32}P-dCTP-labeled *BvGer*171 overnight at 42 °C in NorthernMax Prehyb/Hyb buffer (Ambion). Post-hybridization washes (twice, 15 min each) in 2 × SSC, 0.1% SDS followed with 0.5 × SSC, 0.1% SDS preceded exposure to X-ray film (3 days). Filters were stripped and re-hybridized with an actin probe (as a control) following the same procedure. The actin clone was obtained from the sugar beet EST collection (Genbank accession no. AWO63023).

Reverse transcription-PCR (RT-PCR)

Total RNA (1 μg) from each of USH20 and ACH185 filter-paper and solution-germinated seedlings were reverse transcribed using RT-for-PCR (Clontech) as per manufacturer's instructions. Specific primer pairs to each full-length recovered germin cDNA were designed, synthesized and used to amplify their cognate cDNAs from 1 μg of total RNA using dNTP mix (50 μM), ^{33}P-dATP (50 nM, 2 μCi) and Advantage Klen*Taq* polymerase mix (2 units) (Clontech). Gene specific primers were designed from accession nos. AF10016 (*BvGer165*), AF10017 (*BvGer171*) and AF10018 (*BvGer172*) as shown:

– *BvGer*165: Forward: 5'-TGTACCCGGAAACAATGTA; Reverse: 5'-TGTTGTCATCATACACT
– *BvGer*171: Forward: 5'-GTTTCCCTTAGCTTGCAAAGTA; Reverse: 5'-CATTTGCACAAACTTCCAAAG
– *BvGer*172: Forward: 5'-GTTTCCCTTAGCTTGCAAAAATA; Reverse: 5'-TATCATTCAATACTGCAAAGGA.

PCR cycling was 1 min at 95 °C, 22 cycles of 30 s at 94 °C, 1 min at 50 °C, 1 min at 72 °C; and final extension for 10 min at 72 °C. Fragments of 515 bp, 831 bp and 850 bp, amplified with gene specific primers to *BvGer*165, *BvGer*171 and *BvGer*172, respectively, were verified by sequencing. Actin at (675-bp fragment amplified with primers Forward = 5'-TGTGAGCAACTGGGATGA and Reverse = 5'-GGTAACATCGTGCTCAGT) was used as a control for RT-PCR to verify amplification and loading equivalency. Amplification products were separated in 6% non-denaturing polyacrylamide, dried, and autoradiographed overnight. The specificity of each primer pair was confirmed by the lack of cross-amplification with non-target BvGer plasmid clones, and by re-sequencing the products amplified from experimental samples.

Oxalate oxidase assay

Crude extracts of oxalate oxidase were prepared from 4-day old filter paper, and solution-germinated USH20 and ACH185 seedlings, via the method of Pundir and Nath (1984) with slight modifications. Germinated seedlings (15 g) were excised from the ruptured pericarp and homogenized in 15 ml of distilled water at 4 °C. The homogenate was squeezed through a double layer of cheesecloth and centrifuged at 4 °C, 15,000 g for 40 min. $(NH4)_2SO_4$ crystals were added to the supernatant to obtain 0–30% and 30–65% saturation fractions, and crude protein extracts precipitated at 4 °C overnight. The precipitate from 30 to 65% fractions was collected by centrifugation at 4 °C 10,000 g for 40 min, and the pellet was re-dissolved in 0.05 M sodium succinate pH 5.0 and stored at 4 °C.

Oxalate oxidase activity assays were performed in 10-ml stoppered glass tubes wrapped in carbon paper using 0.53 mg/ml of crude protein, 40 mM of sodium succinate pH 5.0, 2 mM of oxalic acid and 1 mM of $FeSO_4$; incubated at 37 °C for 10 min and then mixed with 1 ml of color reagent (0.05 g of 4-aminophenazone, 0.1 g of phenol and 50 units of horseradish peroxidase per 100 ml of 0.4 M sodium phosphate buffer pH 7.0). Absorbance at 520 nm for each sample was determined after 25 min of color

development at room temperature (Thompson et al. 1995). Values from two independent experiments were averaged.

Direct peroxide assay

H_2O_2 extraction procedures were according to Warm and Laties (1982). Seedlings with emerged radicles (4-day germinated) were thoroughly washed in sterile distilled water, blot-dried in filter paper and then carefully excised from the thick coat without wounding the seedling. Ten seedlings were homogenized, individually, in 1.5-ml micro-centrifuge tubes, with 0.1 ml of chilled 5% trichloroacetic acid (TCA). Crude extracts were centrifuged at 4 °C for 15 min at 12,000 g. Colored components in the supernatant were removed by adding 10 mg of Dowex anion exchange resin (AG 1 × 8) to the mixture followed by centrifugation at 10,000 g for 2 min. Supernatant was further diluted to 0.2 ml with 5% TCA, and a 10-μl aliquot from each seed sample was diluted to 100 μl with 0.25 M sodium phosphate, pH 7.4, and used for H_2O_2 quantification using the Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) hydrogen peroxide assay kit (Molecular Probes). Total nmol H_2O_2 was determined after 30-min incubation of the reaction mixture in the dark at room temperature by measuring fluorescence (using a 560 nm excitation filter) at 590 nm.

Catalase activity assay

Crude protein extracts were prepared following Warm and Laties (1982). One g of 4-day old germinated seedlings was prepared for extraction as described in the H_2O_2 assay procedure. Seedings were homogenized by grinding in a chilled mortar and pestle with 2.5 ml of 40 mM Tris-HCl, pH 7.0. The homogenate was filtered through Miracloth (CalBiochem) and centrifuged at 4 °C for 20 min at 12,000 g. An aliquot containing 100 μg of total protein was diluted to 125 μl with 0.5 M Tris-HCl, pH 7.5, and assayed using the Amplex Red catalase assay kit (Molecular Probes). Catalase activity in each sample was determined after 30-min incubation in the dark at room temperature by measuring absorbance at 563 nm.

Macroarray

Ten ng of purified insert DNA was spotted on a nylon membrane using a 96-well dot-blot apparatus and fixed to the membrane by UV cross-linking. Probes were prepared by reverse transcription of 750 ng of poly-A+ RNA from 4-day old USH20 seedlings germinated on moist filter paper, or in either water, 150 mM of NaCl or 88 mM of H_2O_2 solutions. cDNA probe synthesis and ^{32}P-dATP-labeling was performed using the Strip-EZ RT kit (Ambion). Filters were hybridized with the labelled total cDNA probe at 42 °C for 16 h using the ULTRAhyb hybridization buffer (Ambion). Blots were washed twice at 50 °C in 2 × SSC, 0.1% SDS for 15 min and then twice in 0.5 × SSC, 0.1% SDS for 30 min, autoradiographed for 4 days on the same sheet of film, and images were scanned and analyzed. Analysis consisted of estimating pixel density values in a 400 pixel^2-area centered on each dot using Adobe Photoshop. Each dot value was divided by the background pixel value calculated from the average of five 400 pixel^2-areas with no signal on each blot, to give a proportional difference in signal intensity between a signal (dot) and the background. Proportional differences greater than 0.25 (e.g. 64 pixel values) between background and signal were taken as an estimate (presence vs absence, rather than quantitative) of the moderately abundant transcript levels reported here.

Results

Differences in field stand counts during emergence were consistent over three growing seasons between the same seedlots of sugar beet hybrids. In all cases the hybrid variety USH20 out-performed hybrid ACH185 (Fig. 1, and data not shown). The best performing commercial quality seedlots of these two hybrids were chosen for further analyses.

TeKrony and Hardin (1966) showed that hydrogen peroxide-treated sugar beet seeds gave rise to more vigorous seedlings and resulted in a higher proportion of germinated seedlings than did non-treated seeds. In our hands, continuous incubation of seeds in hydrogen peroxide showed similar results, as compared to continuous incubation in water or any other solute tested to-date (Fig. 1, and McGrath et al. 2000). Germination in water alone showed the same relative emergence rank of varieties and seedlots tested in the field. Hydrogen peroxide stimulated germination in both hybrids such that differences between them were statistically insignificant.

To examine the role(s) of H_2O_2 in promoting sugar beet seed germination, we focused on 4-day old seedlings germinated on moist filter paper vs seedlings germinated in solution. As different solutes may evoke different responses, we initially focused on solutions containing purified water, and purified water with single solute additions of NaCl, mannitol or H_2O_2. Subsequent to identifying a potential oxalate oxidase involved in germination (see below), oxalic acid was included in solution germination assays, as were treatments supplemented with hydrogen peroxide (Fig. 1).

Differential display-PCR on USH20 seedlings was done to assess transcript differences (presence vs ab-

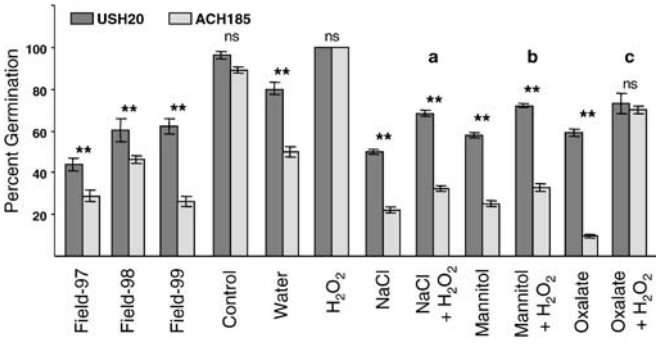

Fig. 1 Comparisons of USH20 and ACH185 commercial sugar beet varieties with higher and lower seedling vigor, respectively. Field emergence at 15 days after planting over 3 consecutive years. Control seeds were germinated on moist filter paper. Solution germination was in de-ionized water, 88 mM of (0.3%) of hydrogen peroxide, 150 mM of NaCl, 200 mM of mannitol, or 120 mM of oxalic acid (pH 4.0), and in each supplemented with 88 mM of H_2O_2. *Asterisks* indicate statistically significant *t*-tests between hybrids at $p \leq 0.01$. *Letters* indicate significant differences between treatments with and without supplemental hydrogen peroxide (*t*-test, $p \leq 0.01$). *Error bars* are the standard error of the mean within each treatment

sence). Of 50 primer combinations tested, only 5% (40/807) of the amplified fragments differed across treatments (filter paper, water, NaCl, mannitol and H_2O_2). Each of these 40 differentially amplified fragments was cloned and sequenced. Among these sequences was a 440-bp fragment (P44) seen in water, salt and mannitol treatments, but not in H_2O_2 or on moist filter paper. The nucleotide and predicted amino-acid sequences of P44 were similar to germin and germin-like protein genes. These were further examined because some germins encode an oxalate oxidase protein (E.C. 1.2.3.4) that catabolizes oxalic acid to CO_2 and H_2O_2. A functional oxalate oxidase would provide an in vivo source of hydrogen peroxide, mimicking responses seen with H_2O_2 in solution, and may in part, have explained seedling vigor.

Fourteen germin-like protein (GLP) gene clones were recovered from a solution stress-germinated USH20 seedling cDNA library probed with P44, and each was fully sequenced. Three distinct groups were found, represented by *BvGer*165, *BvGer*171 and *BvGer*172, each full length and the longest representative of its group. All three encoded a predicted protein composed of 208 amino-acid residues. *BvGer*165 showed 83% amino-acid identity (81% nt identity in coding, 40% in non-coding regions) and 85% amino-acid identity (82% nt identity in coding, 52% in non-coding regions) with *BvGer*171 and *BvGer*172, respectively. *BvGer*171 and *BvGer*172 showed 92% amino-acid identity (95% nt sequence identity in coding, 69% in non-coding regions). An mRNA fragment of approximately 0.9 kb was detected on Northern blots, equivalent to the size of the GLP cDNAs isolated. All three BvGer clones share greater similarity at the predicted polypeptide level with Subfamily-3 type GLPs (Carter and Thornburg 1999) than with true germins.

Poly-A+ RNA was isolated from USH20 seedlings, and from these Northern blots were probed with *BvGer*171. *BvGer*171 hybridized more intensely with RNA isolated from USH20 seedlings germinated in water, NaCl, and mannitol relative to filter paper or hydrogen peroxide germinated-seedlings, while hybrid ACH185 showed a weak and no increase in signal intensity in these treatments (data not shown). A faint signal was seen from each treatment in ACH185, suggesting cross hybridization of BvGer GLP classes, but not differential regulation of GLP transcripts. Gene-specific primers were designed to individually identify transcript abundance levels of the three GLP classes. *BvGer*165, but not the other tested GLP transcript representatives, exhibited an induced pattern of expression similar to those observed on Northern blots (Fig. 2). That is, *BvGer*165, but not *BvGer*171 or *BvGer*172, expression was induced in water, NaCl, and mannitol-germinated USH20 seedlings, but not in filter paper or H_2O_2-germinated seedlings. A large increase in signal intensity was seen with oxalic acid-germinated USH20 seedlings, and a lesser increase was seen in similar ACH185 seedlings (Fig. 2). *BvGer*165 was not induced markedly in ACH185 seedlings germinated under stress. We concluded that *BvGer*165 represented a stress-inducible GLP (defining stress here as germination in solution) in the better stress emerging hybrid USH20, and that this gene (allele) was not expressed in response to stress in ACH185.

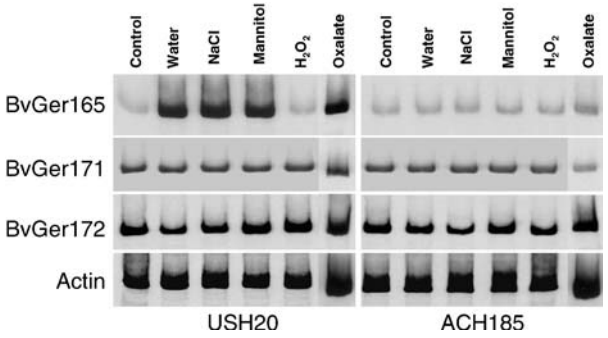

Fig. 2 RT-PCR of USH20 (high emerger) and ACH185 (low emerger) varieties using gene-specific primers for each of the three GLP classes represented by full-length clones *BvGer*165, 171 and 172. β-actin was used as RT-PCR and a loading control

Reduction in germination of the poor-emerging ACH185 in oxalic acid was dramatic relative to other stress treatments tested, while that of USH20 was not as markedly affected (Fig. 1). Relief from oxalate stress by H_2O_2 was as equally dramatic in ACH185, and less so in USH20. A strong induction of GLP expression by oxalate in hybrid USH20 was seen on Northern blots (data not shown). Seeds germinated in NaCl and mannitol also showed reduced germination, and this reduction could be partially compensated by the addition of hydrogen peroxide (Fig. 1). From USH20 RNA gel blots, the GLP transcript signal was high in NaCl and mannitol, and reduced in these treatments supplemented with H_2O_2. This result suggested that external hydrogen peroxide concentrations influenced GLP transcript abundance, as the sole change in these assay conditions was the presence or absence of hydrogen peroxide.

Since oxalate exerted a pronounced effect on germination with and without H_2O_2, and expression of a putative oxalate oxidase GLP responded to stress, oxalate oxidase enzyme activity was examined from crude extracts of 4-day old seedlings. In hybrid USH20, oxalate oxidase activity exceeded the control (filter paper) germinated seedlings in all treatments, except H_2O_2 (Fig. 3), with NaCl and oxalic acid exhibiting the strongest activity increases. In contrast, enzyme activity in ACH185 was relatively constant across treatments, except in seeds germinated in oxalic acid, where a modest but significant increase in activity over control levels was noted, and in mannitol where a modest but significant decrease in enzyme activity was measured. These results were consistent with changes in GLP transcript levels with the exception of the decrease of ACH185 oxalate activity in mannitol.

If oxalate oxidase was active, an increase in H_2O_2 in vivo would be expected. Hydrogen peroxide concentrations were measured within 4-day old germinated

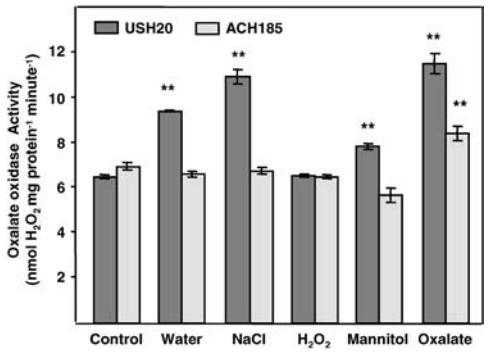

Fig. 3 Oxalate oxidase activity in USH20 and ACH185 relative to germination treatment. *Asterisks* indicate a statistically significant *t*-test between hybrid treatment and control filter paper treatment at $p \leq 0.01$

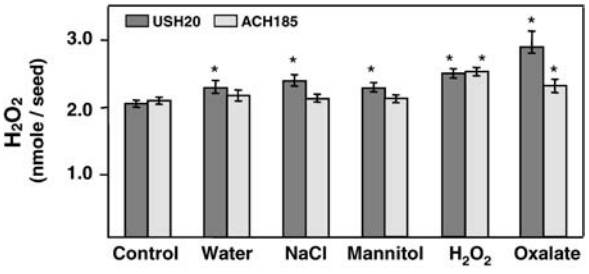

Fig. 4 Mean H_2O_2 content from stress germination treatment tests in sugar beet varieties USH20 and ACH185 in 4-day old germinated seedlings (mean of ten seedlings ± SE). The *asterisk* indicates the treatment mean significantly different from the filter paper mean ($p \leq 0.10$)

seedlings. Relative to the control (filter-paper), H_2O_2 concentrations did not vary dramatically between treatments. However, all USH20 solution-germinated seedlings showed statistically significant higher H_2O_2 levels than filter-paper-germinated seedlings (Fig. 4). Interestingly, oxalate (and H_2O_2)-germinated ACH185 seedlings also had significantly higher in vivo H_2O_2 levels. Significant differences in H_2O_2 concentration were evident between hybrids, except for seedlings germinated in H_2O_2. It should be noted that extensive washing procedures were employed to remove adhered solutes from seedlings; thus measured levels were considered indicative of internal H_2O_2 concentrations. With the exception of hydrogen peroxide-germinated seedlings where H_2O_2 could have diffused to an equilibrium value within seedling tissue, these results were consistent with increased GLP transcript abundance and increased oxalate oxidase enzyme activity in the better emerging USH20.

A catalase enzyme assay was used as a proxy for potential increased degradation of H_2O_2. Catalase activity did not vary significantly across treatments, and did not vary between the two hybrids (Fig. 5). A general increase in catalase activity was seen with increasing H_2O_2 incubation concentrations (data not shown), but the increases were not significantly different from filter-

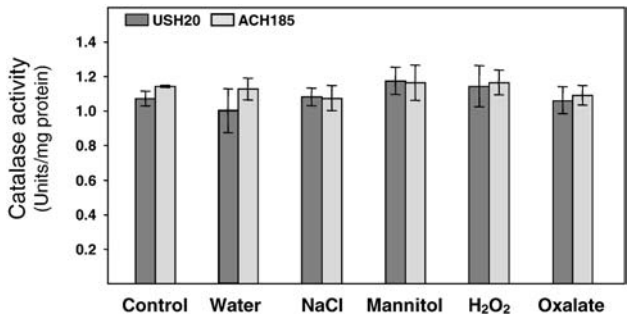

Fig. 5 Catalase activity (mean of three replicates ± SE) in crude protein extracts from 4-day old germinated seedlings. Differences were not significant

paper germination controls. An implication of these results is that in vivo levels of H_2O_2 rise as a consequence of increased H_2O_2 production, in stress-germinated USH20, with little or no change in degradation of H_2O_2 in germinating seedlings, at least due to catalase activity.

Ninety six cDNA clones with at least 400 bp of nucleotide sequence information were selected, non-randomly on the basis of their perceived role in abiotic stress responses, from the available sugar beet seedling ESTs (http://ncbi.nlm.nih.gov/dbEST), and included *BvGer*165 as a positive control. All clones were derived from solution-stressed germinated seedling ESTs from USH20. Clones were blotted onto nylon filters and probed with labeled poly-A+ RNA from USH20 seedlings germinated on filter paper or in solution (i.e. water, NaCl or H_2O_2). The goal was to gain preliminary insight on the question of hydrogen peroxide-inducing expression of many genes, or simply providing an 'ideal' environment similar to control filter paper germination since there appeared little difference in germination percentages (Fig. 1). Only 36 of the 96 clones, including *BvGer*165, showed a detectable signal above background (Table 1). Of these, the majority (30) were not detected from filter paper-germinated seedling RNA. Of the 30 that showed signal strength differences, the majority (17) were 'induced' in the solution environment per se (e.g., water, salt and peroxide), nine were 'induced' in response to salt treatment (with six showing overlap with H_2O_2) and four were specifically 'induced' by hydrogen peroxide treatment. Note that the germin gene followed its expected pattern of expression.

Hydrogen peroxide apparently induced expression of a number of stress-related genes, similar in number and with significant overlap with NaCl (and water)-stressed seedlings, and thus represents a treatment in vitro and potentially in vivo, which effects global gene expression in response to stress environments during germination. Determining whether the *BvGer*165 gene product is the actual effector of enhanced stress germination in USH20 will require demonstration of an oxalate oxidase activity for this specific GLP.

Table 1 Identity and characteristics of sugar beet stress-germinated EST clones that showed relatively large autoradiographic signal intensities on a 96-feature macroarray. ns = not significant at this level of analysis (defined here as values of 0.25 or less, see text)

dbEST ID	Putative function	Relative intensity of signal			
		Control	Water	NaCl	H_2O_2
AW697745	CPEP phosphomutase	1.00	1.00	1.00	1.00
BG932913	Sorghum water-induced cDNA	0.97	1.00	1.00	1.00
BG932915	At hypothetical protein	0.80	1.00	1.00	1.00
BG932918	Cytochrome P450 monooxygenase	0.67	1.00	1.00	1.00
AW697733	Alcohol dehydrogenase	0.64	0.98	0.74	0.49
AW697743	Phosphatidylinositol 3-kinase	0.32	0.26	0.38	0.30
BG932914	Glycogen starch synthase	ns	0.85	0.88	1.00
BE590455	ABC-type transport protein	ns	0.77	ns	ns
AW697769	Glyceraldehyde dehydrogenase	ns	0.77	0.82	1.00
AW697755	Tubulin, beta chain	ns	0.70	0.77	0.96
AW697750	HSP80	ns	0.69	0.92	0.70
AW777178	Translation factor	ns	0.55	0.72	0.65
AF310016	Germin/oxalate oxidase	ns	0.53	0.78	ns
AW697779	Homolog of barley B12 protein	ns	0.53	0.96	0.50
BF011249	MYB-related protein	ns	0.51	0.53	0.46
AW697754	Casein kinase	ns	0.47	0.46	0.75
AW777187	Ferritin (iron and drought induced)	ns	0.46	0.77	0.82
AW777151	Protein kinase	ns	0.45	0.79	0.35
AW697775	At hypothetical protein	ns	0.42	0.42	0.26
AW697774	ADOMET synthethase	ns	0.41	0.84	0.71
BE590446	Malate synthase	ns	0.37	0.29	0.71
BG932912	Histidine decarboxylase	ns	0.29	0.69	0.72
AW697742	dnaK-type molecular chaperone	ns	0.26	0.67	ns
AW697776	At hypothetical protein	ns	ns	0.86	0.30
AW697739	At hypothetical protein	ns	ns	0.48	0.26
BG932916	Unknown	ns	ns	0.42	ns
BE590354	Glutathione S-transferase	ns	ns	0.33	ns
BF011164	Alpha-amylase	ns	ns	0.32	0.53
BF011243	O-methyl transferase	ns	ns	0.29	0.42
AW697730	IPP isomerase	ns	ns	0.28	0.26
BG932917	Aquaporin (salt induced)	ns	ns	0.28	0.39
BE590335	Porin	ns	ns	0.26	ns
BF011145	Na+/H+ exchanger protein	ns	ns	ns	0.39
AW777195	At hypothetical protein	ns	ns	ns	0.36
AW777201	Membrane protein	ns	ns	ns	0.31
BE590435	14-3-3-like protein	ns	ns	ns	0.29

Discussion

In the good emerger, but not the poor one, increased hydrogen-peroxide levels, increased oxalate-oxidase activity and increased abundance of germin-like protein transcripts provide evidence for a role for in vivo regulation of H_2O_2 in a stress-response pathway. A model to explain the difference in stress germination between the good emerging USH20 and the comparatively poor emerging ACH185 can be constructed along the following lines. Simply, inclusion of hydrogen peroxide in the germination environment is sufficient to erase validated germination percentages between hybrid USH20 and hybrid ACH185; thus hydrogen peroxide is an effector of enhanced germination. Expression of a putative oxalate oxidase (*BvGer*165), increased oxalate-oxidase enzyme activity and increased H_2O_2 levels, all suggest that hydrogen peroxide is an effector of enhanced stress germination in vivo, at least for USH20. Thus, ACH185 appears incompetent in producing sufficient hydrogen peroxide in vivo, at least in the same way as USH20, and thus shows less stress germination percentages in the absence of hydrogen peroxide. Of particular significance is the observation that USH20 and ACH185 show equivalent germination in oxalate plus H_2O_2, unlike other H_2O_2-rescued solute germination regimes. This suggests oxalate and H_2O_2 most likely share a common biochemical pathway, or are otherwise aspects of the same mechanism. Specifically, the lack of induction of stress-specific GLP *BvGer*165 in hybrid ACH185 would prevent the oxidation of oxalate to H_2O_2, and an exogenous supply of H_2O_2 rectifies this deficiency. This model predicts that *BvGer*165 is indeed an oxalate oxidase, expressed during germination, and thus a dicot model for the wheat and barley oxalate oxidase germins characterized to-date (Bernier and Berna 2001).

From the simple addition of hydrogen peroxide to sugar beet seedlings during germination, it appears that the physiological and biochemical ramifications of H_2O_2 in seedlings are quite complex. H_2O_2 is beneficial on seed germination in a number of diverse plant species (TeKrony and Hardin 1966; Chien and Lin 1974; Hsiao and Quick 1984; Snow 1985; McGrath et al. 2000). Mechanisms for enhanced H_2O_2 germination may include oxidation of germination inhibitors, oxidation of physical or other chemical restraints on germination and antibiosis to seedling pathogens (Heydecker et al. 1971; Chien and Lin 1974; McGrath et al. 2000). However, it is still not

known if H_2O_2 per se, or some product thereof, would be the primary effector for enhanced germination. One possibility is that molecular oxygen plays a strong role, since H_2O_2 spontaneously decomposes to O_2 and water, but this appears unlikely since constant addition of molecular oxygen to seeds germinating in water shows no effect on germination percentage (data not shown). Nevertheless, H_2O_2 appears to initiate a chain of biochemical events that link it with enhanced germination of sugar beet seedlings.

A sugar beet gene was recovered with high similarity to a class of proteins generally referred to as germin-like proteins, only some of which have been shown to possess oxalate oxidase activity (Carter and Thornburg 1999; Bernier and Berna 2001). Germin, as originally described in cereals, is a discrete marker of early seedling growth (Thompson and Lane 1980). Germin-like proteins have been found in all plants examined and are a subset of the conserved, functionally diverse cupin superfamily of proteins that, in plants, includes at least three separate groups (Khuri et al. 2001). At least 12 GLP genes reside in the *Arabidopsis* genome, and the role(s) of the enzymes encoded by these genes in dicots is still being debated (see Lane 2000; Membré et al. 2000). Germin-like proteins are involved in biotic and abiotic stress responses, and are primarily regulated at the transcriptional level (Berna and Bernier 1999). Germin is a water-soluble, pepsin-resistant, SDS-insensitive, thermostable, hexameric cell-wall glycoprotein that functions as an oxalate oxidase (Dumas et al. 1993; Lane et al. 1993, 1994; Woo et al. 1998). Beet germin has been shown to exhibit thermostability, but does not cross react with wheat germin antibodies (Lane 2000).

High levels of insoluble calcium oxalate in seed balls was suggested to cause inhibition of sugar beet germination, and pre-emergence loss in sugar beet (Miyamoto 1957). Little variation in calcium oxalate was reported between varieties in that study, however. Demonstration here of a stress-induced putative oxalate oxidase in a good, but not poor, emerging hybrid suggests that the fate of calcium oxalate is more important for germination than its presence or absence. If germin-like oxalate oxidase activity resulted in dissolution of calcium oxalate crystals, then both H_2O_2 and Ca^{2+} would be liberated. Like H_2O_2, Ca^{2+} has an important role as a catalyst for the biochemical cross-linking of cell-wall polymers during plant development (Carpita and Gibeaut 1988), as well as integrating developmental and environmental responses (reviewed in Trewavas and Malho 1998). Since MAPKs and Ca^{2+} are important components of abiotic stress-signal transduction pathway(s), release of both calcium and H_2O_2 during emergence may initiate signalling cascades resulting from oxalate catabolism (Lane 1994; Franceschi 1989; Desikan et al. 1999; Bowler and Fluhr 2000; Kovtun et al. 2000; Neill et al. 2002). Consistent with this interpretation are preliminary results of RNA gel blots of USH20 stress-germinated seedlings probed with MAPK (data not shown).

It is likely that other biochemical processes during germination could affect field emergence and stand establishment. Even with regard to GLP expression, it was evident that transcript levels in the poor emerging variety were responsive to some stress treatments, particularly oxalic acid and mannitol, implying another layer of control beyond that afforded by oxidation of oxalate to H_2O_2. The relatively poor emerging ACH185 was the dominant variety grown in Michigan from 1990 through 1995, and its emergence was commercially acceptable, but not as good as the good-emerging dominant USH20 variety it supplanted. Other layers of control beyond GLP expression may be related to the physiological status of the true seed, which may explain some of the variability in stress germination seen in different seedlots of the same variety (McGrath et al. 2000). Recent results suggest a temperature-dependence on germination of these hybrids in solution (data not shown). In any case, the enhancement of germination by H_2O_2 does not result in 100% germination under other solution stresses, nor does any variety emerge to 100% of its potential in the field. At least one target for enhanced emergence has been identified here, demonstrating that biochemical mechanisms leading to enhanced emergence in sugar beet are at least partially accessible experimentally.

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