Sugar Beet Germination: Phenotypic Selection and Molecular Profiling to Identify Genes Involved in Abiotic Stress Response

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Abstract

Emergence and stand establishment are critical concerns of sugar beet growers worldwide, and abiotic stresses potentially limit the types of varieties that can be grown productively. This project seeks to develop information that will be useful in selecting and breeding sugar beet for enhanced emergence in saline conditions. We developed a simple bioassay using seeds submerged in salt water to screen 147 germplasm lines for their ability to germinate in 150 mM NaCl as compared with 0.3% H_2O_2, and used the ratio as a predictor of field emergence potential (FEP). Simultaneously, we surveyed gene expression during salt germination in order to identify potential molecular breeding targets. Expressed Sequence Tags (ESTs) were obtained from 3,119 transcripts of 4-day old seedlings germinated in saline and H_2O_2 solutions. Results suggest that 1-carbon metabolism is an important process in stress germinating seedlings. Results also indicated that germplasm differs in germination under salt stress, and in at least one germplasm, differential gene expression is observed. Results from the salt germination assay appear to be consistent with tolerance at the whole plant level, at least for some tested lines. This information will allow subsequent breeding and physiological studies on sugar beet salt tolerance, for instance in identifying highly salt tolerant sugarbeet germplasm that could be used to help remediate saline soils.

INTRODUCTION

Early season growth (e.g. the first 10 weeks) is critical to stand establishment in sugar beet (*Beta vulgaris*) (Durr and Boiffin, 1995; Kneebone, 1976). Stand establishment is perennially among the highest concerns for sugar beet growers, along with weed control and seedling diseases. Although the balance of biotic and abiotic stress often changes during this time, along with the rapid development of the plant's photosynthetic capacity and of the root as a carbohydrate sink organ, the initial stand achieved appears acutely influenced by abiotic stresses of low soil moisture and salinity (Agarwala and Mehrotra, 1979; Ghoulam and Fares, 2001). Breeding for improved emergence is problematic because not only the field emergence environment but also seed production profoundly influences seed performance, leading to low (or no) heritability estimates for seedling vigor traits. Durrant and Gummerson (1990) reported that selection for increased laboratory germination was accompanied by a general enhancement of field emergence.

Wild germplasm accessions (*Beta vulgaris* spp. *maritima*) have often been collected within 5 m of sea level on sand and shale beaches throughout its natural range. Tremendous molecular genetic diversity exists in the wild germplasm relative to the crop (Baranski et al., 2001; Jung et al., 1993; McGrath et al., 1999; Mita et al., 1991; Nagamine et al., 1989; Wang et al., 1999). Perhaps not surprisingly, genetic variability

exists for salt tolerance in beets (Ghoulam et al., 2002; Freytag et al., 1990; Uno et al., 1996). Ghoulam and Fares (2001) concluded that effects of salinity on germination and growth are a specific effect of ionic toxicity and not principally due to osmotic adjustment in sugar beet. Deploying this diversity toward improving stand establishment is a long-term goal, and may help reclaim the productivity of saline soils for general agriculture, for example in Nile Delta, where accumulation of ions in the harvested root could reduce salt content in the soil. Beets are more susceptible to salinity stress during emergence and stand establishment (Maas, 1990), which can limit beets use for land reclamation purposes, and increasing germination in saline environments is a first step towards such phyto-remediation while maintaining crop productivity.

An artificial stress (submerged germination in aqueous solutions) was used to evaluate differences in germination and response to different stresses (McGrath et al., 2000). That study also demonstrated a positive effect of hydrogen peroxide on sugar beet germination, and subsequently it was shown that a germin-like protein with putative oxalate oxidase function, whose product in part is hydrogen peroxide, was expressed only under solute stress and only in a good emerging variety (de los Reyes and McGrath, 2003). In this study, two approaches were taken. First we screened germplasm to identify populations that exhibit tolerance to germination under various stresses including submergence, alone and in combination with various solutes, in this case NaCl. The second approach was to characterize genes expressed at 4-days after germination under the same conditions, in this case focusing only on one variety. In the former, new germplasm resources for seedling vigor enhancement were sought for deployment in breeding for high salinity agricultural systems such as found in areas of the Nile Delta. In the latter, we hoped to identify additional genes or biochemical processes that contribute to seedling vigor, in this case from a variety known to exhibit good vigor (de los Reyes et al., 2003). Here we report the results from screening 113 germplasms for tolerance to germination in saline solution, and present an overview of genes, as ESTs, expressed during stress germination in solution.

MATERIALS AND METHODS

Plant Material

Sugar beet varieties USH20 (Coe and Hogaboam, 1972) and ACH185 (American Crystal Sugar, Moorehead, MN) were used as controls. All molecular work used USH20 as the source germplasm. Sugar beet SR80 (PI607898) was used for early stress-germination selection experiments. An additional 168 diverse germplasm accessions were tested for potential donors of salt tolerance genes (Table 1), and consisted of 111 sugar beet breeding materials held in the East Lansing, MI USA germplasm enhancement program and 59 Plant Introductions obtained from the U.S. National Plant Germplasm System (http://www.ars-grin.gov/npgs/).

Germination

Salt stress germination in solution was performed as described in 150 mM NaCl (McGrath et al., 2000). Percentage germination (defined by radicle emergence) was determined at 48 hr and 96 hr, typically with three replications of 25 seeds each. For larger initial screenings, however, only a single replicate was done, and in a few instances where seed quantity was limiting, only 10 seeds were tested, and in each of these cases follow-up analyses in replicated trials were done as above for the most promising germplasms. Control germination was performed in 0.3% H_2O_2 and gives equivalent results as filter-paper germination assays (McGrath et al., 2000). Field Emergence Potential (FEP) was calculated as the total number of seeds germinated in NaCl divided by the number germinated in H_2O_2. This ratio of stress to non-stress percent germination represented the tolerance of salt stress during germination.

Molecular Analyses

Large scale RNA isolation was from 2,000 seeds germinated in solution (ca. 0.5 ml solution / seed). Solutions tested included 150 mM NaCl, 200 mM mannitol, 18 MOhm water or 0.3% (88 mM) H_2O_2. Control treatment was seedlings germinated on filter paper. Analysis of differential gene expression was performed by comparing mRNA fingerprints generated by the differential display-reverse transcription polymerase chain reaction (Delta Differential Display, Clontech, Palo Alto CA). Candidate differentially expressed cDNAs were cloned and their inserts were sequenced, and their expression of was confirmed via northern blot analyses. For cDNA library construction, three treatments were used (150 mM NaCl, 200 mM mannitol, and 0.3% H_2O_2). Total RNA was isolated from 4 day-old germinating seedlings. Equal amounts of mRNA (2 ug) from each solution-germination regime were pooled to construct a cDNA library in Lambda Uni-Zap (Stratagene, La Jolla, CA). From the primary library, 100,000 phages were mass excised as pBluescript phagemids, individual colonies were picked and their nucleotide sequences determined. Bidirectional sequencing was done on a portion of the ESTs, however the majority of EST sequence was derived from the 5' end of the cDNA. Subtracted cDNA probes were prepared by biotin-streptavidin subtraction using the Subtractor kit (Invitrogen, Carlsbad, CA). mRNA samples (e.g. salinity stressed) were used as testers for subtraction against the control or un-induced mRNA driver (no salinity stress). The subtracted cDNA pool was labeled with ^{32}P-dCTP and used as probes against colony lifts, and hybridized clones were sequenced.

All sugar beet sequence information was deposited in GenBank. The putative identity of individual ESTs was determined by their alignment with sequences in genome databases (e.g. GenBank) using BLAST search algorithms encoded in mpiBLAST (Darling et al., 2003). A threshold e-value of 10^{-20} or better was used to assign putative function(s). Databases queried to assign putative functions included comparison with the GenBank non-redundant collection (nr, as of 8/15/2006) as well as the Arabidopsis protein database (TAIR proteins v. 6). Statistical analyses were accomplished with the aid of the JMP software package (SAS Institute, Cary, NC), generally implementing Oneway ANOVA and Student's *t* statistical procedures.

RESULTS AND DISCUSSION

Abiotic stress in sugar beet fields within the first few weeks after emergence generally leads to stand loss due to seedling mortality, and while stress applied later in the season can impact yield, plants often appear healthy otherwise. Stand loss impacts yield, albeit indirectly, since remaining beets compensate by growing larger, which creates problems of crown height variability that translate into less efficient removal of leaves prior to harvest, and higher impurities at the factory that reduce sucrose recovery. Below an economic threshold (e.g. 60-80 beets / 30 m of row, 0.75 m row spacing), poor emergence and stand loss requires replanting the crop, with a host of possible consequences including lower yield. In many instances, lack of water is the most serious stress on beets early in the season since the seed is embedded in a corky fruit and this propagule is generally planted shallow (i.e. 2-3 cm), a region subject to rapid drying. Thus, abiotic stress tolerance during germination and early development is desirable for all growers.

Germplasm Screening

Germplasm tested initially under non-replicated conditions is listed in Table 1, along with their calculated Field Emergence Potential (FEP), sorted from highest FEP and arranged with respect to *bona fide* sugar beet germplasm (111 entries; Table 1, columns 1 and 2) and wild and non-sugar beet germplasm (59 entries; Table 1, Column 3). FEP is the simple proportion of seeds germinated under a stressful condition (NaCl in this case) as the measure of vigor divided by the number of seeds germinated in a non-stress condition (H_2O_2 in this case) as the measure of viability. FEP among sugar beet lines ranged from 0 to 0.64 (mean = 0.11, std. dev. = 0.15), while that of the non-sugar beet

germplasm ranged from 0 to 0.90 (mean = 0.30, std. dev. = 0.26). Only 5% of sugar beet germplasm had an FEP > 0.50 (i.e. stress germination 50% of total germination) versus 22% among the non-sugar lines. Nearly 50% of sugar beet lines failed to germinate under NaCl, but all seedlots had viable seed (data not shown). Conversely, only 19% of wild materials failed to germinate under salt stress.

Results from replicated trials of 16 sugar and 56 non-sugar beet lines selected from the non-replicated trial are shown in Figure 1. Mean sugar beet FEP was 0.33 (std. dev. = 0.04), ranging from 0.21 to 0.51, with reasonable correspondence between the single replication (Table 1) and this three rep test (r = 0.79). Mean non-sugar beet FEP was higher (0.48, std. dev. = 0.04) ranging from 0.02 to >1.00, but the correspondence between single and three replication results was lower (r = 0.41), likely due to the greater differences in seed quality in the small seedlots obtained of these non-sugar accessions. For the non-sugar beet results, FEP exceeded 1.00 in four instances, however the results as presented here were limited to 1.00 because, in each of these instances, the number of seeds was limiting and only 10 seeds per replication were tested for those accessions. Salt could have a germinating promoting effect in some germplasm, since the majority of families in the Caryophyllales are native to stressful environments. Overall, these results suggest that non-sugar beet germplasm might contribute additional salt tolerant germination capacity beyond what might be currently available within the sugar beet germplasm.

From six accessions (PI266100, PI140360, PI169023, PI165485, PI357357, PI518160), seeds germinated in salt were planted (20 plants per accession) and these families were grown in the greenhouse, vernalized, and each family was allowed to inter-pollinate in isolation from pollen from the other families. Seed was harvested from each single plant of each family, and a single replication FEP test was performed (Fig. 2). In most instances, a range of FEP values was observed, and in all but one instance (family 04B014 derived from PI518160), the family FEP mean was greater than the parental FEP mean, suggesting a response due to salt germination selection. Individuals within families ranged in FEP values, from 0.0 to 1.0, depending on the family. Not all individuals survived to produce seed.

One salt-selected germination family (04B002 derived from Ames3051) was tested in a replicated fashion (Fig. 3). The progeny FEP mean (0.31) was less than the parent Ames3051 (0.51), however two of the seven family members producing enough seed for this replicated test had higher FEP values than the parent, one significantly (04B002-18, $p = 0.011$), again suggesting selection progress for stress germination in salt solution. Further evidence of a response due to selection was a germination experiment with breeding line SR80, where seeds germinating in water 24 – 96 hrs post-imbibition were planted and inter-pollinated within their respective families (Table 2). In this case, seeds germinating within the first 24 hr incubation gave rise to plants with significantly higher water-germinated FEP values than seeds from plants selected at other time points and the original parent seedlot.

Finally, preliminary experiments suggest that, at least in some instances, higher salt tolerant germination predicts salinity tolerance during the first 9 weeks of growth (Table 3). For three accessions (PI562600, PI562601, PI562604 each collected along the Upper Nile, Egypt), seedlings were germinated in 150 mM NaCl and then transplanted to sand culture. Control plants were watered with water, and treated plants were watered with 200 mM NaCl. Under these conditions, no significant differences were observed in their fresh or dry weights between control and treated plants or between accessions, except between sampling dates (3, 6, and 9 weeks). *Bona fide* sugar beets remain to be tested using this protocol, as the original control accessions tested failed to yield results due to seedling mortality.

Molecular Analyses

Molecular analyses primarily consisted of nucleotide sequencing Expressed Sequence Tags (ESTs). 3,119 ESTs have been characterized to date, and these sequences

arise from two sources and three methodological approaches. The two approaches used were differential display and cDNA library analysis, all from 4-day old seedlings geminated in various conditions. The three methodological approaches were fragment excision from polyacrylamide gels (54 ESTs), subtractive cDNA hybridization that results in enrichment for less abundantly expressed genes (1,003 ESTs total consisting of 632 stress-subtracted and 371 H_2O_2-subtracted ESTs), and sequencing these as well as cDNA clones picked at random (2,062 ESTs). The primary consideration in obtaining these ESTs was to generate sequence data from expressed genes to be used for genetic mapping markers. Thus, the transcript abundance analyses reported here were secondary; however they appear useful as initial predictors for focusing on the more important biochemical events during stress germination.

Differential display revealed that of 807 transcript-derived fragments (TDFs, using 50 primer combinations), 767 were expressed in all germination treatments of Filter Paper (control), NaCl, 200 mM mannitol, H_2O_2, and water, and thus represent constitutively expressed genes during sugar beet germination. Of 49 differentially expressed TDFs that were cloned and sequenced, three were differentially expressed specifically in filter paper germinated seedlings, 12 specifically in NaCl, two in mannitol, 8 in H_2O_2, 13 in water, and 11 in more than one treatment (e.g. three in NaCl and H_2O_2, and 8 in NaCl and mannitol). Thus, most genes do not appear to be differentially regulated during germination at the 4-day old steady-state measurements done here, and a modest 6.1% (49/807) of TDFs appear to be differentially regulated.

Each of the 3,119 ESTs was manually assigned a biological role, and each biological role was further subdivided into a molecular function, where known. Each of the four classes of EST sets constructed (Table 4) had different numbers of ESTs, and the proportion of ESTs with a particular biological role was compared between libraries. For the biological role "Metabolism", the proportion of ESTs in each set was similar, suggesting that directed (i.e. differential display), subtracted, and random sequencing approaches lead to similar conclusions, perhaps with the exception of the Subtracted-Peroxide set which had the highest proportion of "Metabolism" ESTs. The differential display approach seemed to provide the best enrichment scheme for ESTs assigned to "Stress Response", "Cell Wall & Cytoskeleton", and "Transport" categories, suggesting these processes may yield more clues as to the nature of stress responses during germination. Fewer ESTs with an "Unknown" function were seen in the random cDNA sequencing set (Table 4) than the others, suggesting one function of directed and subtraction strategies is to add complexity to the nucleotide sequence dataset, which in this case was desired. The de-emphasized biological roles in the random sequenced set appeared to relate to "Protein Synthesis, Modification, and Turnover" (e.g. processes affecting protein homeostasis), "Signaling", and "Transcription", since these were proportionally detected more frequently in the un-selected transcript pool.

Molecular functions were ascribed to each EST, and summarized either as a proportion of the total number of ESTs or as a proportion of their respective biological roles (Table 5). The most frequent molecular functions were assigned as components of ribosomes (15.9% of all ESTs, 59.9% of ESTs assigned to the "Protein Synthesis, Modification, and Turnover" biological function category) or otherwise unknown (24.7% of all ESTs), predominantly with similarities to genes from other plant species (75.2% of "Unknown" biological function). Genes whose biological role is to generally participate in a stress response ("Stress", 5.7% of all ESTs) were highly represented, as were ESTs whose predicted gene functions participate in one-carbon metabolism ("Metabolism – One Carbon", 4.1% of all ESTs) (Table 5). The use of "Metabolism – One Carbon" as a category here was based on the opportunity to follow the putative flow of carbon molecules from catabolism to anabolism during heterotrophic growth, and essentially includes molecular functions involved with the generation and use of actetyl Co-A (e.g. methyl transferases, S-adenosyl-L-methionine synthetase, etc.).

Subdivision of biological roles and molecular functions relative to their respective EST sets (not including DD-PCR ESTs, where too few sequences were present for a full

analysis) revealed differences in the proportion of ESTs for some molecular functions in each set. For instance, “Cell Wall & Cytoskeleton” roles with molecular functions as cytoskeleton (ca. 33%, Table 6) and membrane (ca. 38%) destined proteins appeared to be enriched using the method of cDNA subtraction relative to random EST sequencing (13.1% and 22%, respectively), whereas histone ESTs appeared to be underrepresented (ca. 7% vs. 20.8%; Table 6). For 15 of the 36 molecular function categories (41.7%), similar proportions were evident across all EST sets (e.g. “Metabolism – Amino Acid” and “Metabolism – Glycolysis”). These biological roles and molecular functions may play a relatively small role in the response to stress during germination. For the remaining 21 molecular function categories, 10 had different proportions between the subtracted and random EST sets, but the subtracted set was either consistently higher or lower than the random set, and of these 10, five were higher (e.g. “Metabolism-TCA cycle” and “Signaling-Phosphatase”) and five lower (e.g. “Metabolism-Oxidative Phosphorylation” and “Signaling-Calcium”) than the respective random set proportion. Such genes and their biochemical processes may be involved in a general response to stress during germination, and breeding selection targeting these processes may promote broad enhancement of germination in a wide range of stress environments.

Differential responses to specific treatments were evident for 11 of the 36 molecular function categories assigned in Table 6. Eight of these appeared to be responsive to the hydrogen peroxide treatment greater than the salt treatment (i.e. molecular functions for cell wall, protease, ribosomal, kinase, WD40, one-carbon, light harvesting, and nucleic acids). Of these seven, transcript abundance appeared to increase for protease, one carbon, and nucleic acids, while the remainder showed a relative decrease. For two of the 11 differential responses, the salt treatment was most affected, and these were molecular functions ascribed as DNA binding and vacuolar ATPases, the latter function being increased as might be expected by cells needing to sequester NaCl in vacuoles. Finally, one molecular function category, lipid metabolism, showed elevated transcript levels in salt and reduced levels in hydrogen peroxide, relative to the random ESTs. These results suggest that hydrogen peroxide is affecting many more biochemical pathways than the salt treatment. Hydrogen peroxide may be operating to stimulate these biochemical pathways, or perhaps another explanation is that physiological stages of germination are transitioned more quickly relative to salt, as part of the growth promoting effect of H_2O_2.

In contrast with the broad generalizations of biological role and molecular function, specific transcripts showed variable proportions from each EST set in all but four of the 32 examples where transcript abundance could be readily identified (i.e. present at >5 instances in at least two of the three EST sets) (Table 7). For instance, expression of alpha-amylase gene(s) represented 26.6% of carbohydrate metabolism-assigned transcripts from the non-subtracted EST set, 66% of subtracted H_2O_2 transcripts, and no (NaCl) stress-subtracted transcripts were detected. This comparison is interesting because it suggests that hydrogen peroxide treatment induces carbohydrate catabolism as an additional source of carbon and energy for heterotrophic growth. As expected, subtraction procedures enriched isocitrate lyase transcripts relative to the random EST set (Table 7). Interestingly, differences in EST abundance in their respective EST sets for abundant transcripts centering on the TCA cycle (e.g. malate dehydrogenase, malate synthase, and phosphoenolpyruvate carboxykinase) were observed. In both subtractions, the catabolic function of phosphoenolpyruvate carboxykinase (converts oxalate to phosphoenolpyruvate) was markedly increased, accompanied by a marked decrease in anabolic function malate synthase (converts acetyl Co-A to malate), again suggesting that carbon flow through the TCA cycle and one-carbon metabolism is important for heterotrophic growth. In contrast, malate dehydrogenase was specifically enriched only in the hydrogen peroxide subtracted EST set (Table 7). Deducing the significance of this finding, as well as observed differences in proportions of other putative EST gene functions, is complicated by a less than full understanding of central metabolism in plants. For instance, the role of phosphofructokinase (PFK) as a regulatory step in plants is less

stringently regulated in plants than animals (Buchanan et al., 2000), and the apparent differential down-regulation of PFK in the subtracted EST sets may suggest that insights can be gained by further examining this germination bioassay.

At this level of analysis, these results suggest a difference in metabolism between salt- and H_2O_2-germinated seedlings, with the peroxide germinated seedlings having an advantage of access to carbohydrates relative to salt stress germinated seedlings. This could be the result of stress acting on the specific gene products, or as a general result of delayed development in the case of salt stress. In summary, stress during germination caused a reduction in germination. Biochemical changes could be detected with this reduction. Selection for enhanced stress tolerance during germination appears to be feasible, since gains were observed in progeny of salt germinated seedlings. Salt- and H_2O_2-germination show very different results, and molecular information supports different metabolic capacities operating in each situation. The facile nature of this germination bioassay and new molecular tools such as microarrays will allow global gene expression testing of the differences observed here. Metabolic profiling may be needed to follow the flow of carbon and energy during sugar beet germination.

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Literature Cited

Agarwala, S.C. and Mehrotra, N.K. 1979. Salt tolerance of some sugarbeet varieties during early stages of growth. Indian J. Plant Physiology 22:167-172.

Baranski, R., Grzebelus, D., Frese, L. and Baril, C. 2001. Evaluation of genetic diversity in a garden beet germplasm collection using RAPD technique. Acta Hort. 546:165-169.

Buchanan, B., Gruissem, W. and Jones, R.L. 2000. Biochemistry and Molecular Biology of Plants. Am. Soc. Plant Physiologists, Rockville, MD. p.660.

Coe, G.E. and Hogaboam, G.J. 1971. Registration of USH20 sugarbeet. Crop Sci. 11:942.

Darling, A.E., Carey, L. and Feng, W. 2003. The design, implementation and evaluation of mpiBLAST. Proceedings of ClusterWorld 2003, June 24-26, San Jose, CA.

De los Reyes, B.G. and McGrath, J.M. 2003. Cultivar-specific seedling vigor and expression of a putative oxalate oxidase germin-like protein in sugar beet (*Beta vulgaris* L.). Theoretical and Applied Genetics 107:54-61.

De los Reyes, B.G., Myers, S.J. and McGrath, J.M. 2003. Differential stress-induction of glyoxylate cycle enzymes as a marker for seedling vigor in sugar beet (*Beta vulgaris*). Molecular Genetics and Genomics 269:692-698.

Durr, C. and Boiffin, J. 1995. Sugarbeet seedling growth from germination to first leaf stage. Journal of Agricultural Science (Cambridge) 124:427-435.

Durrant, M.J. and Gummerson, R.J. 1990. Factors associated with germination of sugar-beet seed in the standard test and establishment in the field. Seed Science and Technology 18:1-10.

Freytag, A.H., Wrather, J.A. and Erichsen, A.W. 1990. Salt tolerant sugarbeet progeny from tissue cultures challenged with multiple salts. Plant Cell Reports 8:647-650.

Ghoulam, C. and Fares, K. 2001. Effect of salinity on seed germination and early seedling growth of sugar beet (*Beta vulgaris* L.). Seed Science and Technology 29:357-364.

Ghoulam, C., Foursy, A. and Fares, K. 2002. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. Environmental and Experimental-Botany 47:39-50.

Jung, C., Pillen, K., Frese, L., Fahr, S. and Melchinger, A.E. 1993. Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". Theor. Appl. Genet. 86:449-457.

Kneebone, W.R. 1976. Some genetic aspects of seed vigor. J. Seed Tech. 1:86-97.
Maas, E.V. 1990. Relative Salt Tolerance of Herbaceous Crops. Results shown at http://www.ussl.ars.usda.gov/pls/caliche/SALTT42A, Accessed 11/30/2006.
McGrath, J.M., Derrico, C.A. and Yu, Y. 1999. Genetic diversity in selected, historical USDA sugarbeet germplasm releases and *Beta vulgaris* ssp. *maritima*. Theor. Appl. Genet. 98:968-976.
McGrath, J.M., Derrico, C.A., Morales, M., Copeland, L.O. and Christenson, D.R. 2000. Germination of sugar beet (*Beta vulgaris* L.) seed submerged in hydrogen peroxide and water as a means to discriminate cultivar and seedlot vigor. Seed Science and Technology 28:607-620.
Mita, G., Dani, M., Casciari, P., Pasquali, A., Selva, E., Minganti, C. and Piccardi, P. 1991. Assessment of the degree of genetic variation in beet based on RFLP analysis and the taxonomy of Beta. Euphytica 55:1-6.
Nagamine, T., Todd, G.A., McCann, K.P., Newbury, H.J. and Ford-Lloyd, B.V. 1989. Use of restriction fragment length polymorphism to fingerprint beets at the genotype and species levels. Theor. Appl. Genet. 78:847-851.
Uno, Y., Kanechi, M., Inagaki, N., Sugimoto, M. and Maekawa, S. 1996. The evaluation of salt tolerance during germination and vegetative growth of asparagus, table beet and sea aster. J. Japan. Soc. Hort. Sci. 65:579-585.
Wang, M., Goldman, I.L. and Wang, M. 1999. Genetic distance and diversity in table beet and sugar beet accessions measured by randomly amplified polymorphic DNA. J. Am. Soc. Hort. Sci. 124:630-635.

Tables

Table 1. List of accessions tested, un-replicated, in salt and hydrogen peroxide solutions, ranked by Field Emergence Potential (FEP), with breeding lines in first two columns and Plant Introduction lines in third column.

Entry	FEP	Entry	FEP	Entry	FEP
EL-A012150	0.64	EL-A010088	0	PI165485	0.90
USH20-377	0.60	EL-A010098	0	PI232889	0.90
EL-A012163	0.56	EL-A010153	0	PI562600	0.80
EL-A012149	0.52	EL-A010250	0	PI140360	0.80
EL-A012858	0.52	EL-A010251	0	PI355963	0.80
EL-A012191	0.44	EL-A010252	0	PI546388	0.80
EL-A012088	0.40	EL-A010253	0	Ames3051	0.80
EL-A012157	0.40	EL-A010254	0	PI 562601	0.70
EL-A012168	0.40	EL-A010255	0	PI171518	0.60
EL-A012172	0.40	EL-A010256	0	PI357361	0.60
EL-A012086	0.36	EL-A010257	0	PI285593	0.55
EL-A012855	0.36	EL-A010258	0	PI 562591	0.50
EL-A012089	0.32	EL-A010259	0	PI 562602	0.50
EL-A012189	0.32	EL-A011986	0	PI116906	0.45
EL-A010162	0.28	EL-A011987	0	PI171519	0.45
EL-A012090	0.28	EL-A012007	0	PI266101	0.45
EL-A009716	0.24	EL-A012009	0	PI518160	0.45
EL-A011971	0.24	EL-A012056	0	PI 562604	0.40
EL-A012158	0.24	EL-A012057	0	PI141918	0.40
EL-A013493	0.24	EL-A012068	0	PI518439	0.40
EL-A009847	0.20	EL-A012069	0	Ames 2661	0.40
EL-A012063	0.20	EL-A012070	0	PI178837	0.35
EL-A012084	0.20	EL-A012071	0	PI357357	0.35
EL-A012152	0.20	EL-A012087	0	PI 562599	0.30
EL-A012181	0.20	EL-A012197	0	PI169023	0.30
EL-A012187	0.20	EL-A012205	0	PI179174	0.30
EL-A012200	0.20	EL-A013472	0	Ames 3049	0.30
EL-A013491	0.20	EL-A013473	0	PI220165	0.25
EL-A012153	0.16	EL-A013475	0	PI266100	0.25
EL-A012178	0.16	EL-A013476	0	PI562582	0.20
EL-A012859	0.16	EL-A013480	0	PI562586	0.20
EL-A010146	0.12	EL-A013481	0	PI562588	0.20
EL-A012072	0.12	EL-A013486	0	PI562589	0.20
EL-A012083	0.12	EL-A013488	0	PI117114	0.20
EL-A012085	0.12	EL-A013489	0	PI176875	0.20
EL-A012159	0.12	EL-A013495	0	PI518159	0.20
EL-A012174	0.12	EL-A013500	0	Ames 3039	0.20
EL-A012183	0.12	EL-A013501	0	Ames 3047	0.20
EL-A012194	0.12	EL-A013502	0	PI 518437	0.15
EL-A013522	0.12	EL-A013503	0	PI546437	0.15
EL-A004969	0.08	EL-A013504	0	PI183211	0.10
EL-A009717	0.08	EL-A013506	0	PI562581	0.10
EL-A012073	0.08	EL-A013512	0	PI562587	0.10
EL-A013474	0.08	EL-A013513	0	PI562593	0.10
EL-A013499	0.08	EL-A013515	0	PI562594	0.10
EL-A013507	0.08	EL-A013516	0	PI562603	0.10
EL-A013514	0.08	EL-A013517	0	PI169030	0.10
EL-A013521	0.08	EL-A013518	0	PI176423	0.05
EL-A004968	0.04	EL-A013519	0	PI562579	0
EL-A012060	0.04	EL-A013520	0	PI562580	0
EL-A012164	0.04	EL-A013523	0	PI562584	0
EL-A013478	0.04	ACH185	0	PI562585	0
EL-A013490	0.04			PI562590	0
EL-A013492	0.04			PI562595	0
EL-A013496	0.04			PI562596	0
EL-A013508	0.04			PI562597	0
EL-A013510	0.04			PI105335	0
EL-A009827	0			PI232892	0
EL-A009828	0			PI518340	0

Table 2. Selection results for germinating SR80 seedlings in water. At each time, germinating seedlings were removed, planted, and allowed to flower. FEP was calculated on the progeny. Results followed by the same letter were not significantly different statistically.

Entry	Mean FEP	Std Dev	*
SR80 (24 hr)	0.62	0.23	a
SR80 (48 hr)	0.33	0.15	b
SR80 (72 hr)	0.22	0.06	b
SR80 (96 hr)	0.31	0.19	b
SR80-parent	0.32	0.15	b
Grand mean	0.36		
F-ratio	3.43 *		
LSD (0.05)	0.25		

Table 3. Average fresh and dry weights (FW and DW, respectively) of three wild accessions watered with 200 mM NaCl or water (control) after 3, 6, and 9 weeks after emergence. sd = standard deviation.

Treatment	Age (weeks)	FW / plant mean (g)	sd	DW/plant mean (g)	sd	DW / FW mean (g)	sd
control	3	0.070	0.010	0.006	0.001	0.080	0.017
NaCl	3	0.069	0.018	0.005	0.002	0.071	0.014
control	6	0.123	0.012	0.012	0.002	0.097	0.008
NaCl	6	0.114	0.012	0.010	0.003	0.087	0.014
control	9	0.518	0.202	0.037	0.015	0.070	0.003
NaCl	9	0.449	0.291	0.042	0.032	0.083	0.027

Table 4. Definition and distribution of EST sets classified according to biological role. N = number of ESTs in the EST set.

Biological Role	DD-PCR (%)	Subtracted Peroxide (%)	Subtracted Stress (%)	cDNA library (%)	Overall
Unknown	27.8	30.2	31.0	21.6	27.7
Metabolism	18.5	28.3	23.7	20.6	22.8
Protein synthesis, modification, turn-over	14.8	16.2	20.9	30.4	20.6
Stress Response	14.8	8.9	6.3	5.3	8.8
Cell Wall & Cytoskeleton	11.1	5.9	7.8	8.1	8.2
Transport	5.6	3.5	2.2	3.1	3.6
Signaling	3.7	3.0	4.3	5.5	4.1
Development	0.0	1.3	1.1	1.3	0.9
Transcription	3.7	2.7	2.7	4.1	3.3
N=	54	371	632	2,062	3,119

Table 5. Molecular functions assigned to the biological roles, considered as a proportion of all ESTs (total) or only the relevant biological role (biological).

Biological Role	Molecular Function	Number	% of Biological	% of total
Cell Wall & Cytoskeleton	Cell Wall	66	26.9	2.1
	Cytoskeleton	62	25.3	2.0
	Histone	39	15.9	1.3
	Membrane	57	23.3	1.8
	Membrane - porin	21	8.6	0.7
Signaling	Calcium	20	13.1	0.6
	GTP-binding	34	22.2	1.1
	Kinase	43	28.1	1.4
	Phosphatase	17	11.1	0.5
	14-3-3	10	6.5	0.3
	WD40	10	6.5	0.3
	Other	19	12.4	0.6
Transcription	Transcritpion Factor	85	74.6	2.7
	DNA binding	29	25.4	0.9
Protein synthesis,	RNA-binding	80	9.7	2.6
modification, and turn-over	Translation	100	12.1	3.2
	Ribosomal	495	59.9	15.9
	Protein folding	8	1.0	0.3
	Ubiquitin	51	6.2	1.6
	Protease	93	11.2	3.0
Metabolism	Glycolysis	67	9.7	2.1
	Oxidative Phosphorylation	36	5.2	1.2
	TCA cycle	45	6.5	1.4
	Energy	12	1.7	0.4
	ATP synthase	15	2.2	0.5
	One-Carbon	127	18.4	4.1
	Amino Acid	49	7.1	1.6
	Carbohydrate	45	6.5	1.4
	Sugar	32	4.6	1.0
	Lipid	46	6.7	1.5
	Nucleic acids	16	2.3	0.5
	Nitrogen	8	1.2	0.3
	Phosphorous	11	1.6	0.4
	Metal	10	1.5	0.3
	Hormone	9	1.3	0.3
	Secondary	45	6.5	1.4
	ReDox	76	11.0	2.4
	Light harvesting	29	4.2	0.9
	Other	11	1.6	0.4
Transport	ATPase Vacuolar	27	28.7	0.9
	Sugar	5	5.3	0.2
	Protein	7	7.4	0.2
	Amino Acid	4	4.3	0.1
	Nitrogen	3	3.2	0.1
	Phosphorous	3	3.2	0.1
	ABC	2	2.1	0.1
	Adenylate	2	2.1	0.1
	Other	15	16.0	0.5
	Putative	26	27.7	0.8
Development	Auxin responsive	2	5.3	0.1
	Calcium related	16	42.1	0.5
	Circadian	2	5.3	0.1
	Gibberellin responsive	3	7.9	0.1
	Putative	15	39.5	0.5
Stress Response	Stress	177	93.2	5.7
	Transposase	13	6.8	0.4
Unknown	Plant	578	75.2	18.5
	Unknown	27	3.5	0.9
	Low E-value	164	21.3	5.3
Total		3119		

Table 6. Molecular functions assigned to each EST set.

Biological activity	Molecular function	N=	%	Biological activity	Molecular function	N=	%	EST set
Cell Wall & Cytoskeleton	Cell Wall	47	28.0	**Metabolism**	Amino Acid	29	6.8	cDNA library
		4	18.2			9	8.6	subtracted - H_2O_2
		13	26.5			10	6.7	subtracted - stress
	Cytoskeleton	22	13.1		ATP synthase	11	2.6	cDNA library
		7	31.8			1	1.0	subtracted - H_2O_2
		17	34.7			3	2.0	subtracted - stress
	Histone	35	20.8		Carbohydrate	30	7.1	cDNA library
		2	9.1			6	5.7	subtracted - H_2O_2
		2	4.1			6	4.0	subtracted - stress
	Membrane	37	22.0		One-Carbon	77	18.2	cDNA library
		9	40.9			29	27.6	subtracted - H_2O_2
		17	34.7			20	13.3	subtracted - stress
Protein synthesis, Modification, and Turn-over	Protease	60	9.6		Energy	8	2.0	cDNA library
		15	25.0			0	0.0	subtracted - H_2O_2
		18	13.6			4	2.7	subtracted - stress
	Ribosomal	406	64.8		Glycolysis	41	9.7	cDNA library
		17	28.0			9	8.6	subtracted - H_2O_2
		69	52.3			17	11.3	subtracted - stress
	RNA-binding	55	8.8		Hormone	6	1.4	cDNA library
		6	10.0			0	0.0	subtracted - H_2O_2
		15	11.4			3	2.0	subtracted - stress
	Translation	62	9.9		Light Harvesting	18	4.2	cDNA library
		17	28.0			2	1.9	subtracted - H_2O_2
		20	15.2			9	6.0	subtracted - stress
	Ubiquitin	37	5.9		Lipid	29	6.8	cDNA library
		4	7.0			2	1.9	subtracted - H_2O_2
		9	6.8			14	9.3	subtracted - stress
Signaling	14-3-3	10	8.8		Metal	6	1.4	cDNA library
		0	0.0			1	1.0	subtracted - H_2O_2
		0	0.0			3	2.0	subtracted - stress
	Calcium	17	15.0		Nitrogen	3	0.7	cDNA library
		1	9.0			1	1.0	subtracted - H_2O_2
		2	7.4			3	2.0	subtracted - stress
	GTP-binding	25	22.1		Nucleic acids	7	1.7	cDNA library
		3	27.0			6	5.7	subtracted - H_2O_2
		6	22.2			3	2.0	subtracted - stress
	Kinase	34	30.1		Oxidative Phosphorylation	25	6.8	cDNA library
		1	9.0			3	2.9	subtracted - H_2O_2
		7	25.9			7	4.7	subtracted - stress
	Phosphatase	10	8.8		Phosphorous	6	6.8	cDNA library
		2	18.0			5	4.8	subtracted - H_2O_2
		4	14.8			0	0.0	subtracted - stress
	WD40	8	7.1		ReDox	59	6.8	cDNA library
		0	0.0			6	5.7	subtracted - H_2O_2
		2	7.4			11	7.3	subtracted - stress
Transcription	DNA binding	23	27.1		Secondary	26	6.8	cDNA library
		3	30.0			7	6.7	subtracted - H_2O_2
		3	17.6			12	8.0	subtracted - stress
	Transcription Factor	62	72.9		Sugar	13	6.8	cDNA library
		7	70.0			6	5.7	subtracted - H_2O_2
		14	82.4			10	6.7	subtracted - stress
Transport	ATPase Vacuolar	10	15.6		TCA cycle	21	6.8	cDNA library
		2	15.4			10	9.5	subtracted - H_2O_2
		3	21.4			14	9.3	subtracted - stress

Table 7. Putative functions for 32 more highly expressed genes whose expression could be compared across EST sets.

Biological activity	Putative Gene Function	%	Biological activity	Putative Gene Function	%	EST set
Carbohydrate Metabolism	alpha-Amylase	26.6	**Stress**	Alcohol Dehydrogenase	3.7	cDNA library
		66.0			6.0	subtracted - H_2O_2
		0.0			0.0	subtracted - stress
Amino Acid Metabolism	Alanine Amino Transferase	6.8		Ascorbate peroxidase	28.8	cDNA library
		11.0			50.0	subtracted - H_2O_2
		30.0			45.0	subtracted - stress
	Glutathione S-transferase	15.2		Germin-like Proteins	7.3	cDNA library
		16.6			3.0	subtracted - H_2O_2
		0.0			12.5	subtracted - stress
	Adenosylhomocysteinase	10.4		Heat Shock Protein 70	9.2	cDNA library
		31.0			9.0	subtracted - H_2O_2
		11.0			7.5	subtracted - stress
	S-adenosyl-L-methionine synthetase	18.1		Polyphenol Oxidase	5.5	cDNA library
		17.0			18.0	subtracted - H_2O_2
		20.0			5.0	subtracted - stress
Glycolysis	Pyruvate Kinase	5.2	**ReDox**	B12D protein (SOD?)	25.4	cDNA library
		7.0			0.0	subtracted - H_2O_2
		10.0			36.0	subtracted - stress
	Aldolase	19.5	**Cell Wall & Cytoskeleton**	Actin	27.3	cDNA library
		22.0			42.8	subtracted - H_2O_2
		17.6			0.0	subtracted - stress
	Enolase	7.3		Aquaporin	21.6	cDNA library
		22.0			11.1	subtracted - H_2O_2
		11.8			0.0	subtracted - stress
	Glyceraldehyde 3-phosphate dehydrogenase	21.9		Luminal Binding Protein	13.5	cDNA library
		22.0			33.3	subtracted - H_2O_2
		29.4			0.0	subtracted - stress
	Phosphofructokinase	19.5		Tubulin	36.4	cDNA library
		11.0			14.3	subtracted - H_2O_2
		5.9			41.0	subtracted - stress
	Phosphoglucomutase	14.6	**P-Energy Metabolism**	Nucleoside Diphosphate Kinase	100.0	cDNA library
		0.0			0.0	subtracted - H_2O_2
		5.9			50.0	subtracted - stress
Oxidative Phosphorylation	Cytochrome c	48.0	**Carbon Metabolism**	RuBisCO	38.5	cDNA library
		33.3			33.3	subtracted - H_2O_2
		86.0			20.0	subtracted - stress
	NADH dehydrogenase	16.0		Isocitrate Lyase	19.0	cDNA library
		66.6			40.0	subtracted - H_2O_2
		14.3			50.0	subtracted - stress
	Inorganic Pyrophosphatase	83.3		Malate Dehydrogenase	19.0	cDNA library
		100.0			40.0	subtracted - H_2O_2
		0.0			21.0	subtracted - stress
Translation	Elongation Factor 1-alpha	27.4		Malate Synthase	19.0	cDNA library
		47.0			0.0	subtracted - H_2O_2
		60.0			7.0	subtracted - stress
Development	Translationally Controlled Tumor Protein	46.0		Phosphoenolpyruvate carboxykinase	3.9	cDNA library
		0.0			14.0	subtracted - H_2O_2
		57.0			10.0	subtracted - stress

Figures

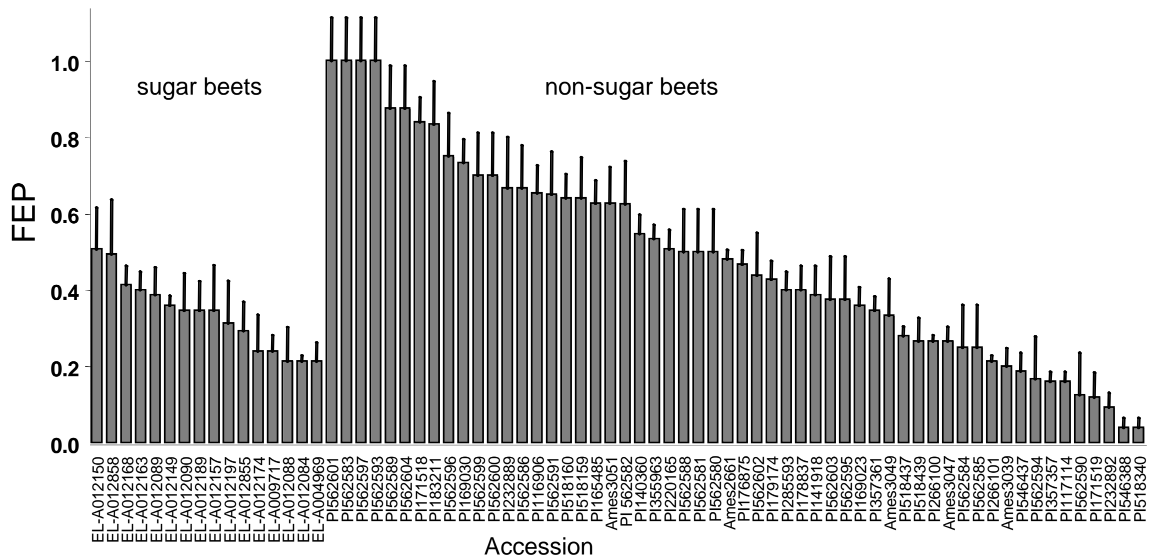

Fig. 1. Distribution of FEP among accessions selected from those in Table 1 in replicated solution germination trials. Error bars are standard deviations.

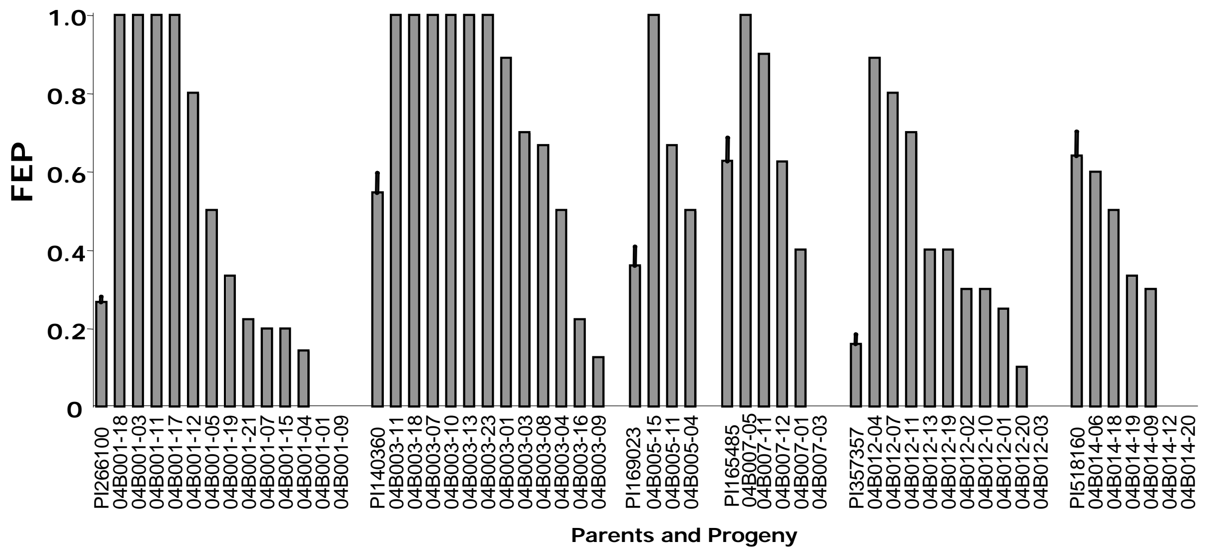

Fig. 2. Distribution of FEP among progeny from accessions selected for salt germination capacity compared to the parent in un-replicated solution germination trials.

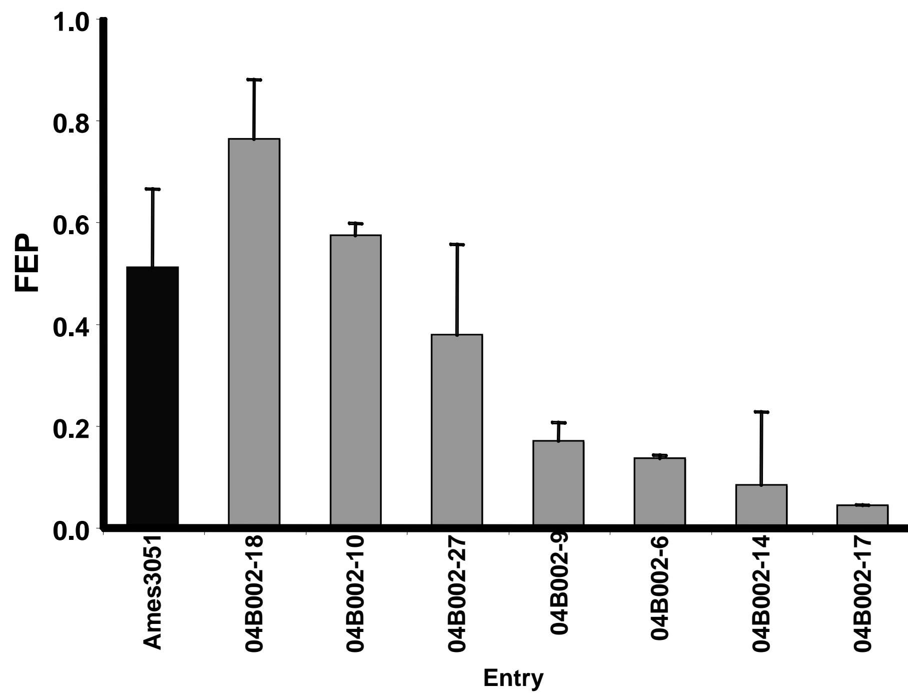

Fig. 3. Distribution of FEP from accession Ames3051 whose progeny were selected for salt germination capacity in replicated solution germination trials. Error bars are standard deviations.