SEEDLING VIGOR IN *BETA VULGARIS*: THE ARTISTRY OF GERMINATION

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Abstract:

Seedling vigor and stand establishment are two problems that growers have struggled with for decades. The initial conditions that a germinating seed encounters, and its ability to deal with them, affect the rate at which germination occurs, the rate of mobilization of stored energy reserves that the seedling uses to withstand adverse environments post emergence, and the overall ability of the seedling to survive. To understand differences in vigor, information on gene expression and imbibition of true (naked) seed and fruits was collected in the lab to begin creating a scaffold upon which vigor differences might be explained. Four varieties (two legacy, one cms and one commercial) were tested when germinated under stressed (H_2O) or non-stressed (H_2O_2) conditions, both physically using imbibition data and genetically using gene expression data. Results demonstrated that the speed and number that germinated for each variety were different, and the imbibition times were similar both in water and hydrogen peroxide. The weight of the true seed did not vary between varieties. Overall trends and patterns of varieties, when comparing them between treatments and time points, showed a range of patterns of expression for most genes tested.

Introduction:

Seedling vigor, defined here as a seedling's inherent ability to successfully cope with adverse environmental conditions, is key to reaching a full mature stand. The genetics of sugar beet fitness, and in particular, seedling vigor, are poorly understood and only account for 30% of the variation seen (Durrant and Gumerson 1990). Selection for improved fitness by choosing vigorous seedlings has been ineffective, which could be a result of the low heritability or an indication of a multi-component system. Developing molecular markers to assess a population's overall ability to respond to its environment provides valuable insights into the genetics of sugar beet fitness and tools to assess a germplasm's fitness and can streamline breeding efforts. In particular, evaluating germplasm fitness at the pre-emergence level may be most useful since many mechanisms of vigor are likely instigated prior to the emerged seedling stage. A limiting factor in looking at the earliest germination events is there are no visible changes prior to the eruption of the radicle, making pre-emergent germination a difficult area to study.

Seed scientists generally accept that there are three main phases of seed germination: imbibition, resumption of biochemical processes, and radical elongation or growth. While this model is accepted across species, there is no data to support or reject this for sugarbeets, and more importantly, there is no information about how or when these transitions, so vital to germination and vigor, might occur. Previous work (McGrath et al. 2000) focused on post emergence at 96 hours, and demonstrated that there was a difference in gene expression between USH20 (a legacy commercial hybrid that shows good field emergence) and ACH185

(another legacy commercial hybrid with less vigor under field conditions). The vigor difference was attributed, in part, to expression of an oxalate oxidase (GLP165, germin-like protein 165; de los Reyes & McGrath 2003) in USH20 but not in ACH185 under stress conditions. These initial seedling vigor studies demonstrated that the germination vigor increased when seeds were treated with hydrogen peroxide. The increased vigor was also correlated with an increase in stored lipid metabolic reserve mobilization, putatively supplying energy for growth and to overcome stress (de los Reyes & McGrath 2003). Expression analyses at 96 hours suggested that 26% of genes are used in processes of catabolism (energy production) and 74% are used in growth for USH20 (McGrath et al. 2008).

Materials and Methods:

Plant Material and Gene Analyses: Seed of SP6822 (EL-A015030), ACH185 (EL-A012206), SD-Low genetic / High vigor (EL-A022441), and C869cms (EL-A013483) were germinated in solutions of either H_2O or 0.3% H_2O_2. Samples were collected at 0, 24, 48, 72, and 96 hours, flash frozen in liquid nitrogen and tissue was ground. RNA was extracted using the Machery-Nagel NucleoSpin RNA spin columns (Cat. # 740 949.10) and first strand cDNA synthesis was performed using the Invitrogen SuperScript III Reverse Transcriptase as per manufacturer' s protocol. Gene specific oligonucleotides, designed from beet ESTs showing high sequence similarity to Arabidopsis genes involved in germination, were synthesized (IDT) and initially tested on GenomiPhied cDNA (GE Healthcare) to find those genes most likely to show differences between sample time points. Oligos that showed presence/absence differences were then used to test for gene expression differences using quantitative PCR (qPCR; Stratagene MX4000). qPCR reactions used the KAPA Sybr Master Mix kit per the manufacturer's protocol. Each sample included an internal ROX control and an 18S ribosomal RNA (rRNA) was used as a housekeeping gene. C_t values were used as the measure of gene expression change (lower numbers indicate higher expression).

Physical Analyses: For imbibition studies, true seed (without the fruit) and fruited seed were germinated on filter paper. To extract true seeds from the fruits, seed was soaked overnight in water. Once the pericarp had softened, pressure was applied to the seed using the flat end of a 15 mL Falcon tube. After the seed cap (operculum) was loosened, a dental pick was used to pluck out the true seed. True seeds were allowed to dry overnight at room temperature before imbibition treatments were started. Each treatment was replicated three times (with the exception of SP6822 in the true seed treatment which was replicated twice). For the true seed imbibition study, beet seeds were weighed to obtain a starting dry weight and placed onto moistened filter paper in germination boxes. Weights were taken twice daily and seeds were checked for radicle emergence.

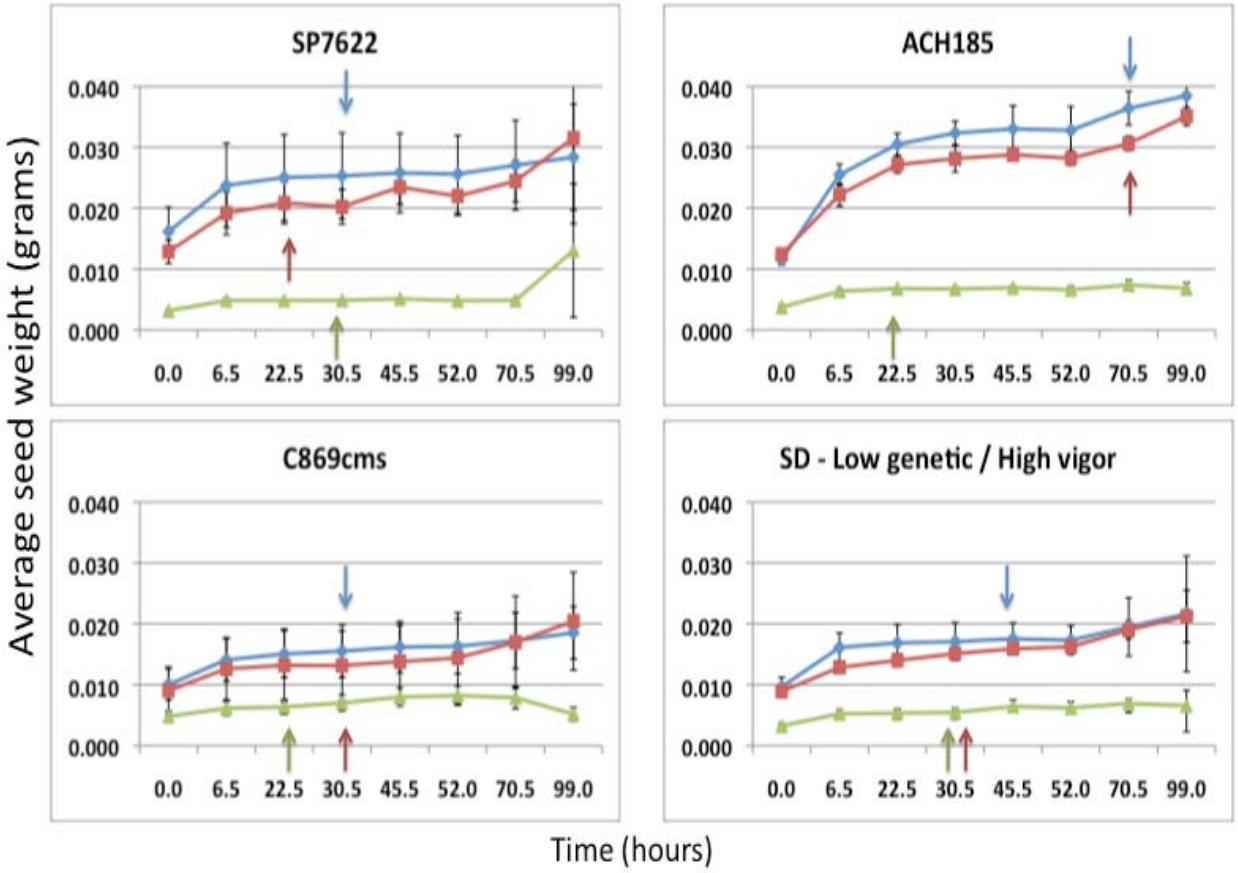
Fruited seeds were weighed to obtain a dry starting weight and then germinated on filter paper in germination boxes with either H_2O or 0.3% H_2O_2. Each treatment was replicated three times. Seeds were weighed twice daily from 0 to 99 hours. Seeds were removed from the filter paper, blotted to remove excess water and weighed. The seeds were also checked concurrently for radicle emergence. At the end of the study (>99 hr), the total

number of seeds germinated and a final weight were taken for both fruited and true seeds. Weight values were plotted over time for both fruited and naked seeds.

Results and Discussion:

Imbibition and Germination of Fruits and True Seeds: True seeds completely imbibed by 6.5 hours after placing them on water-soaked filter paper (Figure 1). Under these conditions, there was no apparent difference in the rate of imbibition between varieties tested. Radical emergence occurred between 22.5 and 30.5 hours after treatment had began for all good emerging varieties. SP6822 showed a spike in weight at 70.5 hours, unlike other varieties tested that either remained constant or decreased in weight. This may indicate growth in SP6822 at 70.5 hours versus radicle elongation. Naked seed growth was defined by elongation of radicles and greening of cotyledons. Following the accepted seed germination phases, imbibition is complete by 8 hours, resumption of biochemical processes from 8 to 22 hr and growth / radicle elongation occurring from 22 hr onwards in sugar beet.

FIGURE 1: Imbibition time-course. True seed (green triangles), Water germinated fruit (blue diamonds), Hydrogen peroxide germinated fruit (red squares). Arrows indicate first sign of radicle elongation. Fruited seeds had an increased amount of water uptake and retention, as a result of the increased tissue of the pericarp, in comparison to naked true seeds.



There was no significant difference between the weight of the true seed for each variety tested (dry weight average 3.5 mg), but the weights of the fruited seeds varied. The

amount of water absorbed by the fruit was dependent on the starting size of the fruit and whether the seed was polished. SP6822 was partially polished and multigerm (dry weight average 16 mg), C869 was an unpolished monogerm seed (dry weight average 13.4 mg), ACH185 was a multigerm unpolished seed (dry weight average 11 mg) and EL-A022441 was a polished monogerm seed variety (dry weight average 6 mg). Imbibition completion, for fruited seeds, ranged from 6.5 to 22 hours.

For C869cms, imbibition was complete at 6.5 hours, and was not dependent on treatment as both H_2O_2 and H_2O imbibed at the same rate (Figure 1). The first radical emerged was at 30.5 hours, also regardless of treatment. The total number of germinated seeds varied depending on the treatment, H_2O total germination was 44 seeds of 107 tested, while in H_2O_2 59 germinated of 107 seeds tested, a difference of 41% vs. 55% respectively. In SP6822, the pollen parent for the legacy good emerging variety USH20, imbibition times were also comparable between treatments, taking place within the first 22 hours for both H_2O_2 and H_2O. Radical emergence differed between the treatments in this variety. The H_2O_2 treatment had emergence at 22.5 hr with a total number germinated of 185 out of 231 seeds (80%). The H_2O treatment however, had radical emergence at 30.5 hours with a germination of 46 out of 132 seeds (34.8%). EL-022441, the European commercial low genetics/high vigor variety completed imbibition by 6.5 hours. This was also independent of the treatment. It also showed a range of radical emergence between 30.5 and 45.5 hr, independent of treatment. The total number germinated however did show a difference between the treatments, H_2O was 29 germinated of 188 seeds (15.4%) and H_2O_2 was 167 out of 230 (72.6%).

Overall, the germplasms tested showed a range of development from 0 to 99 hours. In particular, the time to radical emergence did not vary greatly between the good emerging varieties, but the time to complete imbibition and the number that germinated did vary. The H_2O_2 replications of all varieties showed a slight, decrease in weight (translated to water uptake), in comparison to the H_2O treatment, but this was not significantly different. In general, imbibition of fruit was complete by 22 hr regardless of the treatment and resumption of biochemical processes transitioned to growth by 30.5 hr (except for the variety ACH185). ACH185 showed very little germination and lots of variance in the imbibition study. Its imbibition was completed at 22.5 hr, and this showed no variance between treatments or replications. Radical emergence occurred in one replication of the H_2O treatment at 70.5 hr, no other reps germinated by 99 hr. In the H_2O_2 treatment, radical emergence also started at 70.5 hr, characterized by a spike in growth at that time (Figure 1). There was consistent radical emergence throughout all three replications of the H_2O_2 treatment at 70.5 hr. Because of the increase in vigor when treated with H_2O_2 shown by this variety, ACH185 was also used for qPCR analyses. Beet EST-based oligos, chosen for their diversity in function, were tested. Data were evaluated, operating under assumptions from the imbibition study that 0 to 22.5 hours was the imbibition phase, 22.5 to 70.5 hr was resuming biochemical processes, and 70.5 hr onwards was the onset of growth for this particular variety.

Gene Expression Surveys:

Gene expression was measured in ACH185 under H_2O_2 conditions from 0 to 96 hours. Gene expression was estimated by the C_t value, a logarithmic scale that indicates the cycle number where real time measurements of the amount of DNA amplification in the Polymerase Chain Reaction (PCR) becomes detectable, thereby estimating the DNA

concentration in the original DNA sample. Since the DNA that was added is derived from expressed genes, and a series of specific genes were assayed (Table 1), the copy number of the original transcripts can be estimated, and followed over time. It should be noted that lower C_t values indicate a higher level of gene expression.

Genes chosen for assay were chosen on their basis to potentially be involved during sugar beet germination, or as controls, or at random. Representative samples are shown in Figure 2. Generally, the overall trend in gene expression was low expression (or low but detectable presence of transcripts) at 0 hours and a steady increase up to 96 hours regardless of the gene assayed. This may indicate that the resumption of biochemical processes is actually occurring simultaneously with imbibition, and not in a linear fashion as previously thought.

TABLE 1: Genes assays during imbibition and germination.

Gene	Locus ID	Gene Name	Role in Arabidopsis
ARP4	AT1G18450	Actin Related Protein	Chromatin architecture

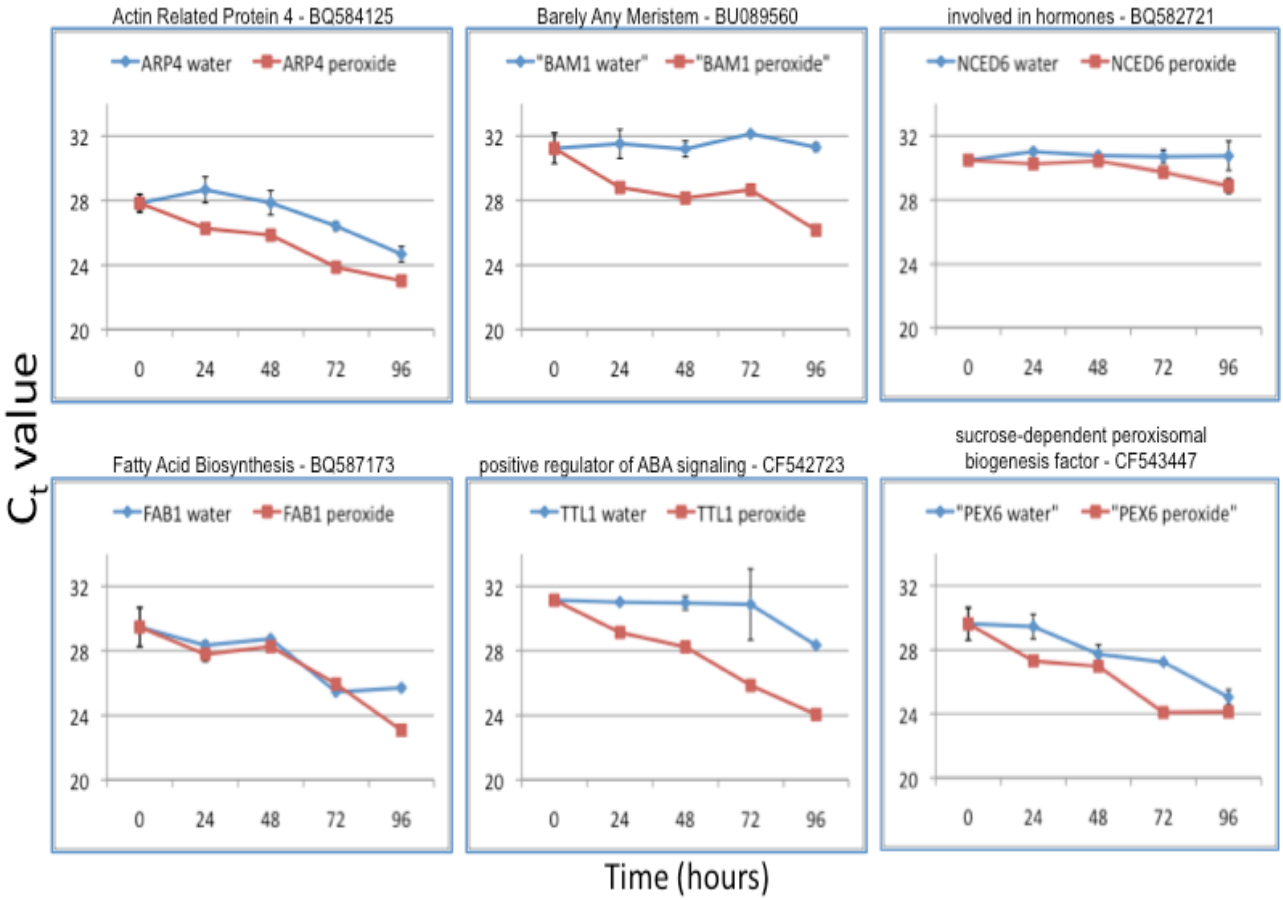


FIGURE 2: Gene expression in ACH185 fruits on H_2O_2 saturated filter paper.

Certain genes showed differences in the speed of increase in expression over time when compared between treatments. *BAM*1 (*Barely Any Meristem* 1), a gene involved in meristem development and growth showed an increase in the speed and level of expression in

the H_2O_2 treatment when compared with H_2O, which did not appear to increase in expression during germination in the weak emerger (Figure 2). It will be interesting to see whether this gene is more active in a stronger emerger. *CAT*2 (*Catalase* 2) is a gene involved in stress response and H_2O_2 sensitivity during germination in Arabidopsis, and showed high expression in both H_2O and H_2O_2 treatments (data not shown). *FAB*1, involved in fatty acid biosynthesis, showed a fairly consistent expression level between treatments at early time points, with a jump in expression occurring around 48 hr, and only a significant difference in expression between H_2O and H_2O_2 at 96 hr. This increase in *FAB*1 coincided with radicle emergence occurring around 70.5 hr. *ARP*4, *Actin Related Protein* 4, a gene involved in establishing chromatin architecture as well as structural components of the cytoskeleton, showed a slow yet steady increase in gene expression over time, but significantly higher in H_2O_2 treated seeds than in H_2O germinated seeds, which may be related to seedling vigor. *NCED*6, a gene involved in abscisic acid (ABA) biosynthesis that is an inhibitor of germination, transcript levels did not change or vary significantly until 96 hr, as might be expected. This gene may be useful as a control for future experiments. A regulator of ABA action, TTL1, however, showed a drastic difference between H_2O and H_2O_2, with constant level in water until 72 hr but a constant increase in expression in H_2O_2 over time. This may be another gene involved in the expression of seedling vigor. *PEX*6, a gene involved in peroxisome biosynthesis, showed a relative constant rate of increase in both treatments.

Gene expression data coupled with physical data of water uptake and growth over time depicts the seed as a dynamic system, undergoing changes between 0 and 96 hours. Most important is the knowledge that imbibition and the resumption of biochemical processes generally appears to be a concurrent and not sequential series of events, although specific genes may show different behavior. Seedling vigor is still an unfolding story. This study serves as the first framework available to illuminate the genetics of seedling vigor.

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