TRANSCRIPTOMES OF SEEDS GERMINATING AT TEMPERATURE EXTREMES

Safa Alzohairy[1] and J. Mitchell McGrath[2*]

[1]Plant Breeding, Genetics, and Biotechnology Program, Dept. of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing, MI 48824. [2]USDA-ARS, Sugarbeet and Bean Research Unit, 1066 Bogue St., East Lansing, MI 48824.

Introduction:

Temperature stress on plants is defined as any drop (cold stress) or rise (heat stress) in temperature that causes reversible or irreversible inactivation of physiological processes or lethal injury in plants. In general each plant has an optimum temperature to grow and develop and any deviation than the optimum temperature is considered as temperature stress. Under such conditions plants may adapt themselves with changes in morphological, physiological, and biochemical processes in order to cope with temperature changes. Heat stress is often overlooked since it is often in combination with drought stress which also influences crop growth, development, and yield processes. Cold stress affects biochemical, molecular, and metabolic processes, as well, and adversely affects plant growth, and development, and limits plant productivity.

Germination is crucial to developing healthy, vigorous, and productive field populations of sugar beets. Despite planting high-quality, technically-augmented seed for growers with very high germination (>92%), field emergence and persistence continues to hover at ~60% in Michigan. Previous research suggests this difference is the result of stress during germination in the field. Of stresses that could be imposed, moisture, temperature, and impedance (i.e.. the inability of the seedling to emerge through physical constraints such as crusting or tight seed) are likely three important factors that reduce sugar beet emergence and stand establishment. The

East Lansing USDA-ARS sugar beet program has focused on stress responses during germination. To date, we have identified some biochemical pathways related to moisture availability that appear to influence seed germination and seedling vigor in ways that can perhaps improve emergence potential. However, we still do not understand stress germination responses in such a way that might allow us to increase genetic gains for traits related to emergence, seedling vigor and stand establishment, a goal for the 'one seed – one beet' concept.

One way to identify additional genes involved in stress germination response is to examine expression of all genes during germination in different environments. The identification of genes expressed during a stress has become much more facile and affordable in recent years, and part of our goal is to produce a catalog of expressed genes during sugar beet seed germination. In this particular paper, we sought to extend and expand our previous moisture stress responses during germination to temperature stress responses, as measured at the level of gene expression.

Materials and Methods:

Sixty-four East Lansing breeding lines (Table 1) plus SP6822 were initially screened for germination at temperature extremes that could be expected under field conditions (e.g. 10 ^{o}C for early spring planted beets and 40 ^{o}C for late summer planted beets). Germination experiments were carried out in two replicates, first with 10 seeds for the population, and then with 3 replications of 25 seeds for confirmation. Seeds were placed in flasks with 15 ml 0.3% H_2O_2 and incubated with shaking (120 rpm) in ambient lighting, with solutions changed daily. Germinated seeds were counted after 96 h. Germination was defined as radicle protrusion in these experiments.

Table 1: Germplasm examined for temperature stress in solution.

Accession ID	Identifier	Germ. type	Fertility	Seed Generation	Lineage	Main Trait
EL-A021481	EL54 Hero M- ms	breeding	segregating	release	(SP6822-4 X 625-4)	Aphanomyces
EL-A021725	pEL60	breeding	self sterile	IC-1	(95HS2/sel) x 07-5E	Nematode (cyst)
EL-A021738		breeding	self sterile	IC-2	EL50 mix EL55	Cercospora
EL-A021739		breeding	self sterile	IC-2	EL50/2 x 2007 GH 33B	Cercospora
EL-A021740	EL60	breeding	self sterile	IC-2	Rhiz, rz, Trad EL, Cerc sln	Cercospora
EL-A021744	Low water elites	breeding	self sterile	IC-2	95HS2/sel & SR96/sel	elites - low water
EL-A021841	HS elites	breeding	self sterile	IC-2	SR rz Rhizoc	elites
EL-A021842	SR96 sel //	breeding	self sterile	IC-2	SR96/sel	elites
EL-A022406		breeding	self sterile	IC-1	PI 266100 germ test seln	salt tolerant germ
EL-A022410		breeding	self sterile	IC-1	PI 518160 germ test seln	salt tolerant germ
EL-A022411		breeding	self sterile	IC-1	PI 357361 germ test seln	salt tolerant germ
EL-A022413		breeding	self sterile	IC-1	PI 232889 germ test seln	salt tolerant germ
EL-A022418		breeding	self sterile	IC-1	PI 169030 germ test seln	salt tolerant germ
EL-A022420		breeding	self sterile	IC-1	PI 355963 germ test seln	salt tolerant germ
EL-A022426	C40 HSx	breeding	self sterile	IC-1	C40 HS x SR & Logan	SR
EL-A022459		breeding	self sterile	IC-3	SR Suc RZM IC2	SR
EL-A013484	C869 O-type	parent	SF	source	C869	CMS & O-type
EL-A013698	EL55 "Old Seed"	breeding	self sterile	IC-2	00B041	Seed longevity
EL-A015031	SP7322	genetic	self sterile	source	SP6822 (seedling vig. Stud.)	Trad EL
EL-A015033	USH20	genetic	self sterile	source	USH20(seedling vig. Stud.)	Trad EL
EL-A022776	EL64, pEL63	breeding	self sterile	IC-1	(Salinas nema. x 07-5E/24A)x08-5E	Nematode (Cyst)
EL-A022799	EL56	breeding	self sterile	IC-2	NaCl germ-high Ames 3051	salt tolerant germ
EL-A022804		breeding	SF	IC-1	(EL51 F3 ms CAPT.) x SR & wilds	Rhizoctonia
EL-A022805		breeding	self sterile	IC-1	HS elit. (+ low water x nema - 4 pl.)	Nematode (Cyst)
EL-A022806	lo. wat.&HS elit.	breeding	self sterile	IC-1	HS elites & (low water x nema)	Nematode (Cyst)
EL-A022807	mix. - low water	breeding	self sterile	IC-1	low water x nema	Nematode (Cyst)
EL-A022808	08 mix - Cerc	breeding	self sterile	IC-1	previous OP mat. mix (+some SF's)	Cercospora
EL-A022809	EL57, SF Mix. "B"	breeding	SF	IC-1	self fertile mixer, broad SF base	EL
EL-A023046		hybrid	SF	F1	C869CMS x 08-33A	salt tolerant germ
EL-A023335	C842x	capture	SF	F1	C842cms x 07-5E (nematode)	salt tolerant germ
EL-A023353	C842x	capture	SF	F1	C842cms x 07-5E (nematode)	salt tolerant germ
EL-A023567	C842x	capture	SF	F1	C842cms x 07-5E (nematode)	salt tolerant germ
EL-A024953	SR98 x Cerc	breeding	self sterile	IC-1	FC mix	Rhizoctonia
EL-A024956		breeding	self sterile	IC-1	EL50/2	Cercospora
EL-A024963		breeding	self sterile	IC-1	Bay city (nema w/ salt)	nematode & salt
EL-A024966		breeding	self sterile	IC-1	SR w/ salt (elites & low water)	salt tolerant germ
EL-A024969	SR101	breeding	self sterile	IC-1	SR (elites) w/Rhiz	SR
EL-A024974		breeding	self sterile	IC-1	SR w/EL	SR
EL-A024975		breeding	self sterile	IC-1	SR (low water) w/EL	SR
EL-A024982		breeding	self sterile	IC-3	06 bay city sln's	Nematode (Cyst)
EL-A024983	SR99	breeding	self sterile	IC-2	(95HS2/sel) x 07-5E	Nematode (Cyst)
EL-A024984		breeding	self sterile	IC-3	04B134 (Bvm nema / sel Bay City)	Nematode (Cyst)
EL-A024999		RIL	SF	F-2	(C869cms x PI562591)(salt)	salt tolerant germ
EL-A027007	EL63	breeding	self sterile	IC-1	(Sal. nema x 07-5E/24A)x08-5E	Nematode (Cyst)
EL-A027008		breeding	self sterile	IC-1	PI 357361 germ test seln	salt tolerant germ
EL-A027009		breeding	self sterile	IC-1	SR80 germ x salt tol.	Nematode (Cyst)
EL-A027010	EL64	breeding	self sterile	IC-1	low water x nema	low water nema
EL-A027011		breeding	self sterile	IC-1	06 bay city sln's IC 07 5E nema	Nematode (Cyst)
EL-A027012		breeding	self sterile	IC-1	(Sal. Nema. x 07-5E/24A)x08-5E	Nematode (Cyst)
EL-A027013		breeding	self sterile	IC-1	(95HS2/sel) x 07-5E	Nematode (Cyst)
EL-A027014		breeding	self sterile	IC-1	M6-2	Nematode (Cyst)
EL-A027017	EL65	breeding	self sterile	IC-1	Bay City sln x 08-5E (nema) = EL58	Nematode (Cyst)
EL-A027136		breeding	self sterile	IC-1	PI 518160 germ test seln	salt tolerant germ
EL-A027137		breeding	self sterile	IC-1	M1-4	Nematode (Cyst)
EL-A027138		breeding	self sterile	IC-1	PI 232889 germ test seln	salt tolerant germ
EL-A027140		breeding	self sterile	IC-1	CN927-202 5927-202 NN? x 08-5E	Nematode (Cyst)
EL-A027141		breeding	self sterile	IC-1	(Sal. Nema. x 07-5E/24A)x08-5E	Nematode (Cyst)
EL-A027147		breeding	self sterile	IC-1	PI 140360 germ test seln	salt tolerant germ
EL-A027149	SR98x	breeding	self sterile	IC-1	rhizoc - SR98 - many for selection	Rhizoctonia
EL-A027150	Group6 - Nema	breeding	self sterile	IC-1	Nematode group	Nematode (Cyst)
EL-A027151	Group7 - Salt	breeding	self sterile	IC-1	Salt tolerant selections group	salt/nema/rhizoc
EL-A027152	SR100	breeding	self sterile	IC-3	(L.W./HS elit.)xearly nema selns	Nematode (Cyst)
EL-A029768	EL59	breeding	self sterile	IC-2	Sclerotuim r. toler. x 08-5E (nema)	Nematode (Cyst)
EL-A029769	EL61	breeding	self sterile	IC-3	2008 Brabrant nema selections. mix	Nematode (Cyst)

Transcriptome experiments followed standard methods and bioinformatic analyses as described below. RNA was isolated from seeds of two germplasms at three temperatures 10, 20 ^{o}C, or 41 ^{o}C, sequenced on an Illumina HiSeq 2500 instrument in 150 nt paired end mode. .

<u>Results and discussion</u>

Preliminary screening of 65 East Lansing breeding lines at 10, 20 ^{o}C, or 40 ^{o}C in solution showed that variation was present for germinability across these temperatures. The 20 ^{o}C temperature was considered as optimal, and the one from which other temperatures would be compared according to their temperature tolerance profiles. Wide variation was seen for low temperature germination, with the best low temperature germination being seen in SP6822 (EL-A015031) (Figure 1). High temperature germination did not vary as much as expected from optimal, which was surprising. Further experiments demonstrated a sharp reduction at 41 and 42 ^{o}C (Figure 2), with no germination observed at 45 ^{o}C.

Figure 1: Initial survey of 65 East Lansing breeding lines at 3 constant temperatures in solution after 96 hours. X-axis is number of germinated seeds. 10 ^{o}C (black), 20 ^{o}C (red), or 40 ^{o}C (green).

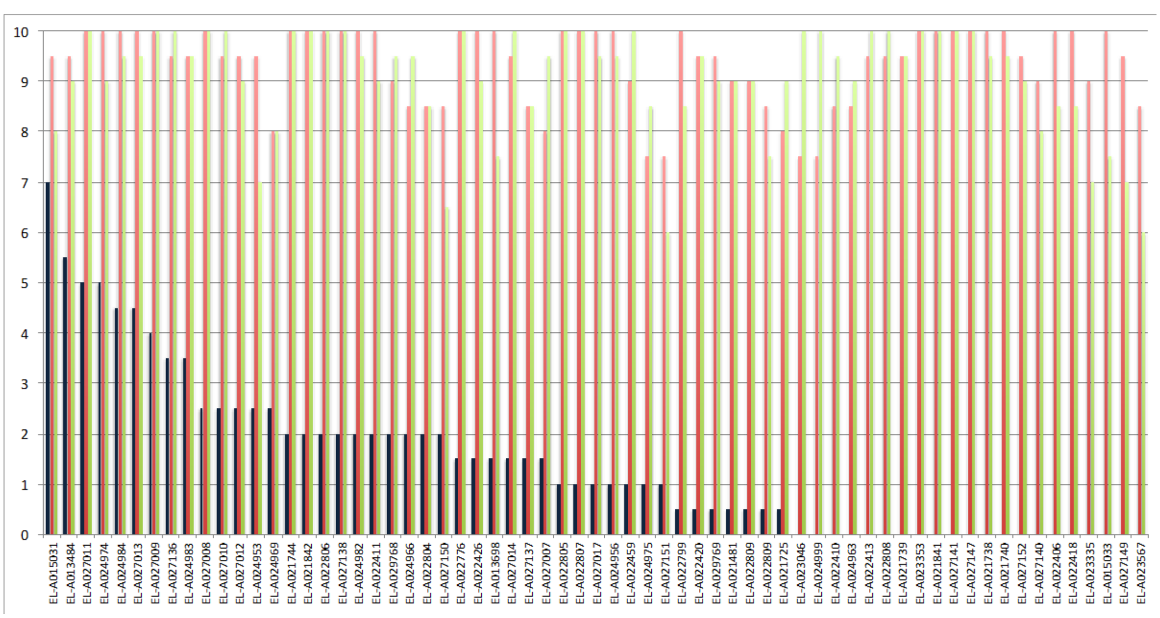
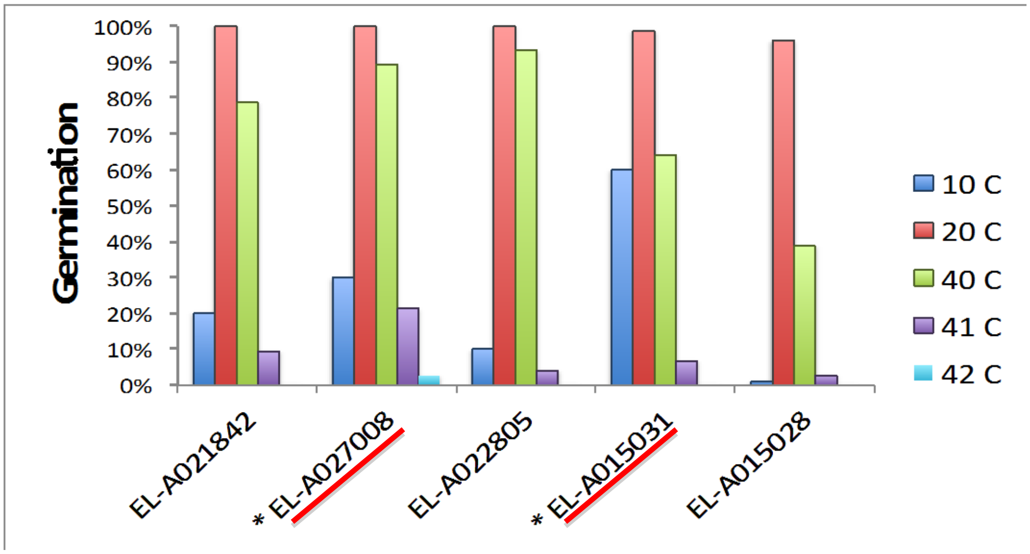


Figure 2: Solution germination summary combined across multiple experiments with different temperatures using five lines identified from preliminary tests. Underlined germplasm indicates lines selected for gene expression studies.



Transcriptomes:

RNA was isolated from germinated seedlings (96 hr, 0.3% hydrogen peroxide) from two germplasms, each with 3 treatments (10, 20, or 41 ^{o}C, without replication). Germplasm EL-A015031, as the cold tolerant representative, is a 2004 increase of SP6822, the pollen parent of formerly widely grown Michigan hybrid US H20. Germplasm EL-A027008, chosen for the high temperature representative, is maternally derived from PI 357361, which was initially selected for salt tolerant germination, then intercrossed with 29 similarly selected PI's after field evaluation, and finally intercrossed with five East Lansing smooth-root breeding populations. On the Illumina HiSeq 2500 platform, two flowcells of150 bp paired-end sequences were obtained using the six treatments, resulting in over 270 million reads in aggregate and >225 million reads after quality filtering (Table 2).

Table 2: Statistics of raw RNA-seq reads from each germplasm and treatment.

Accession	Name	Temp (°C)	Germination (%)	No. of RNAseq reads	No. of clean reads	%
EL-A015031	SP6822	10	60	36,376,646	30,303,259	83
EL-A027008	TBA	10	14	57,616,766	47,997,276	83
EL-A015031	SP6822	20	100	50,089,616	41,081,978	82
EL-A027008	TBA	20	100	53,313,856	44,390,182	83
EL-A015031	SP6822	41	7	32,504,844	27,304,247	84
EL-A027008	TBA	41	21	44,695,764	37,528,103	84
Total				274,597,492	228,605,045	83

Reads were assembled in aggregate using the Trinity algorithm, resulting in some unusual results of exceptionally long transcripts (Table 3, left panel), but in general the assembly was well supported with mapping >90% of the assembled transcripts (using BBmap) against the RefBeet 1.1 and C869-0.4 whole genome assemblies (Table 3, right panel). For each treatment, paired-end reads were directly mapped to the C869-0.4 assembly using BBmap, resulting in 75% of reads mapping to the C869-0.4 genome.

Table 3: Aggregate Trinity transcriptome read assembly and assembly mapping statistics via BBmap to two draft genomes.

Input reads	228,605,045
Number of contigs output	309,981
contigs > 300 nt	232,519
contigs > 1,000 nt	118,621
contigs > 10,000 nt	267
Mean contig size (nt)	1,170
Median contig size (nt)	635
N50 contig length (nt)	2,133
L50 contig	52,504

Input Parameters:	
Key Length (kmer)	13
Max Indel	16,000
Minimum Score Ratio	0.56
Reads Used	866,795

Genome	RefBeet 1.1		C869-0.4	
	% of reads	% of bases	% of reads	% of bases
Reads mapped	95.0%	96.2%	93.7%	95.0%
unambiguously	93.5%	95.4%	86.7%	88.6%
ambiguously	1.5%	0.8%	7.0%	6.3%
perfectly	17.5%	14.8%	15.9%	13.3%
Substitutions	60.7%	0.3%	62.8%	0.3%
Deletions	55.2%	67.8%	55.7%	66.6%
Insertions	16.9%	0.2%	18.6%	0.2%
N's	0.5%	0.0%	4.7%	0.1%

Differential expression of transcripts, determined using Tophat and Cufflinks, that mapped to the draft genomes were compared between germplasms and treatments to identify characteristics associated with differentially expressed genes (Table 4). A higher percentage of genes were differentially up-regulated in EL-A015031 relative to EL-A027008 at the lower temperatures, but the converse was true at the highest temperature. With respect to temperature comparisons, it was evident that more genes were differentially up-regulated at 10 °C relative to 20 °C in both germplasms. No other clear differences were observed.

Table 4: Summary of differentially regulated genes observed between treatments and germplasms.

Comparison				
Germplasm	Temperature °C	No. diff_exp genes	Up regulated (%)	Down regulated (%)
EL-A015031 vs EL-A027008	10	95	**69.7**	17.4
EL-A015031 vs EL-A027008	20	156	**51.3**	27.9
EL-A015031 vs EL-A027008	41	161	21.7	**78.3**
Treatment				
EL-A015031	10 vs 20	583	**66.7**	33.3
EL-A027008	10 vs 20	476	**70.0**	30.0
EL-A015031	10 vs 41	571	*52.5*	*47.5*
EL-A027008	10 vs 41	454	35.2	**64.8**
EL-A015031	20 vs 41	510	*54.7*	*45.3*
EL-A027008	20 vs 41	641	*52.6*	*47.4*

Individual transcripts were functionally annotated with Arabidopsis similarities. The distribution of transcripts by predicted function was similar between germplasms, with some major differences shown by individual genes (Figure 5). Further analyses of these genes in are progress.

Figure 5: Differential gene expression by TAIR gene annotation.