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Highlight

Oligogalactolipid production is a response to severe cold in many land plant lineages. It occurs during times of membrane damage and can be reproduced in multiple species by cytosolic acidification.

Abstract

Severe cold, defined as a damaging cold beyond acclimation temperatures, has unique responses, but the signaling and evolution of these responses are not well understood. Production of oligogalactolipids, which is triggered by cytosolic acidification in Arabidopsis (*Arabidopsis thaliana*), contributes to survival in severe cold. Here, we investigated oligogalactolipid production in species from bryophytes to angiosperms. Production of oligogalactolipids differed within each clade, suggesting multiple evolutionary origins of severe cold tolerance. We also observed greater oligogalactolipid production in control samples instead of temperature-challenged samples of some species. Further examination of representative species revealed a tight association between temperature, damage, and oligogalactolipid production that scaled with the cold tolerance of each species. Based on oligogalactolipid production and transcript changes, multiple angiosperm species share a signal of oligogalactolipid production initially described in Arabidopsis, cytosolic acidification. Together, these data suggest that oligogalactolipid production is a severe cold response that originated from an ancestral damage response that remains in many land plant lineages and that cytosolic acidification may be a common signaling mechanism for its activation.

Keywords and Abbreviations

Keywords

Abiotic stress, acidification, angiosperms, damage, membrane, oligogalactolipid, phylogeny, severe cold.

Abbreviations

Trigalactosyldiacylglycerol (TGDG), SENSITIVE TO FREEZING 2 (SFR2), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), thin-layer chromatography (TLC), differentially expressed genes (DEGs), gene ontology (GO)

Introduction

As the climate changes, weather extremes are becoming more common. One such extreme phenomenon is temperature stress, which affects the production of many worldwide staple crops. However, losses caused by temperature stress are difficult to predict because they occur in a non-linear fashion (Schlenker & Roberts, 2009). Cold stress has a large influence on crops originating from tropical environments and grown in temperate climates, such as maize (*Zea mays* L.). For many years, studies have been conducted on such crops to better understand how they survive cold temperatures (Sellschop & Salmon, 1928). Cold stress includes both chilling stress (temperatures above 0°C) and freezing stress (temperatures below 0°C), with adaptation to each of these stresses being species specific (Lyons, 1973).

One adaptative strategy used by plants in response to low-temperature stress relies on lipid remodeling of membranes (Uemura *et al.*, 1995; Welti *et al.*, 2002; Li *et al.*, 2008; Moellering *et al.*, 2010). Glycerolipids constitute the bulk of the membrane lipids, consisting of a polar head group attached to two fatty acid "tails" through a glycerol backbone. Head group size, in addition to the level of saturation in the fatty acid tails, affects how a lipid bonds within the membrane, thus changing the physical properties of the entire membrane (Melser *et al.*, 2011). In response to stress, lipid remodeling targets both the fatty acid tails and the lipid head group. The saturation level of fatty acid tails changes in response to low-temperature stress, with fatty acids becoming more unsaturated after low-temperature exposure (Hugly & Somerville, 1992; Miquel *et al.*, 1993; Steponkus, 1996). Unsaturation weakens hydrophobic interactions of the hydrocarbon tail group, ultimately decreasing the temperature at which the membrane transitions to a gel phase. Changes in lipid head groups have also been observed (Raju *et al.*, 2018). For example, SENSITIVE TO FREEZING 2 (SFR2) and ACYLATED GALACTOLIPID- ASSOCIATED PHOSPHOLIPASE 1 (AGAP1) in Arabidopsis (*Arabidopsis thaliana*) both convert monogalactolipids into other lipids during freezing challenge (Moellering *et al.*, 2010; Nilsson *et al.*, 2015; Barnes *et al.*, 2016). Head groups influence membrane permeability, and the extent of physical space needed by head and tail groups modulates the effectiveness of weak hydrophobic interactions between lipids, thus modifying membrane flexibility and temperatures at which the membrane undergoes phase transition (Melser *et al.*, 2011).

SFR2 resides on the outer envelope of the chloroplast (Fourrier *et al.*, 2008), where it is required for remodeling lipid head groups in response to severe cold (Moellering *et al.*, 2010), which we define as a damaging cold beyond acclimation temperatures. SFR2 converts monogalactosyldiacylglycerol (MGDG) to oligogalactolipids, including digalactosyldiacylglycerol (DGDG) and trigalactosyldiacylglycerol (TGDG). Lipids with three or more galactose head groups result uniquely from SFR2 activity and are not biosynthesized through any other known mechanism. The reaction involves removal of a galactose from one MGDG and subsequent linkage to another galactolipid, leading to the release of a diacylglycerol (Moellering et al., 2010; Roston et al., 2014). This diacylglycerol is then converted into triacylglycerol (TAG) through a multistep pathway. Fatty acids derived from MGDG are found in lipid droplets of TAG released after SFR2 activation (Barnes *et al.*, 2016). SFR2 is constitutively present in Arabidopsis, but the accumulation of the unique TGDG lipid product is only observed following SFR2 activation after exposure to sub-zero freezing stress (severe cold) (Thorlby *et al.*, 2004; Barnes *et al.*, 2016).

The activity responsible for the production of TGDG has previously been studied under the name galactolipid-galactolipid galactosyltransferase (GGGT) (Dorne *et al.*, 1982; Heemskerk *et al.*, 1983) and was later attributed to *SFR2*. Much of the initial work was performed in isolated chloroplasts from spinach (*Spinacia oleracea*), where TGDG accumulates in response to the chloroplast isolation procedure and to ozone fumigation (Dorne *et al.*, 1982; Heemskerk *et al.*, 1983, 1986; Sakaki *et al.*, 1985, 1990). TGDG accumulation is also associated with the response to other processing stresses and environmental cues, including protoplast isolation in common bean (*Phaseolus vulgaris*) and Arabidopsis (Webb & Williams, 1984; Barnes *et al.*, 2019) and chloroplast isolation and wounding in Arabidopsis (Vu *et al.*, 2015). Increased amounts of TGDG and a concomitant reduction in MGDG are observed in the desiccation plants rock violet (*Boea hygroscopica*) and blue gem (*Craterostigma plantagineum*) during the transition to the desiccated form (Navari-Izzo *et al.*, 1994; Sgherri *et al.*, 1994; Gasulla *et al.*, 2013). Moreover, drought and salinity stress cause TGDG accumulation in tomato (*Solanum lycopersicum*), which was directly attributed to SFR2 through the use of mutant lines, as in studies of severe cold in Arabidopsis (Wang *et al.*, 2016).

Cold is a stress that is relative to each species and has multiple definitions. Chilling stress is a type of cold stress that is non-damaging. It induces physiological changes that then allow acclimation to additional cold. Sensing of initial chilling is well characterized and has been reviewed extensively (Thomashow, 1999, 2001, 2010). Briefly, initial chilling is sensed at the plasma membrane through a flux of calcium ions (Knight *et al.*, 1991, 1996); the resulting calcium spike causes changes in gene expression, including that of the *C-REPEAT/DRE BINDING FACTOR* (*CBF*) and *COLD-REGULATED* (*COR*) transcription factor genes (Fowler & Thomashow, 2002; Catala *et al.*, 2003; Chinnusamy *et al.*, 2007; Doherty *et al.*, 2009). These transcriptional changes produce alterations in cellular membranes (Zhao *et al.*, 2016), the accumulation of solutes within the cell (McKown *et al.*, 1996), and metabolic rebalancing (Schulze *et al.*, 2012). Exposure to lower temperatures then induces severe cold stress, from a damaging level of cold. In freezing-tolerant plants, this includes freezing stress. Studies of severe low-temperature stress across multiple species have shown that sub-zero acclimation, consisting of a period of below-zero non-lethal temperature, is distinct from initial chilling sensing and prepares plants for additional damaging temperatures (Castonguay *et al.*, 1993; Monroy *et al.*, 1993; Livingston, 1996; Herman *et al.*, 2006; Le *et al.*, 2008, 2015; Espevig *et al.*, 2011; Takahashi *et al.*, 2019). Indeed, many of the changes that occur during sub-zero acclimation are different from those observed during initial chilling sensing and involve modulation of numerous genes not induced during initial cold treatment, as well as proteome- and cell wall-specific changes (Takahashi *et al.*, 2019). Little is known about how severe cold is sensed or transduced. Our previous work identified cytosolic acidification as one signaling mechanism employed by Arabidopsis that results in TGDG accumulation (Barnes *et al.*, 2016), but it is unknown whether other species use the same mechanism.

Studies of closely related species that grow in both temperate and tropical climates have allowed a better understanding of how the evolutionary origin of low-temperature tolerance shapes species distribution and defines their effective growth areas. Investigation of temperature tolerance in the Pooideae subfamily of the grasses indicated that tolerance to initial cold is potentially more ancient than tolerance to drought (McKeown *et al.*, 2016; Das *et al.*, 2021). Furthermore, severe cold tolerance appears to have evolved more recently than tolerance to initial cold (McKeown *et al.*, 2016; Das *et al.*, 2021). Differences to severe cold tolerance even exist at the subspecies level in

maize, when highland and lowland maize landraces are compared (Barnes *et al.*, 2022). An improved understanding of the evolution of severe cold signals will allow the engineering of increased tolerance in crops.

To explore the evolution of severe cold responses, we used TGDG production as a marker and investigated its activation through cellular acidification. We examined TGDG production across bryophytes, gymnosperms, and angiosperms under both routine growth conditions and severe cold. We show here that TGDG production correlates with temperature and cellular acidity in multiple species and compare transcriptional changes induced by severe cold and artificial acidification treatments.

Materials and Methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*, Columbia-0 [Col-0]) plants were grown on a mixture of Sungrow Propagation Mix soil and Turface at 22°C under a 16-h-light/8-h-dark photoperiod. Plants were grown for 3 to 4 weeks before cold acclimation at 6°C under a 12-h-light/12-h-dark photoperiod for 1 week. Garden peas (*Pisum sativum* 'Little Marvel') were grown in vermiculite at 22°C under a 16-h-light/8-h-dark photoperiod. Pea plants were grown for 2 to 3 weeks under normal conditions before acclimation at 6°C under a 12-h-light/12-h-dark photoperiod for 1 week. Maize (*Zea mays* 'B73') and sorghum (*Sorghum bicolor* 'BTx623') were both grown in chambers under a 16-h-light/8-h-dark photoperiod with 29°C during the day and 22°C at night on standard greenhouse soil mix (8:8:3:1 [w/w/w/w] peat moss: vermiculite: sand: screened topsoil, with 7.5:1:1:1 [w/w/w/w/] Waukesha fine lime, Micromax, Aquagro, and Green Guard per cubic yard). Acclimation was carried out at 16°C for 1 week for both maize and sorghum, and maize was additionally acclimated for 3 days at 6°C. Wheat (*Triticum aestivum* 'Overland') was also grown in standard greenhouse soil at 21°C under a 16-h-light/8-h-dark photoperiod before cold acclimation at 6°C under a 12-h-light/12-h-dark photoperiod for 1 week. All plants for all treatments were moved into low-temperature stress at the end of their respective day for treatment in the dark.

Plants for phylogeny tests, excluding trees, were grown under greenhouse conditions at 24°C on standard greenhouse soil mix, as described above, under a 12-h-light/12-h-dark photoperiod. *Physcomitrium patens* was grown on PpNH4 Moss Medium (Caisson Laboratories, Smithfield, UT, USA), and *Spirodela polyrhiza* was grown on Schenk and Hildebrandt (Sigma Aldrich, Inc., St. Louis, MO, USA.) basal salt medium with pH adjusted to 5.8 using potassium phosphate. The moss and duckweed were grown at 22°C under a 16-h-light/8-h-dark photoperiod in a growth chamber. Tree samples were collected from the University of Nebraska-Lincoln campus; leaves were sampled in the early fall before any nights approaching or below freezing had occurred. Plants were obtained from diversity and teaching greenhouses at the University of Nebraska-Lincoln. Species that were not already grown by the University of Nebraska-Lincoln were obtained through cuttings or seeds from the USDA-GRIN database.

Lipid analysis

Lipids were extracted from plant tissues using a modified Bligh and Dyer method and thin-layer chromatography (TLC) analysis as described (Wang and Benning, 2011). Lipids were loaded onto EMD60 plates (Millipore, Burlington, MA, USA) and subsequently resolved in a solvent system of chloroform:methanol:acetic acid:water (85:20:10:4, v/v/v/v) as described (Barnes *et al.*, 2016). Samples frozen in water to match ion leakage low-temperature treatments were allowed 30 min of thawing so that the water could be removed, and the lipids extracted. α-Naphthol stain was used to detect galactolipids on the chromatogram as described (Wang & Benning, 2011).

TGDG levels were calculated relative to DGDG, as DGDG is not responsive to severe cold temperatures and makes up approximately 25% of most plant membranes. ImageJ with the FIJI plug-in densitometry function (Schindelin *et al.*, 2012, 2015; Schneider *et al.*, 2012) was used for quantification of DGDG and TGDG levels, with fractional TGDG/DGDG ratios based on the gray value of each TLC spot measured (Rouser *et al.*, 1966).

Phylogenetic analysis of TGDG accumulation

SFR2-like proteins were identified using Phytozome and NCBI Blast. Relationships in the cladogram were based on version 13 of the Angiosperm Phylogeny Website (http://www.mobot.org/mobot/research/apweb/) and other available phylogenomic analyses (Wickett *et al.*, 2014; Gitzendanner *et al.*, 2018).

Plant material was sampled by taking six leaf discs of 8 mm in diameter. For plants with irregular leaves, such as needles or small fronds, tissue samples equivalent to the weight of six leaf discs were collected with a razor blade. Corresponding fresh and frozen samples were collected, with samples of each equivalent to six discs. Lipids from fresh samples (controls) were immediately extracted for lipid analysis as described above. "severe cold challenged" samples were obtained from plants cooled gradually over 24 h from 6°C to –20°C in a refrigerated circulator (Figure 1A). Several plant varieties were sourced from the U.S. National Plant Germplasm System.

Ion leakage

Plants used for ion leakage were grown as described above, and all plants were cold acclimated for 1 week at the appropriate temperature. Ion leakage was determined using a refrigerated circulator (AP15R-40, VWR, Radnor, PA, USA) with leaf pieces or punches floated onto 3 mL of ddH_2O. For Arabidopsis, an entire leaf was used. For pea, a leaflet was used after removing the mid-vein. For sorghum, three leaf punches of 8 mm in diameter were used. Samples were collected from the second true leaf, except for Arabidopsis, where rosette leaves were sampled, with care taken to use older, expanded leaves and avoid cotyledons. Different plants were then chilled to temperatures sufficient to induce stress in the respective species. Stress was imposed on Arabidopsis as previously described (Warren *et al.*, 1996), and similar conditions were used for pea. Briefly, samples were exposed to an initial equilibration at 2°C for 30 min, nucleation was initiated with a ddH_2O ice chip at –1°C for 1 h, and subsequent chilling occurred at a rate of –2°C/h (Figure 1B). Samples for sorghum were collected at temperatures from 0°C to –4°C. Following a 30-min equilibration at 0°C, samples were cooled from 0°C to –1°C at a rate of –2°C/h, and ice nucleation was initiated at –1°C and held for 1 h before subsequent chilling at a rate of –1°C/h from –1°C to –3°C and then –2°C/h from –3°C to –4°C (Figure 1B). The slower chilling between –1°C and –3°C allowed for sampling in 0.5°C steps.

After chilling, leaf samples were incubated at 4°C overnight. The temperature was then raised to room temperature, and the samples were shaken at 250 RPM for 15 min (Warren *et al.*, 1996). This set of samples constituted the initial conductivity measurement, which was performed using an Accumet AB200 (Fisher Scientific, Hampton, NH, USA). For total conductivity measurements,

samples were heated to 65°C for 30 min to release all electrolytes, then cooled down to room temperature and shaken at 250 RPM for 15 min. To calculate leakage, the percentage relative to total ions was calculated, plotted, and fit to a sigmoidal curve (Warren *et al.*, 1996)). At each temperature point, an equivalent sample for lipid analysis and fractional TGDG accumulation was collected for all species. For Arabidopsis and sorghum, an additional and equivalent sample was also taken for transcriptome analysis.

Whole plant freezing tests

Cold-acclimated soil-grown plants were treated as indicated in Figure 1C. After cold acclimation, each species had multiple plants treated overnight at varying temperatures (pea, 0°C, –2°C, and –4°C; maize, 2°C and 0°C; sorghum, 2°C and 0°C; wheat, 0°C, –2°C, –5°C, and –10°C). For all tests, ice chips were added on the soil surface to induce ice nucleation 1 to 2 h after being introduced to the treatment temperature if it was at or below 0°C. Leaf lipid samples for TGDG accumulation were collected from the second true leaf after 16 h of freezing by extra-sharp double-sided razor blade directly into extraction buffer, extracted as described above, and analyzed via TLC. Any samples damaged by pressure during sampling from evidence of green seepage from the leaf were discarded. Post-freezing recovery was performed by returning plants to a greenhouse at 24°C for 1 week before photographs were taken.

Cytosolic acidification

All experiments were performed on excised leaves. For Arabidopsis, the leaf was placed into a cup of acid solution or water after having removed the leaf from the rosette of a full--sized 3-week-old plant. For pea, a leaflet was removed, gently scored with a razor blade on its abaxial side and placed in a cup as above. A 20 mM acetic acid solution, adjusted to pH 5 with concentrated KOH, was used for acidification treatment along with water as a control, as described (Barnes *et al.*, 2016). Treatments were conducted for 3 h. Leaves were patted dry before extracting lipids. For sorghum, the sorghum stalk above the soil surface was cut using a new razor blade for each plant and shoots were inserted into a tube containing 20 mM 2,4-dinitrophenol, pH 5, in 18.2% (v/v) methanol, adjusted with KOH, or into 18.2% (v/v) methanol/water as a control. These samples were immediately placed into a humidity chamber for 3 h with a minimum relative humidity of

84%. Leaf punches were taken from the second true leaf to mimic the samples used in ion leakage tests.

RNA-seq data generation and processing

Total RNA was isolated for each sample using a Zymo Quick-RNA Plant Mini-prep Kit (Zymo Research Corp, Irvine, CA, USA), and RNA-seq libraries were prepared according to Illumina TruSeq Sample Preparation V2 using 1 μg of starting total RNA. Libraries were sequenced using a 75-bp paired-end Illumina Miseq instrument at the University of Nebraska Medical Center. Raw reads were deposited in the NCBI SRA (Sequence Read Archive) database under the BioProject ID PRJNA894306. Trimmomatic 0.36 (Bolger *et al.*, 2014) was used to remove low-quality reads and adapters using default parameters. The resulting clean reads from Arabidopsis and sorghum samples were aligned to the *Arabidopsis thaliana* TAIR10 and *Sorghum bicolor* v3.1 genomes, respectively (retrieved from Phytozome v12.0) using GSNAP (2018-03-25) (Wu & Nacu, 2010). Alignment files were converted to bam format using Samtools (v1.9) (Li *et al.*, 2009) and used as input to HTSeq (0.6.1) (Anders *et al.*, 2015) for generation of raw counts per gene.

Differentially expressed genes

The formula design ~= Replicate + Condition in DESeq2 was used to identify differentially expressed genes (DEGs) for each species for both artificial acidification and severe cold treatment using DESeq2 (Love *et al.*, 2014). Two and three biological replicates were employed for the identification of DEGs during temporal and chemical treatment. Any gene in the condition factor (Control vs. Treatment) with an adjusted p-value < 0.05 and absolute Log_2 fold-change > 1 were classified as DEGs. Overall, four gene categories in each species were generated: upregulated by chemical treatment, upregulated by temperature treatment, downregulated by chemical treatment, and downregulated by temperature treatment. To identify differentially expressed orthologs between Arabidopsis and sorghum, a list of corresponding orthologs between each sorghum gene model and the best hit Arabidopsis gene models was retrieved from Phytozome v12.0. To compare with randomly overlapping orthologs in either treatment, only genes with 1:1 orthologs between sorghum and Arabidopsis were considered as background. Sorghum genes in each category were assigned to the best hit Arabidopsis gene models. To determine the significance of co-upregulated or co-downregulated orthologs between Arabidopsis and sorghum, we randomly picked the equal

number of upregulated or downregulated genes in Arabidopsis respectively and tested the number of genes with orthologs in sorghum. The F test was used to determine significance between real overlapping ortholog numbers and permutated overlapping ortholog numbers. In total, 100 permutations were performed.

Gene Ontology Analysis

Lists of genes obtained from DEG analysis were analyzed via the Gene Ontology Resource (Ashburner *et al.*, 2000; Mi *et al.*, 2019). GO Enrichment Analysis was performed, and significantly enriched categories were reported (Gene Ontology Consortium, 2021).

Results

TGDG production has multiple patterns in land plants

In Arabidopsis, SFR2 is always present (Thorlby *et al.*, 2004; Barnes *et al.*, 2016), and its activation leads to TGDG production in response to low-temperature stress. To better understand the similarity of severe cold responses across a set of diverse plant species, we analyzed TGDG accumulation in 43 species after identical low-temperature exposure (Figure 1). Species were chosen for their economic importance, availability of sequenced genomes, or phylogenetic origin, and all 43 species are represented in a phylogenetic tree based on plant evolution (Figure 2A).

Quantification of TGDG levels allowed observation of four patterns of TGDG accumulation (Figure 2B). Production of TGDG was below the detection limit for both control and cold-challenged samples in some species (Figure 2B, shaded oval). These species did not appear to group clearly across phyla or relative cold hardiness. The second pattern of TGDG accumulation is from species tending to have low TGDG levels in control samples and a substantial increase in TGDG levels in cold-challenged samples (Figure 2B). This pattern of TGDG accumulation appeared in some species of eudicots, monocots, basal angiosperms, ferns, and lycophytes. In the third pattern, TGDG levels were higher in control samples than in those challenged with cold (Figure 2B, open icons). The amount of TGDG detected under control conditions in these species, which included wheat (*Triticum aestivum*) and maize, may be caused by wounding damage of the leaf punch during sample collection, masking any increase in TGDG during low temperature challenge. The species having this pattern of TGDG accumulation had no discernable similarities

across phylogeny or cold hardiness. The final pattern of TGDG accumulation was characterized by high amounts of TGDG in both control and cold-challenged samples. Many species exhibiting this pattern were the most anciently diverged lineages of land plants such as mosses and liverworts (Figure 2B). Among all phylogenetic groups, the angiosperms (eudicots, monocots, basal angiosperms) appear to have the strongest differences in TGDG accumulation patterns between species (Figure 2, red/orange/yellow/pink/purple).

Total cellular damage corresponds to TGDG accumulation

The presence of TGDG in control samples in Figure 2 suggested that TGDG may accumulate in response to cellular damage caused by the mechanical wounding from taking a leaf punch. We hypothesized that severe cold stress might also cause sufficient cellular damage to trigger TGDG accumulation, rather than directly inducing the formation of this class of lipids. To assess the association between cellular damage and severe cold stress, we measured ion leakage, a quantitative measure of cell damage (Demidchik *et al.*, 2014), in parallel with TGDG content in Arabidopsis, pea, and sorghum. Prior to severe cold stress, plants were cold acclimated at a temperature appropriate for each species. We then transferred leaf pieces or discs of cold-acclimated plants to a continuous ramp of severe cold in a refrigerated circulator and determined ion leakage and lipid contents at each temperature (Figure 3).

The temperature at which 50% cellular leakage occurs, termed LT_{50}, varied for each species and reflected their low-temperature tolerance (Figure 3A). Arabidopsis results were similar to previously published outcomes (Warren *et al.*, 1996), showing partial leakage from temperatures ranging from –2°C to –10°C (Figure 3A) and an LT_{50} of –5.1°C. In pea, all temperatures below freezing induced some cellular damage, as in Arabidopsis (Figure 3A), but LT_{50} was reached earlier at around –2.5°C. Sorghum leakage occurred rapidly, and exhibited ion leakage values close to total ion leakage at the range of temperatures tested (Figure 3A), resulting in an LT_{50} of –2.2°C.

The speed of the ion leakage assay (Figure 1) provided an opportunity for direct comparisons between TGDG production and the extent of membrane damage, which we assessed at each temperature point. Final TGDG levels were highest in Arabidopsis, while in sorghum and pea, accumulation occurred more modestly with the levels of TGDG nearly 10-fold lower than those

observed in Arabidopsis (Figure 3B). The amount of TGDG occurred in a similar order to the relative cold tolerance levels of the species, and all species accumulated TGDG within the narrow timeframe of the experiment. The inflection points of TGDG accumulation were near the inflection points of ion leakage. The inflection point of TGDG accumulation was –6.0°C (Figure 3B) in Arabidopsis, –1.4°C in sorghum (Figure 3C), and –4.5°C in pea (Figure 3C). These results indicate a tight association between membrane damage and TGDG accumulation, as all species induced TGDG accumulation in the same temperature and time range as membrane damage occurred.

Whole-plant low-temperature treatments suggest that TGDG accumulation in freshly collected excised leaves results from wounding

Because damage may have a role in the severe cold response, we wished to use whole plants to ask if species with TGDG levels higher in control samples than in those challenged with cold in Figure 2 (open icons) accumulate TGDG in response to severe cold. To accurately encompass the variety and divergence of TGDG accumulation patterns illustrated in Figure 2B, we selected pea (*Pisum sativum*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), and wheat (*Triticum aestivum*). Pea had one of the highest relative accumulations of TGDG in the cold. Maize and sorghum are closely related and had similar TGDG levels, with one accumulating more TGDG in cold-challenged samples and one in control samples. Wheat was selected as it had one of the highest accumulations of TGDG in control samples. Each of these species is also of economic importance and has established growth conditions.

Plants were cold-acclimated prior to freezing at temperatures appropriate for their low-temperature tolerance: 6°C for pea and wheat and 16ºC for maize and sorghum. Each freezing schedule was also adjusted to match the cold tolerance of the species. Pea, maize, and sorghum each died at temperatures 1°C lower than shown (Figure 4A). Wheat died at –10°C. We exposed plants to cold stress at the end of the light cycle, which is when plants normally exhibit their maximal cold tolerance levels (Raju *et al.*, 2018), thus mirroring the overnight conditions when freezing would be experienced in a field. Pea and wheat were the most tolerant of freezing of the four species, as they both survived exposure to below-freezing temperatures (Figure 4A), despite being the most disparate in TGDG accumulation in their control and cold-challenged samples (Figure 2).In all plants, TGDG accumulation increased in response to cold challenge (Figure 4B,C). We confirmed

that wheat, maize, and sorghum were very sensitive to wounding stress and that the sample collection method was important. Even when using whole plants, we detected higher levels of TGDG in samples collected during control growth conditions if there was any leaf damage during lipid sampling. As seen in Figure 3, TGDG levels also scaled with low-temperature tolerance. Wheat was the most tolerant species assayed and had the most TGDG accumulation. Sorghum and maize were the least tolerant species and accumulated less TGDG.

Cytosolic acidification mimics a freezing response in Arabidopsis, pea, and sorghum

Next, we asked whether TGDG accumulation is activated by similar mechanisms in TGDG-accumulating angiosperm species that differ in their ability to tolerate cold. We previously developed a protocol that artificially acidified the cytosol to mimic the lipid response and pH change that occurs during an overnight freezing test (Barnes *et al.*, 2016). We thus excised whole leaves from pea and leaf pieces from sorghum, and treated them with low levels of organic acids, processing Arabidopsis plate-grown rosettes concomitantly as a control. In both Arabidopsis and pea, TGDG accumulated in response to acidification within 120 min (Figure 5A, B, D).

In sorghum, sampling the leaves by razorblade or leaf punch caused TGDG accumulation in control samples after just 30 min. We reasoned that the considerable amounts of wax on sorghum leaves (Traore et al., 1989) might prevent them from taking up acid or water. We then transitioned to use whole excised shoots placed vertically to treat the sorghum, and a more hydrophobic organic acid, 2,4-dinitrophenol. After 180 min, we routinely observed TGDG accumulation in the acid-treated sorghum and none in the control (Figure 5C, D). Thus, cytosolic acidification appears to activate TGDG accumulation in multiple species.

Severe cold and acidification treatments invoke overlapping transcriptome responses

We used our new assay for sorghum acidification to ask if sorghum and Arabidopsis would have any similarities in non-TGDG responses to the stimuli of severe cold or acidification. We already knew that lipid changes induced by low-temperature and acid treatments are similar in Arabidopsis (Barnes *et al.*, 2016) and wanted to separate the response to severe cold challenge and response to acid treatment to identify any overlap between treatments or between species. Thus, we exposed Arabidopsis to 0°C or –7°C and sorghum to 0°C or –2.5°C through a quick ramp in temperature

as for the ion leakage samples (Figure 1). In parallel, we exposed plants grown simultaneously to the acidification and control treatments defined above. We then collected samples for RNA-seq analysis. We identified significant differentially expressed genes (DEGs) as any gene in the treatment group with an adjusted *p*-value below 0.05 and an absolute Log_2 fold-change greater than 1 relative to the control group. We first defined eight groups: upregulated or downregulated in response to severe cold in or when acidified in Arabidopsis (Figure 6A), and the same four groups in sorghum (Figure 6B). There was a statistically significant set of genes that were enriched in response to severe cold and acidification when either up or downregulated in each species.

We attempted to define any genes enriched in response to both cold and acidification in both species using orthologous gene sets. There were overlaps between all single stress categories (i.e., upregulation in response to cold in Arabidopsis and sorghum), however, no overlap was detected between the two species' responses to both stresses, likely due to the small numbers of genes and the difficulty of identifying homologs between sorghum and Arabidopsis. Thus we investigated the categories of genes affected in both stresses and both species. To do so we further separated the DEGs into eight groups by upregulation and downregulation in response to treatment. The eight groups were then used to identify Gene Ontology (GO) terms of enrichment for each species (Figure 6C, D). Complete lists of the GO term categories can be found in Supplementary Tables 1-8. We identified multiple GO terms that were significantly enriched in both Arabidopsis and sorghum, under both severe cold and acid treatments (Figure 6C & D, bold), and many more that were species-specific. Importantly, we detected no significant enrichment for GO term categories in biological processes that were related to initial chilling responses such as response to cold, cold acclimation, and response to temperature stimulus among DEGs (Barah *et al.*, 2013). Instead, response to stresses, oxygen-containing compounds, stimulus, and chemicals were significantly enriched in response to both treatments; the same GO categories are enriched in response to wounding and other stresses that cause cellular damage (Reymond *et al.*, 2000; Ding *et al.*, 2013; Mata-Pérez *et al.*, 2015). GO terms for the molecular function and cellular component ontologies, protein binding, cellular periphery, and plasma membrane were significantly enriched in both species. This indicates the importance of membrane dynamics in response to cellular damage and stress. To summarize, there are similarities in non-TGDG responses to the stimuli of severe cold or acidification in multiple species.

Discussion

SFR2 is ubiquitous across the plant kingdom (Fourrier *et al.*, 2008), raising the possibility that its product TGDG might be as well. Our phylogenetic analysis of TGDG production in leaf discs indicated that most plants synthesize and accumulate TGDG (Figure 2), with the most ancestrally diverged species displaying the highest levels of TGDG. It is possible that TGDG production is as ubiquitous as the presence of SFR2, though in some phylogenetically diverse species, it remains below the detection limit in control and severe temperature challenge. Other species, including maize and wheat, appeared to accumulate higher levels of TGDG under control conditions compared to severe cold challenge treatment, which prompted us to test the role of tissue damage, which likely occurred during sample preparation. We compared TGDG production with the extent of membrane damage through ion leakage assays in sorghum, pea, and Arabidopsis (Figure 3). These assays showed tight associations between membrane damage and accumulation of TGDG. We then asked if a subset of species from the initial screen with high accumulations of TGDG in control conditions also accumulated TGDG in response to whole plant cold challenge (Figure 4). They did, and the scale of response matched well with the level of the species cold tolerance. This implied a similar mechanism of activation may be used in all species. Tests of pea, sorghum, and Arabidopsis all showed that acidification can trigger TGDG accumulation independently of temperature (Figure 5) (Barnes *et al.*, 2016). Finally, we detected significant overlaps between DEGs and their associated pathways after acidifying or severe cold treating Arabidopsis and sorghum (Figure 6). Many of these DEGs are not related to initial cold responses and instead are associated with more general cellular stresses and membrane dynamics. Together, this work suggests that the TGDG severe cold response has evolved from an ancestral response, the strength of the response has been modified multiple times in each phylum, and that acidification of the cytosol plays a role in sensing severe cold.

Multiple studies have harnessed plant diversity and phylogeny to understand the loss and gain of tolerance mechanisms to abiotic stresses such as drought and salinity (Bromham *et al.*, 2020; Marks *et al.*, 2021). Costa and colleagues reported that drought stress imposed on vegetative tissue co-opts and reprograms some of the mechanisms used during seed desiccation (Costa *et al.*, 2017). Drought tolerance of vegetative tissues has arisen separately multiple times, suggesting that this trait may emerge from the modification of regulatory regions of existing genes (VanBuren, 2017).

An examination of the phylogeny of salt tolerance reveals a unique pattern. Rather than clusters of salt-tolerant species within families, these species are often located at the tips of a phylogeny, with few common relatives also exhibiting salt tolerance (Bromham *et al.*, 2020). The authors suggest such a pattern may originate from one of three potential reasons: 1) a recent environmental change caused the gain of salt tolerance; 2) salt tolerance is a highly labile trait with frequent loss and frequent gain; or 3) the trait is quick to arise and has a high extinction rate. Currently, our understanding of severe low temperature tolerance is consistent with each of these hypotheses. The angiosperms (Figure 2, Eudicots, Monocots, and Basal Angiosperms) include species that produce high levels of TGDG in response to cold (e.g., *Arabidopsis thaliana*, *Cabomba aquatica*) and no detectable TGDG (*Gossypium raimondii, Ginko biloba*), as do the ferns (high, *Equisetum arvense*, low, *Osmundastrum cinnamomeum*). This is consistent with multiple separate evolutions or losses of severe cold tolerance and may parallel the hypothesis of drought tolerance emergence from modification of existing genes involved in damage. Similarly, TGDG accumulations in response to cold varied widely between species within each phyla (Figure 2), an observation that was confirmed within Angiosperms by whole plant cold challenge assays (Figure 4). This is consistent with the salt tolerance hypotheses for trait lability. Future work could dissect patterns of response further within phyla, as we show that there are differences in TGDG accumulation in each.

The results presented here expand our initial understanding of TGDG accumulation in response to severe cold by demonstrating that TGDG levels broadly scale with plant species' cold tolerance levels and coincide with cellular damage. We previously reported in Arabidopsis that TGDG can accumulate in response to severe cold (Barnes *et al.*, 2016). In Figure 2, we tested a wide range of species, finding that some species accumulate more TGDG in control samples than in response to cold challenge. Figure 3 established a reduced time scale experiment, within which multiple species, Arabidopsis, sorghum, and pea, accumulate TGDG as membranes are damaged. Under control, whole-plant, growth conditions, and species-specific cold challenges, pea, maize, sorghum, and wheat all accumulated TGDG in response to cold challenge (Figure 4). The temperature at which TGDG began to accumulate matched the cold tolerance limits of the species, with maize and sorghum accumulating TGDG at temperatures that would not be considered severe cold by Arabidopsis or pea plants. Wheat and maize had higher levels of TGDG in control

treatments (Figure 4C), which further increased when cold challenged. This finding contrasted with results shown in Figure 2B, in which wheat and maize accumulated more TGDG in control than in cold challenge. Experimental differences between these two sets of cold challenges (Figure 1) explain the accumulation of TGDG in response to cold. The tight association between damage and TGDG production (Figure 3), suggests that the difference in TGDG accumulation in the control samples (Figure 2, Figure 4) is likely in response to damage caused by leaf punching. The central vacuole and the apoplastic spaces of plant leaves are approximately pH 6 (Gao et al., 2004; Martiniere et al., 2013) making them both reservoirs of acidity. Other work supports that when tissues are damaged by pathogens, acidification occurs (Lebrun-Garcia et al., 1999; Roos et al., 2006). This idea is supported by our previous observation that wounding changes cytosolic pH and initiates TGDG accumulation (Vu *et al.*, 2015). Together, these data suggest that a component of the severe cold response is a response to membrane damage, both of which promote TGDG accumulation.

Membrane damage and cytosolic acidification seem to be consistent factors uniting the multiple stresses that activate SFR2 in angiosperms accumulating TGDG. Previous data from multiple reports have indicated that TGDG accumulates in response to various membrane-damaging stresses such as ozone treatment of spinach (Sakaki *et al.*, 1985, 1990), salt and drought stress of tomato (Wang *et al.*, 2016), and protoplast isolation from fava bean (*Vicia faba*) or Arabidopsis (Webb & Williams, 1984; Barnes *et al.*, 2019). Our previous investigation revealed that TGDG accumulation promoted by protoplast isolation is pH dependent (Barnes *et al.*, 2019). Membrane damage occurs in each of these stresses, suggesting a broad similarity in mechanisms across species. Many of the species we tested here showed an increase in TGDG levels in response to severe cold, and at least a subset of these increases appeared to be associated with damage (Figure 2B). Based on this information, we suggest that TGDG accumulation after a membrane damaging event is the ancestral state.

The specifics of how membrane damage and cytosolic acidification are linked to TGDG accumulation remain unknown. The abundance of SFR2, the enzyme that produces TGDG, does not increase in response to severe cold and is not induced by either temperature or pH when tested in a heterologous system (Roston *et al.*, 2014); therefore, post-transcriptional mechanisms must

connect damage and cytosolic acidification to TGDG accumulation. The same mechanisms may underlie the overlapping DEGs responding to severe cold or acidification of Arabidopsis and sorghum (Figure 6A & B). Interestingly, many of the gene categories were associated with response to multiple types of stress, but not cold specifically (Figure 6C & D). This observation suggests a core response to these two stresses.

We used the prevalence of oligogalactolipid accumulation as a tool to better understand the evolution of plant cold tolerance. We linked cytosolic acidification with activation of a severe cold response including TGDG accumulation (Figure 5) and multiple transcriptional changes (Figure 6). We correlated TGDG accumulation with membrane damage over a short time window (Figure 3). We also showed that TGDG accumulation in response to severe low temperature varies in each phyla investigated, likely originating from an ancestral state in which TGDG production was relatively higher (Figure 2). Our results expand our understanding of severe low temperature tolerance across plant species, setting the stage for pursuing many more fundamental questions in stress signaling.

Supplementary Data

Supplementary Table 1. GO terms for Arabidopsis DEGs downregulated in response to acidification.

Supplementary Table 2. GO terms for Arabidopsis DEGs upregulated in response to acidification.

Supplementary Table 3. GO terms for Arabidopsis DEGs downregulated in response to cold.

Supplementary Table 4. GO terms for Arabidopsis DEGs upregulated in response to cold.

Supplementary Table 5. GO terms for sorghum DEGs downregulated in response to acidification.

Supplementary Table 6. GO terms for sorghum DEGs upregulated in response to acidification.

Supplementary Table 7. GO terms for sorghum DEGs downregulated in response to cold.

Supplementary Table 8. GO terms for sorghum DEGs upregulated in response to cold.

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Author contributions

ACB and RLR designed the experiments; ACB, SMS, and JLM carried out the experiments; all authors analyzed the results; ACB and RLR wrote the manuscript; all authors edited the manuscript.

Conflict of interest

James C. Schnable has equity interests in Data2Bio, LLC; Dryland Genetics Co; and EnGeniousAg LLC. He is a member of the scientific advisory board of GeneSeek. We have no other conflicts to disclose.

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Data availability

The sequencing data that support the findings of this study are available in the NCBI SRA (Sequence Read Archive) database under the BioProject ID PRJNA894306. The full GO term analyses are available in the supplementary material of this article.

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Figure Legends

Figure 1. Schematics of low-temperature treatments. A) Temperatures applied to samples for the plant phylogeny described in Figure 2. All species received the same treatment**. B)** Temperatures applied to Arabidopsis (*At*), pea (*Ps*), and sorghum (*Sb*) during ion leakage described in Figures 3 and 6. The line describing pea has been shifted to the right for clarity. Black arrows indicate sample treatment in *At* or *Sb* for comparison to artificial acidification in Figure 6. **C)** Temperature profiles applied to pea (*Ps*), sorghum (*Sb*), maize (*Zm*), and wheat (*Ta*) during whole-plant stresses described in Figure 4. Lines for *Sb*, *Zm*, and *Ta* are shifted to the right for clarity.

Figure 2. Most land plants accumulate TGDG, including species with high cold susceptibility

A) Phylogenetic tree of plant evolution based on version 13 of the Angiosperm Phylogeny website and published phylogenetic trees. Colors indicate to which plant group each species belongs: red, orange, and yellow, eudicots; magenta, monocots; violet, basal angiosperms; plum, gymnosperms; green, ferns; teal, lycophytes; turquoise, mosses; aqua, liverworts. Icons alternate circle and square for ease of visibility. Open icons denote species with more fractional TGDG in control samples, and closed icons denote species with more fractional TGDG in cold challenged samples. **B)** TGDG/DGDG ratio plotted in logarithmic scale for each species described in A. Lipids were quantified in freshly sampled leaves (control, x-axis) or leaves after 24 hours of cold challenge defined in Figure 1 (cold stressed, y-axis). A grey oval indicates no observable TGDG, values in this area indicate the limit of detection in each species. The same color scheme is used in both panels.

Figure 3. Severe low-temperature damage corresponds with TGDG accumulation

A) Ion leakage curves for Arabidopsis, sorghum, and pea. Data are shown as means ± standard deviation. Arabidopsis, n = 8; pea, n = 4–7; sorghum, n = 6. The inflection point of each curve fit is noted. **B)** Fractional TGDG/DGDG ratios for leaf samples treated as in A. Data are shown as means ± standard deviation. The inflection point of the Arabidopsis curve fit is shown. **C)** A reproduction of the data shown in B of sorghum and pea fractional TGDG/DGDG ratios with adjusted y-axis values and the corresponding inflection point for

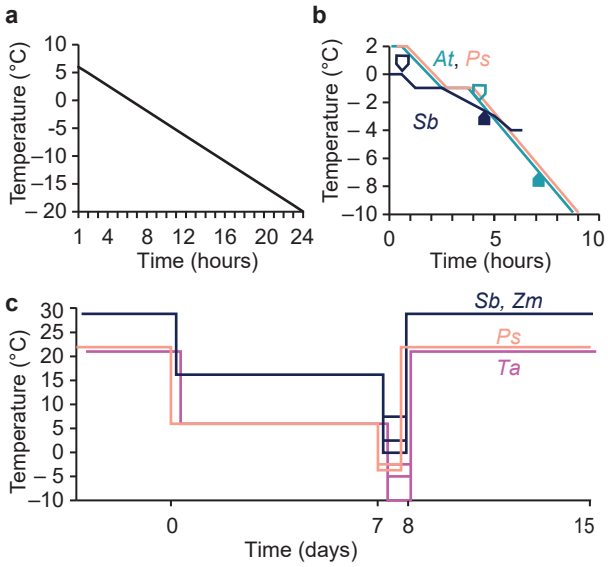
each species. Arabidopsis, n = 6; pea, n = 5–15; sorghum, n = 5. Additional temperature samples for line fit of sorghum were taken from –5°C to –8°C and are shown (n = 2). Additional samples (n = 8) were taken at –20°C for pea to improve curve fitting; these are not shown.

Figure 4. TGDG accumulates in response to low-temperature stress of whole plants
A) Representative photographs of plants grown at the indicated temperatures and **B)** corresponding thin-layer chromatograms stained with α-naphthol for galactose visualization for pea, maize, sorghum, and wheat. Black arrows indicate the position of TGDG; the blue arrow indicates a pigment that is not TGDG; +, positive control of frozen Arabidopsis lipids; –, negative control of freshly sampled Arabidopsis lipids without TGDG accumulation. **C)** Fractional TGDG/DGDG levels are quantified for each species with n ≥3 biological replicates for each species. Averages are indicated with SEM error bars, asterisks indicate a difference between control temperatures by ANOVA corrected for multiple comparisons with Bonferroni's method.

Figure 5. TGDG accumulates in Arabidopsis, pea, and sorghum upon acidification
Thin-layer chromatograms stained with α-naphthol to identify galactose head groups. **A)** Arabidopsis, **B)** pea, and **C)** sorghum leaves were treated and collected at the number of minutes indicated above. "A: denotes acidification by treatment with 20 mM acetic acid, pH 5 for Arabidopsis and pea or 20 mM 2,4-dinitrophenol, pH 5 in 18.2% methanol for sorghum; "C" denotes control treatment of water for Arabidopsis and pea and 18.2% methanol for sorghum; +, positive control of frozen Arabidopsis lipids; –, negative control of freshly sampled Arabidopsis lipids without TGDG accumulation. **D)** Fractional TGDG/DGDG levels quantification of the data shown and replicates, n ≥ 6. Averages are indicated with SEM error bars, and asterisks indicating statistical significance in acidified vs control conditions by t-test with $p < 0.05$. Times below 120 minutes were not statistically significant in pea or sorghum and are not shown.

Figure 6. Severe cold treatment and acidification have overlapping responses in Arabidopsis and sorghum

Venn diagrams representing the number of differentially expressed genes separated into direction of regulation in response to severe cold (blue) or acid (orange) treatments and the overlap between the two for **A)** Arabidopsis and **B)** sorghum. Differentially expressed genes were tested for Gene Ontology (GO) enrichment in biological process, molecular function, or cellular component ontologies separately for both **C)** Arabidopsis and **D)** sorghum. The top 10 GO terms in each ontology with statistically relevant enrichments for both severe cold and acidification are reported. *p*-values are given as the negative natural log. GO categories that are enriched for in both species in response to severe cold and acidification are indicated in bold.



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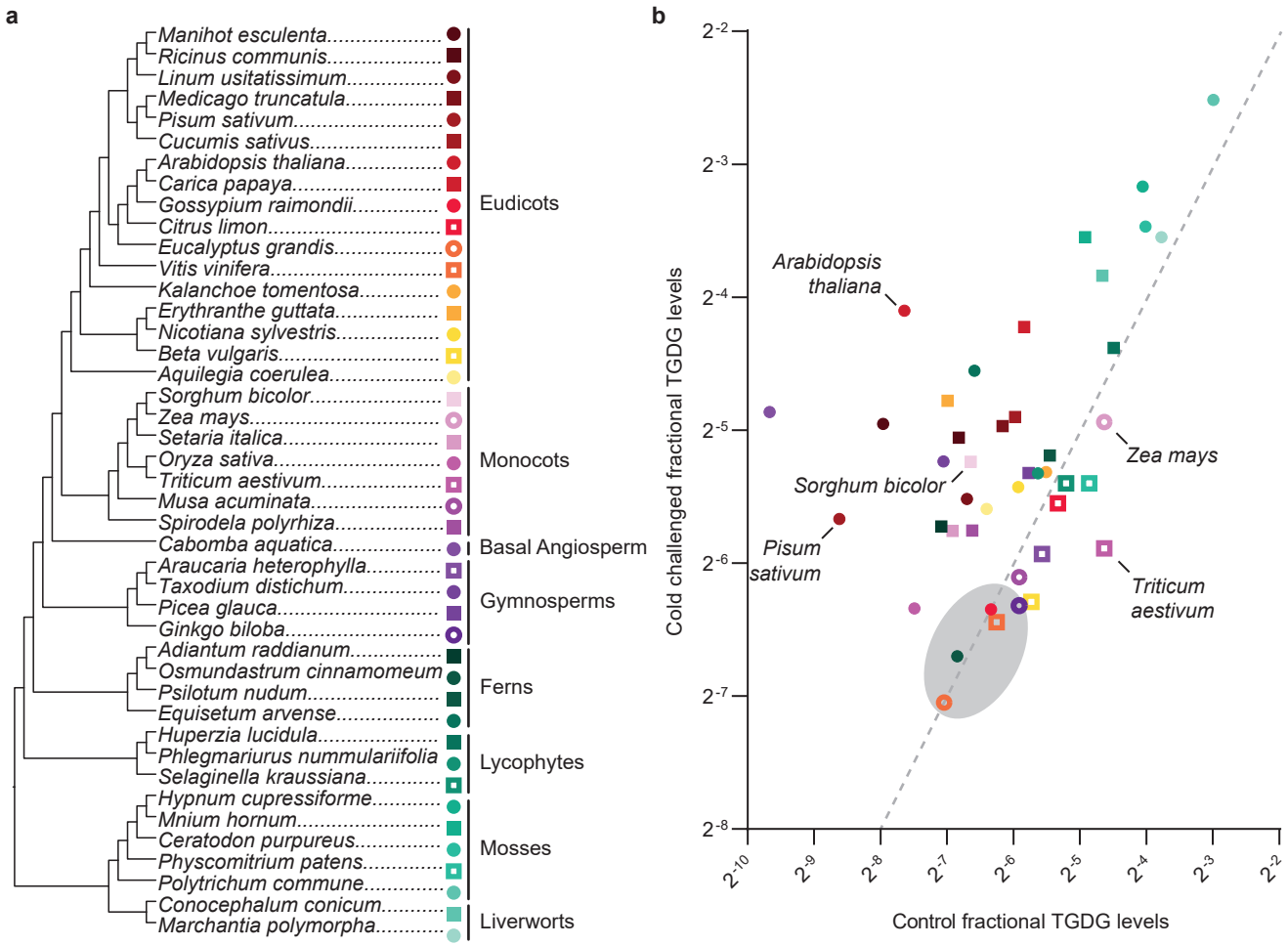

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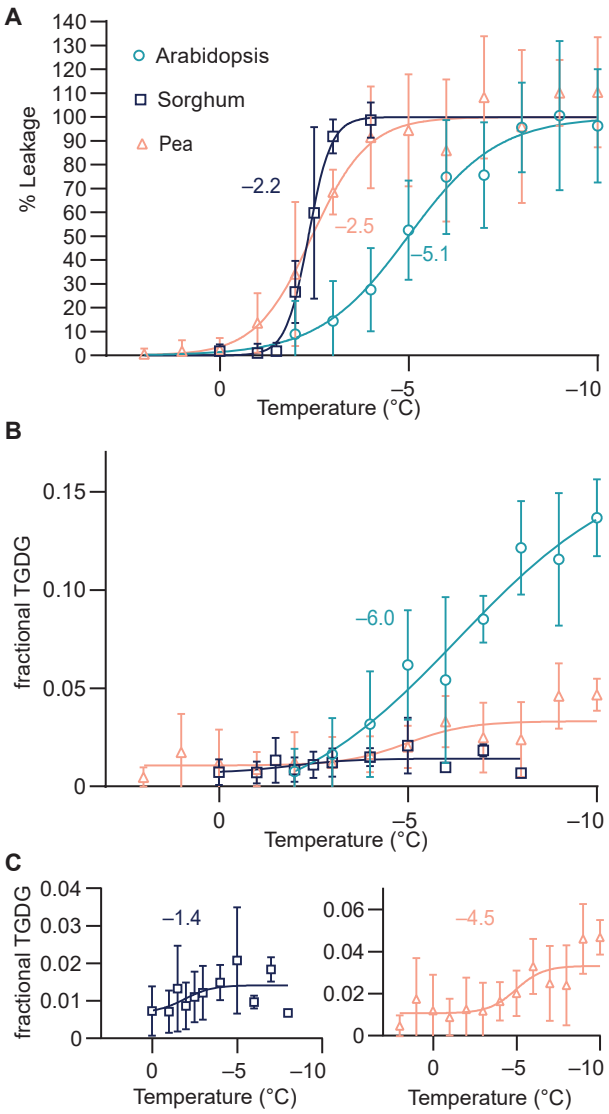

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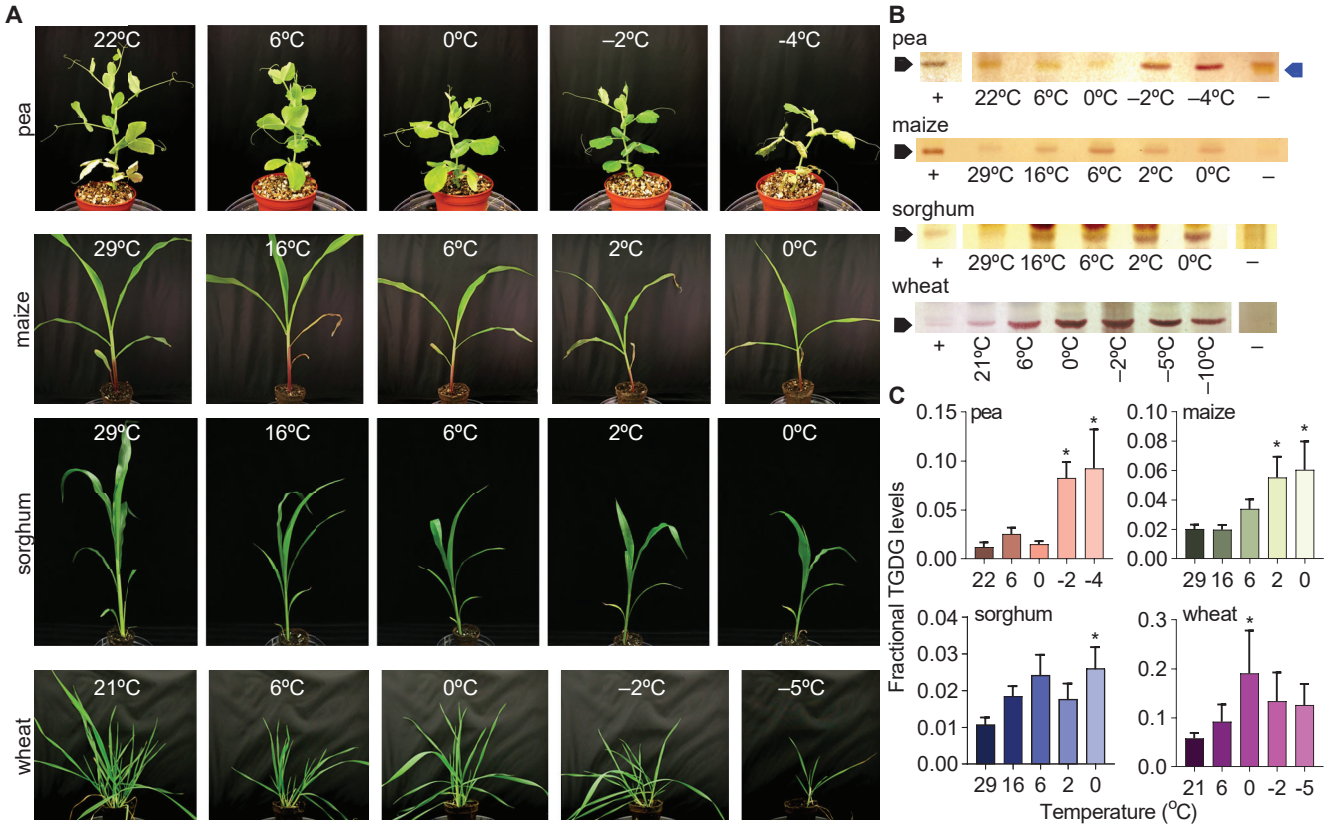

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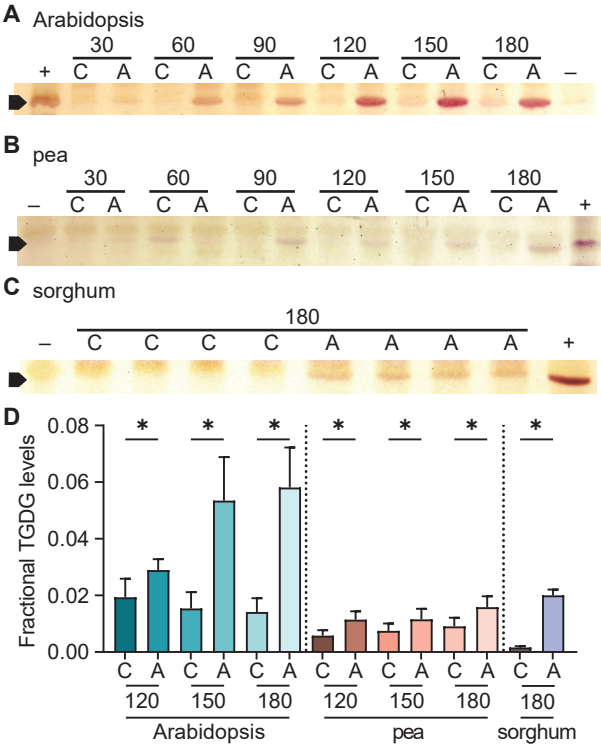

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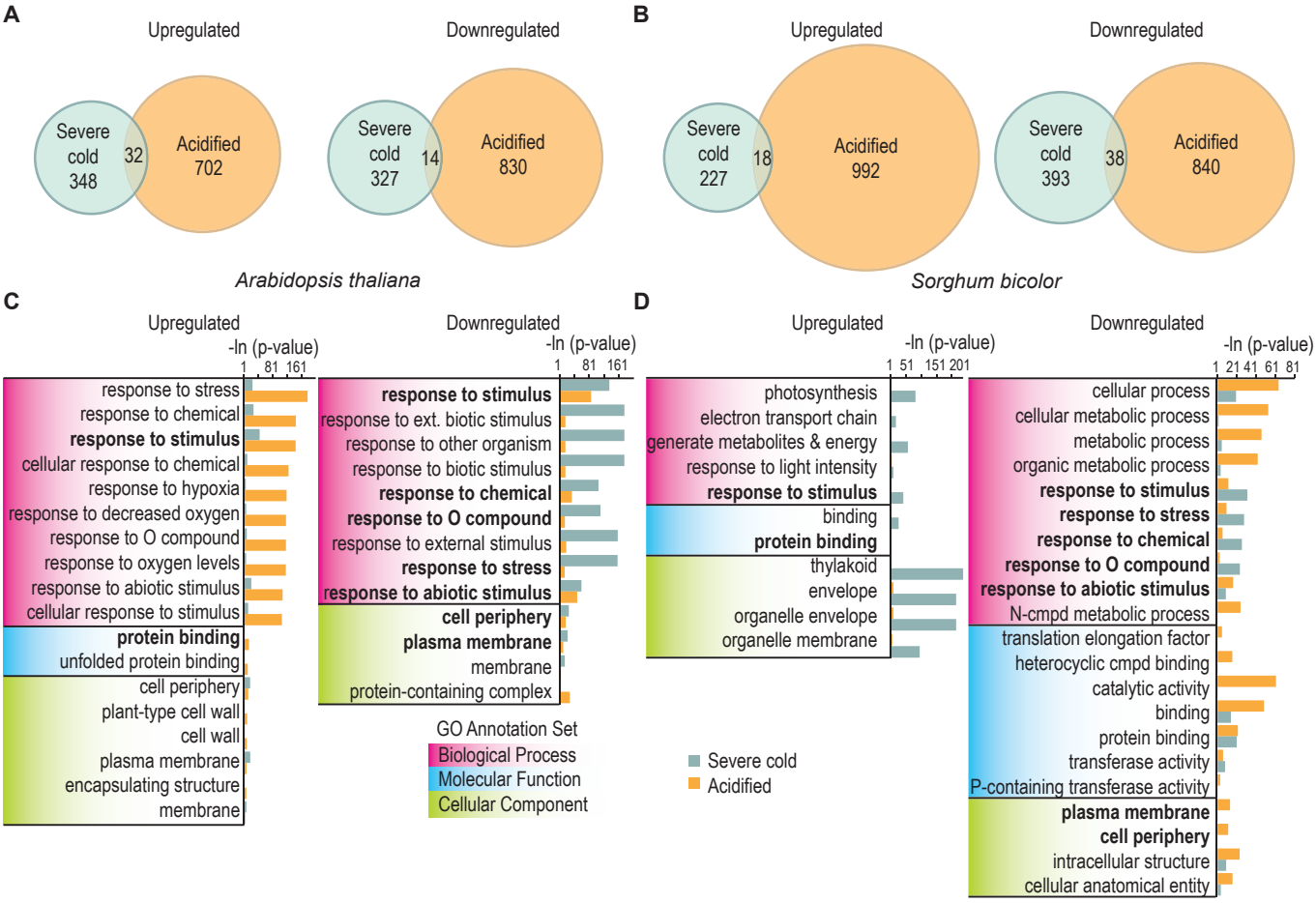

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Biological Process	Gene #	Expected	Over/Under	Fold Enrichment	P-Value
response to stimulus (GO:0050896)	474	280.85	+	1.69	5.58E-38
response to light stimulus (GO:0009416)	160	60.46	+	2.65	1.03E-24
response to radiation (GO:0009314)	161	61.73	+	2.61	3.37E-24
response to abiotic stimulus (GO:0009628)	244	124.18	+	1.96	8.25E-22
response to organic substance (GO:0010033)	214	105.83	+	2.02	8.63E-20
response to chemical (GO:0042221)	261	152.53	+	1.71	1.68E-15
response to endogenous stimulus (GO:0009719)	160	76.73	+	2.09	1.15E-14
response to hormone (GO:0009725)	158	75.71	+	2.09	2.11E-14
response to external stimulus (GO:0009605)	160	89.56	+	1.79	5.46E-09
response to auxin (GO:0009733)	43	11.62	+	3.7	7.79E-09
biological process involved in interspecies interaction between organisms (GO:0044419)	138	75.07	+	1.84	4.07E-08
response to external biotic stimulus (GO:0043207)	137	74.59	+	1.84	5.47E-08
response to other organism (GO:0051707)	137	74.59	+	1.84	5.47E-08
response to biotic stimulus (GO:0009607)	137	74.65	+	1.84	5.55E-08
cellular nitrogen compound metabolic process (GO:0034641)	41	97.8	-	0.42	7.99E-08
response to oxygen-containing compound (GO:1901700)	159	93.18	+	1.71	1.98E-07
response to stress (GO:0006950)	238	159.56	+	1.49	3.60E-07
response to lipid (GO:0033993)	112	58.41	+	1.92	4.36E-07
nucleobase-containing compound metabolic process (GO:0006139)	23	68.64	-	0.34	4.67E-07
nucleic acid metabolic process (GO:0090304)	16	56.63	-	0.28	5.97E-07
defense response to other organism (GO:0098542)	115	63.87	+	1.8	8.27E-06
defense response (GO:0006952)	127	73.68	+	1.72	1.27E-05

response to red or far red light (GO:0009639)	35	11.32	+	3.09	6.12E-05
response to organic cyclic compound (GO:0014070)	56	24.66	+	2.27	1.73E-04
gene expression (GO:0010467)	19	51.74	-	0.37	6.31E-04
response to bacterium (GO:0009617)	73	37.49	+	1.95	6.76E-04
response to wounding (GO:0009611)	54	24.6	+	2.19	9.57E-04
cellular aromatic compound metabolic process (GO:0006725)	48	91.04	-	0.53	1.19E-03
response to light intensity (GO:0009642)	32	11.26	+	2.84	1.41E-03
heterocycle metabolic process (GO:0046483)	46	87.03	-	0.53	2.39E-03
RNA metabolic process (GO:0016070)	14	41.93	-	0.33	2.61E-03
secondary metabolic process (GO:0019748)	52	24.45	+	2.13	3.02E-03
cell wall organization or biogenesis (GO:0071554)	59	29.19	+	2.02	3.15E-03
response to fungus (GO:0009620)	60	30.03	+	2	4.42E-03
response to gibberellin (GO:0009739)	16	3.74	+	4.27	1.13E-02
signaling (GO:0023052)	116	74.68	+	1.55	1.24E-02
cellular response to organic substance (GO:0071310)	80	46.79	+	1.71	2.07E-02
response to alcohol (GO:0097305)	66	36.04	+	1.83	2.14E-02
defense response to fungus (GO:0050832)	45	21.19	+	2.12	2.22E-02
cell communication (GO:0007154)	121	81.14	+	1.49	4.74E-02
lipid metabolic process (GO:0006629)	68	38.85	+	1.75	4.74E-02
organic acid metabolic process (GO:0006082)	99	63.03	+	1.57	4.87E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
RNA binding (GO:0003723)	10	48.9	-	0.2	3.66E-08
mRNA binding (GO:0003729)	5	31.94	-	0.16	1.42E-05

catalytic activity, acting on a nucleic acid (GO:0140640)	5	23.27	-	0.21	2.07E-02
amide transmembrane transporter activity (GO:0042887)	10	1.69	+	5.92	3.79E-02
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
protein-containing complex (GO:0032991)	28	93.4	-	0.3	3.52E-13
cell periphery (GO:0071944)	179	108.1	+	1.66	1.99E-08
intracellular non-membrane-bounded organelle (GO:0043232)	13	51.38	-	0.25	1.32E-07
non-membrane-bounded organelle (GO:0043228)	13	51.38	-	0.25	1.32E-07
plasma membrane (GO:0005886)	148	92.88	+	1.59	1.67E-05
extracellular region (GO:0005576)	149	94.84	+	1.57	4.04E-05
catalytic complex (GO:1902494)	12	40.87	-	0.29	1.11E-04
ribonucleoprotein complex (GO:1990904)	2	20.89	-	0.1	3.41E-04
nuclear lumen (GO:0031981)	7	29.46	-	0.24	1.14E-03
intracellular organelle lumen (GO:0070013)	11	34.83	-	0.32	3.22E-03
membrane-enclosed lumen (GO:0031974)	11	34.83	-	0.32	3.22E-03
organelle lumen (GO:0043233)	11	34.83	-	0.32	3.22E-03
envelope (GO:0031975)	14	38.73	-	0.36	4.66E-03
organelle envelope (GO:0031967)	14	38.73	-	0.36	4.66E-03
membrane (GO:0016020)	203	151.84	+	1.34	8.61E-03
intracellular protein-containing complex (GO:0140535)	5	22.46	-	0.22	1.54E-02
nucleus (GO:0005634)	263	319.94	-	0.82	3.83E-02
plastid stroma (GO:0009532)	6	22.67	-	0.26	4.72E-02

Supplemental Table 1: Significant, down-regulated GO Terms for artificially acidified Arabidopsis.

Biological Process	Gene #	Expected	Over/Under	Fold Enrichment	P-Value
response to stress (GO:0006950)	373	139.52	+	2.67	1.55E-77
response to chemical (GO:0042221)	341	133.37	+	2.56	2.09E-63
response to stimulus (GO:0050896)	477	245.57	+	1.94	3.29E-63
cellular response to hypoxia (GO:0071456)	85	6.31	+	13.47	3.07E-57
cellular response to decreased oxygen levels (GO:0036294)	85	6.36	+	13.36	5.37E-57
cellular response to oxygen levels (GO:0071453)	85	6.39	+	13.31	7.09E-57
cellular response to chemical stimulus (GO:0070887)	201	54.03	+	3.72	1.14E-54
response to hypoxia (GO:0001666)	89	8.53	+	10.44	2.88E-52
response to decreased oxygen levels (GO:0036293)	89	8.74	+	10.19	1.64E-51
response to oxygen-containing compound (GO:1901700)	243	81.48	+	2.98	1.87E-51
response to oxygen levels (GO:0070482)	89	8.79	+	10.13	2.52E-51
response to abiotic stimulus (GO:0009628)	277	108.59	+	2.55	1.76E-47
cellular response to stimulus (GO:0051716)	266	102.23	+	2.6	1.47E-46
cellular response to stress (GO:0033554)	142	32.07	+	4.43	1.93E-44
response to wounding (GO:0009611)	110	21.51	+	5.11	1.50E-38
response to chitin (GO:0010200)	71	8.13	+	8.73	1.26E-36
response to organic substance (GO:0010033)	232	92.54	+	2.51	1.91E-36
response to external biotic stimulus (GO:0043207)	189	65.22	+	2.9	4.12E-36
response to other organism (GO:0051707)	189	65.22	+	2.9	4.12E-36
response to biotic stimulus (GO:0009607)	189	65.27	+	2.9	4.58E-36
biological process involved in interspecies interaction between organisms (GO:0044419)	189	65.64	+	2.88	9.54E-36
response to external stimulus (GO:0009605)	207	78.31	+	2.64	1.23E-34

response to inorganic substance (GO:0010035)	170	55.72	+	3.05	2.21E-34
defense response (GO:0006952)	180	64.43	+	2.79	4.33E-32
response to organonitrogen compound (GO:0010243)	90	17.1	+	5.26	1.06E-31
cellular process (GO:0009987)	552	392.01	+	1.41	1.85E-30
response to nitrogen compound (GO:1901698)	95	20.85	+	4.56	3.93E-29
defense response to other organism (GO:0098542)	159	55.85	+	2.85	1.75E-28
response to fungus (GO:0009620)	103	26.26	+	3.92	6.38E-27
response to oxidative stress (GO:0006979)	81	16.18	+	5.01	7.34E-27
response to alcohol (GO:0097305)	111	31.51	+	3.52	1.42E-25
response to lipid (GO:0033993)	145	51.07	+	2.84	2.18E-25
response to bacterium (GO:0009617)	111	32.78	+	3.39	3.12E-24
response to endogenous stimulus (GO:0009719)	168	67.09	+	2.5	4.23E-24
response to hormone (GO:0009725)	166	66.2	+	2.51	5.57E-24
defense response to fungus (GO:0050832)	82	18.53	+	4.43	6.39E-24
response to abscisic acid (GO:0009737)	102	28.32	+	3.6	6.69E-24
response to osmotic stress (GO:0006970)	92	23.6	+	3.9	1.68E-23
defense response to bacterium (GO:0042742)	98	27.16	+	3.61	6.98E-23
response to temperature stimulus (GO:0009266)	89	22.86	+	3.89	1.37E-22
cellular response to organic substance (GO:0071310)	122	40.91	+	2.98	2.15E-22
response to organic cyclic compound (GO:0014070)	86	21.56	+	3.99	2.25E-22
regulation of response to stress (GO:0080134)	88	23.23	+	3.79	1.51E-21
regulation of defense response (GO:0031347)	79	19.64	+	4.02	1.59E-20
regulation of response to stimulus (GO:0048583)	108	35.26	+	3.06	3.12E-20

response to salt stress (GO:0009651)	70	16.42	+	4.26	3.68E-19
response to acid chemical (GO:0001101)	94	29.19	+	3.22	1.45E-18
response to salicylic acid (GO:0009751)	58	11.61	+	4.99	2.08E-18
response to water (GO:0009415)	90	28.32	+	3.18	2.73E-17
response to water deprivation (GO:0009414)	86	26.39	+	3.26	5.02E-17
cellular response to endogenous stimulus (GO:0071495)	94	31.51	+	2.98	1.87E-16
cellular response to hormone stimulus (GO:0032870)	92	30.56	+	3.01	2.79E-16
hormone-mediated signaling pathway (GO:0009755)	81	24.55	+	3.3	3.57E-16
cell communication (GO:0007154)	150	70.95	+	2.11	2.06E-14
signaling (GO:0023052)	141	65.3	+	2.16	6.21E-14
immune system process (GO:0002376)	52	12.04	+	4.32	8.25E-14
signal transduction (GO:0007165)	138	63.64	+	2.17	8.61E-14
cellular response to oxygen-containing compound (GO:1901701)	85	30.09	+	2.82	2.81E-13
indole-containing compound metabolic process (GO:0042430)	33	4.8	+	6.87	5.08E-13
response to heat (GO:0009408)	40	7.89	+	5.07	3.60E-12
biological regulation (GO:0065007)	319	217.04	+	1.47	1.01E-11
response to cold (GO:0009409)	48	12.48	+	3.84	8.31E-11
response to reactive oxygen species (GO:0000302)	28	4.36	+	6.43	4.28E-10
response to fatty acid (GO:0070542)	53	16.1	+	3.29	1.12E-09
regulation of cellular process (GO:0050794)	250	163.78	+	1.53	2.22E-09
regulation of biological process (GO:0050789)	289	198.43	+	1.46	2.57E-09
response to jasmonic acid (GO:0009753)	52	16	+	3.25	2.94E-09
secondary metabolic process (GO:0019748)	61	21.38	+	2.85	5.62E-09

response to hydrogen peroxide (GO:0042542)	19	2.01	+	9.47	1.09E-08
toxin metabolic process (GO:0009404)	22	3.11	+	7.06	3.50E-08
metabolic process (GO:0008152)	376	285.75	+	1.32	7.12E-08
sulfur compound metabolic process (GO:0006790)	44	14.49	+	3.04	1.29E-06
camalexin metabolic process (GO:0052317)	10	0.42	+	23.68	1.33E-06
camalexin biosynthetic process (GO:0010120)	10	0.42	+	23.68	1.33E-06
toxin biosynthetic process (GO:0009403)	10	0.53	+	18.94	6.86E-06
phytoalexin biosynthetic process (GO:0052315)	10	0.53	+	18.94	6.86E-06
phytoalexin metabolic process (GO:0052314)	10	0.53	+	18.94	6.86E-06
indole phytoalexin biosynthetic process (GO:0009700)	10	0.53	+	18.94	6.86E-06
indole phytoalexin metabolic process (GO:0046217)	10	0.53	+	18.94	6.86E-06
cellular metabolic process (GO:0044237)	311	233.04	+	1.33	7.10E-06
photosynthesis (GO:0015979)	25	5.54	+	4.51	7.42E-06
small molecule metabolic process (GO:0044281)	126	75.41	+	1.67	6.18E-05
organic acid metabolic process (GO:0006082)	99	55.11	+	1.8	9.61E-05
response to molecule of bacterial origin (GO:0002237)	17	3.09	+	5.5	2.05E-04
protein folding (GO:0006457)	22	5.31	+	4.15	2.60E-04
oxoacid metabolic process (GO:0043436)	93	51.55	+	1.8	2.66E-04
cellular response to organic cyclic compound (GO:0071407)	25	6.84	+	3.66	3.28E-04
indole-containing compound biosynthetic process (GO:0042435)	13	1.87	+	6.94	6.90E-04
response to carbohydrate (GO:0009743)	20	4.72	+	4.23	7.40E-04
secondary metabolite biosynthetic process (GO:0044550)	20	4.75	+	4.21	8.03E-04
indole-containing compound catabolic process (GO:0042436)	7	0.34	+	20.4	1.28E-03

protein complex oligomerization (GO:0051259)	11	1.45	+	7.58	2.74E-03
regulation of immune system process (GO:0002682)	19	4.72	+	4.02	2.99E-03
cellular response to salicylic acid stimulus (GO:0071446)	15	2.96	+	5.07	3.06E-03
defense response by callose deposition (GO:0052542)	8	0.66	+	12.12	4.42E-03
aromatic compound biosynthetic process (GO:0019438)	48	22.22	+	2.16	4.65E-03
indole glucosinolate metabolic process (GO:0042343)	7	0.5	+	13.96	9.49E-03
sulfur compound biosynthetic process (GO:0044272)	17	4.22	+	4.03	1.09E-02
generation of precursor metabolites and energy (GO:0006091)	29	10.74	+	2.7	1.13E-02
immune response (GO:0006955)	19	5.25	+	3.62	1.26E-02
cellular response to lipid (GO:0071396)	34	13.91	+	2.44	1.43E-02
cellular response to ethylene stimulus (GO:0071369)	13	2.53	+	5.13	1.43E-02
callose localization (GO:0052545)	9	1.13	+	7.93	1.96E-02
aromatic compound catabolic process (GO:0019439)	20	5.97	+	3.35	2.03E-02
aromatic amino acid family catabolic process (GO:0009074)	7	0.58	+	12.05	2.11E-02
regulation of cellular ketone metabolic process (GO:0010565)	14	3.09	+	4.53	2.28E-02
organic substance metabolic process (GO:0071704)	318	258.85	+	1.23	2.72E-02
organonitrogen compound metabolic process (GO:1901564)	193	143.11	+	1.35	2.77E-02
cellular macromolecule biosynthetic process (GO:0034645)	6	25	-	0.24	2.98E-02
polysaccharide localization (GO:0033037)	9	1.21	+	7.41	3.16E-02
organic cyclic compound metabolic process (GO:1901360)	123	82.56	+	1.49	3.62E-02
innate immune response (GO:0045087)	17	4.7	+	3.62	3.97E-02
defense response by callose deposition in cell wall (GO:0052544)	6	0.42	+	14.21	4.55E-02
organic cyclic compound catabolic process (GO:1901361)	20	6.33	+	3.16	4.58E-02

organic cyclic compound biosynthetic process (GO:1901362)	49	25	+	1.96	4.67E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
catalytic activity (GO:0003824)	317	220.34	+	1.44	1.90E-10
binding (GO:0005488)	372	272.44	+	1.37	2.25E-10
protein binding (GO:0005515)	224	151.11	+	1.48	6.85E-07
unfolded protein binding (GO:0051082)	18	3.01	+	5.98	1.57E-05
oxidoreductase activity (GO:0016491)	76	38.8	+	1.96	1.43E-04
tetrapyrrole binding (GO:0046906)	29	10.58	+	2.74	5.05E-03
ATP-dependent activity (GO:0140657)	42	19.22	+	2.19	1.31E-02
transferase activity (GO:0016740)	135	92.12	+	1.47	1.47E-02
chlorophyll binding (GO:0016168)	7	0.71	+	9.82	3.80E-02
Cellular Component					
cell periphery (GO:0071944)	152	94.52	+	1.61	4.12E-06
cytoplasm (GO:0005737)	470	391.56	+	1.2	4.46E-06
intracellular anatomical structure (GO:0005622)	630	569.43	+	1.11	8.28E-06
plant-type cell wall (GO:0009505)	37	13.59	+	2.72	9.80E-05
cell wall (GO:0005618)	37	14.12	+	2.62	2.36E-04
plasma membrane (GO:0005886)	127	81.22	+	1.56	5.05E-04
external encapsulating structure (GO:0030312)	37	14.75	+	2.51	7.38E-04
membrane (GO:0016020)	180	132.76	+	1.36	1.28E-02
plasmodesma (GO:0009506)	46	23.44	+	1.96	2.17E-02
cell-cell junction (GO:0005911)	46	23.44	+	1.96	2.17E-02
symplast (GO:0055044)	46	23.44	+	1.96	2.17E-02

anchoring junction (GO:0070161)	46	23.44	+	1.96	2.17E-02
cell junction (GO:0030054)	46	23.44	+	1.96	2.17E-02
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
cell periphery (GO:0071944)	152	94.52	+	1.61	4.12E-06
cytoplasm (GO:0005737)	470	391.56	+	1.2	4.46E-06
intracellular anatomical structure (GO:0005622)	630	569.43	+	1.11	8.28E-06
plant-type cell wall (GO:0009505)	37	13.59	+	2.72	9.80E-05
cell wall (GO:0005618)	37	14.12	+	2.62	2.36E-04
plasma membrane (GO:0005886)	127	81.22	+	1.56	5.05E-04
external encapsulating structure (GO:0030312)	37	14.75	+	2.51	7.38E-04
membrane (GO:0016020)	180	132.76	+	1.36	1.28E-02
plasmodesma (GO:0009506)	46	23.44	+	1.96	2.17E-02
cell-cell junction (GO:0005911)	46	23.44	+	1.96	2.17E-02
symplast (GO:0055044)	46	23.44	+	1.96	2.17E-02
anchoring junction (GO:0070161)	46	23.44	+	1.96	2.17E-02
cell junction (GO:0030054)	46	23.44	+	1.96	2.17E-02

Supplemental Table 2: Significant, up-regulated GO Terms for artificially acidified Arabidopsis.

Biological Process	Gene #	Expected	Over/Under	Fold Enrichment	P-Value
response to external biotic stimulus (GO:0043207)	166	30	+	5.53	6.14E-77
response to other organism (GO:0051707)	166	30	+	5.53	6.14E-77
response to biotic stimulus (GO:0009607)	166	30.02	+	5.53	6.89E-77
biological process involved in interspecies interaction between organisms (GO:0044419)	166	30.19	+	5.5	1.56E-76
defense response (GO:0006952)	161	29.63	+	5.43	7.44E-73
response to external stimulus (GO:0009605)	170	36.02	+	4.72	3.39E-69
response to stress (GO:0006950)	216	64.17	+	3.37	6.01E-69
defense response to other organism (GO:0098542)	148	25.69	+	5.76	1.05E-68
response to bacterium (GO:0009617)	112	15.08	+	7.43	1.71E-59
response to stimulus (GO:0050896)	263	112.95	+	2.33	2.22E-59
response to fungus (GO:0009620)	93	12.08	+	7.7	3.23E-49
response to oxygen-containing compound (GO:1901700)	149	37.48	+	3.98	5.27E-49
cellular response to chemical stimulus (GO:0070887)	124	24.85	+	4.99	9.78E-49
defense response to bacterium (GO:0042742)	93	12.49	+	7.44	4.74E-48
response to chitin (GO:0010200)	61	3.74	+	16.31	1.21E-47
response to organic substance (GO:0010033)	156	42.56	+	3.67	1.51E-47
response to chemical (GO:0042221)	184	61.34	+	3	1.22E-46
cellular response to stimulus (GO:0051716)	162	47.02	+	3.45	1.66E-46
defense response to fungus (GO:0050832)	79	8.52	+	9.27	2.40E-46
response to organonitrogen compound (GO:0010243)	76	7.87	+	9.66	1.48E-45
response to organic cyclic compound (GO:0014070)	79	9.92	+	7.97	7.27E-42
response to nitrogen compound (GO:1901698)	77	9.59	+	8.03	6.77E-41

regulation of response to stress (GO:0080134)	79	10.68	+	7.39	1.10E-39
regulation of defense response (GO:0031347)	74	9.03	+	8.19	1.17E-39
response to salicylic acid (GO:0009751)	59	5.34	+	11.05	2.57E-37
cellular response to organic substance (GO:0071310)	96	18.82	+	5.1	1.53E-36
signal transduction (GO:0007165)	117	29.27	+	4	3.55E-36
signaling (GO:0023052)	118	30.03	+	3.93	7.45E-36
regulation of response to stimulus (GO:0048583)	89	16.22	+	5.49	9.88E-36
response to wounding (GO:0009611)	72	9.89	+	7.28	2.72E-35
cell communication (GO:0007154)	118	32.63	+	3.62	1.72E-32
response to endogenous stimulus (GO:0009719)	112	30.86	+	3.63	1.50E-30
response to hormone (GO:0009725)	110	30.45	+	3.61	1.08E-29
response to lipid (GO:0033993)	96	23.49	+	4.09	4.68E-29
cellular response to oxygen-containing compound (GO:1901701)	75	13.84	+	5.42	6.30E-29
cellular response to endogenous stimulus (GO:0071495)	74	14.5	+	5.11	6.56E-27
response to abiotic stimulus (GO:0009628)	137	49.94	+	2.74	9.38E-27
cellular response to hormone stimulus (GO:0032870)	72	14.06	+	5.12	3.84E-26
hormone-mediated signaling pathway (GO:0009755)	64	11.29	+	5.67	4.41E-25
regulation of cellular process (GO:0050794)	166	75.33	+	2.2	2.25E-23
response to jasmonic acid (GO:0009753)	51	7.36	+	6.93	5.67E-23
response to fatty acid (GO:0070542)	51	7.41	+	6.89	7.52E-23
cellular process (GO:0009987)	273	180.3	+	1.51	9.81E-23
regulation of biological process (GO:0050789)	182	91.27	+	1.99	1.02E-21
cellular response to hypoxia (GO:0071456)	34	2.9	+	11.72	5.67E-21

cellular response to decreased oxygen levels (GO:0036294)	34	2.93	+	11.62	7.23E-21
cellular response to oxygen levels (GO:0071453)	34	2.94	+	11.57	8.16E-21
biological regulation (GO:0065007)	188	99.83	+	1.88	8.73E-20
response to hypoxia (GO:0001666)	36	3.92	+	9.18	4.31E-19
response to decreased oxygen levels (GO:0036293)	36	4.02	+	8.96	9.12E-19
response to oxygen levels (GO:0070482)	36	4.04	+	8.91	1.10E-18
response to alcohol (GO:0097305)	62	14.5	+	4.28	4.32E-18
regulation of immune system process (GO:0002682)	27	2.17	+	12.42	9.63E-17
response to acid chemical (GO:0001101)	57	13.43	+	4.25	3.27E-16
response to molecule of bacterial origin (GO:0002237)	23	1.42	+	16.19	3.46E-16
response to water deprivation (GO:0009414)	54	12.14	+	4.45	4.82E-16
response to inorganic substance (GO:0010035)	79	25.63	+	3.08	1.15E-15
response to abscisic acid (GO:0009737)	55	13.03	+	4.22	1.98E-15
response to water (GO:0009415)	55	13.03	+	4.22	1.98E-15
cellular response to organic cyclic compound (GO:0071407)	28	3.14	+	8.91	5.46E-14
cellular response to salicylic acid stimulus (GO:0071446)	20	1.36	+	14.71	4.53E-13
immune system process (GO:0002376)	34	5.54	+	6.14	6.48E-13
systemic acquired resistance (GO:0009627)	20	1.41	+	14.2	8.27E-13
response to osmotic stress (GO:0006970)	46	10.85	+	4.24	1.77E-12
cellular response to stress (GO:0033554)	52	14.75	+	3.53	2.43E-11
response to temperature stimulus (GO:0009266)	41	10.51	+	3.9	1.06E-09
phosphorylation (GO:0016310)	48	14.19	+	3.38	1.28E-09
protein phosphorylation (GO:0006468)	45	12.83	+	3.51	2.30E-09

salicylic acid mediated signaling pathway (GO:0009863)	14	0.97	+	14.42	2.18E-08
response to oxidative stress (GO:0006979)	32	7.44	+	4.3	4.58E-08
regulation of cellular ketone metabolic process (GO:0010565)	15	1.42	+	10.56	2.08E-07
indole-containing compound metabolic process (GO:0042430)	17	2.21	+	7.69	1.05E-06
regulation of small molecule metabolic process (GO:0062012)	15	2.04	+	7.35	2.04E-05
phosphate-containing compound metabolic process (GO:0006796)	53	22.81	+	2.32	4.52E-05
phosphorus metabolic process (GO:0006793)	53	23.36	+	2.27	1.11E-04
response to cold (GO:0009409)	23	5.74	+	4.01	1.28E-04
jasmonic acid mediated signaling pathway (GO:0009867)	13	1.76	+	7.39	2.00E-04
cellular response to lipid (GO:0071396)	24	6.4	+	3.75	2.09E-04
response to salt stress (GO:0009651)	26	7.55	+	3.44	2.97E-04
cellular response to jasmonic acid stimulus (GO:0071395)	13	1.85	+	7.05	3.34E-04
cellular response to fatty acid (GO:0071398)	13	1.89	+	6.86	4.42E-04
gene expression (GO:0010467)	2	20.81	-	0.1	7.59E-04
immune response (GO:0006955)	14	2.42	+	5.8	1.02E-03
innate immune response (GO:0045087)	13	2.16	+	6.02	1.81E-03
oxoacid metabolic process (GO:0043436)	50	23.71	+	2.11	2.92E-03
regulation of response to biotic stimulus (GO:0002831)	13	2.37	+	5.49	4.74E-03
regulation of cell death (GO:0010941)	10	1.36	+	7.35	6.92E-03
regulation of response to external stimulus (GO:0032101)	13	2.55	+	5.1	1.02E-02
nucleobase-containing compound metabolic process (GO:0006139)	7	27.61	-	0.25	1.34E-02
positive regulation of defense response (GO:0031349)	9	1.18	+	7.64	1.67E-02
organic acid metabolic process (GO:0006082)	50	25.35	+	1.97	1.86E-02

indole glucosinolate metabolic process (GO:0042343)	5	0.23	+	21.68	2.60E-02
response to oomycetes (GO:0002239)	9	1.27	+	7.06	3.03E-02
RNA metabolic process (GO:0016070)	2	16.86	-	0.12	3.44E-02
nucleic acid metabolic process (GO:0090304)	5	22.77	-	0.22	4.05E-02
regulation of cell communication (GO:0010646)	18	5.39	+	3.34	4.30E-02
protein modification process (GO:0036211)	58	32.28	+	1.8	4.78E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
ADP binding (GO:0043531)	22	2.08	+	10.6	3.77E-12
kinase activity (GO:0016301)	53	15.73	+	3.37	4.41E-11
protein kinase activity (GO:0004672)	44	12.64	+	3.48	3.15E-09
transferase activity, transferring phosphorus-containing groups (GO:0016772)	54	18.29	+	2.95	3.82E-09
protein serine/threonine kinase activity (GO:0004674)	36	9.26	+	3.89	2.09E-08
phosphotransferase activity, alcohol group as acceptor (GO:0016773)	45	14.56	+	3.09	7.83E-08
transferase activity (GO:0016740)	81	42.37	+	1.91	2.22E-05
adenyl ribonucleotide binding (GO:0032559)	28	8.12	+	3.45	5.37E-05
adenyl nucleotide binding (GO:0030554)	28	8.26	+	3.39	7.45E-05
catalytic activity, acting on a protein (GO:0140096)	63	31.61	+	1.99	2.98E-04
anion binding (GO:0043168)	39	15.53	+	2.51	5.36E-04
purine ribonucleotide binding (GO:0032555)	29	9.7	+	2.99	5.49E-04
purine nucleotide binding (GO:0017076)	29	9.86	+	2.94	7.55E-04
carbohydrate derivative binding (GO:0097367)	30	10.49	+	2.86	8.37E-04
ribonucleotide binding (GO:0032553)	29	10.05	+	2.89	1.11E-03
transmembrane signaling receptor activity (GO:0004888)	12	1.93	+	6.22	2.09E-03

ion binding (GO:0043167)	63	34.04	+	1.85	3.48E-03
signaling receptor activity (GO:0038023)	13	2.6	+	5	7.29E-03
transmembrane receptor protein kinase activity (GO:0019199)	10	1.52	+	6.59	1.01E-02
small molecule binding (GO:0036094)	37	16.45	+	2.25	1.10E-02
binding (GO:0005488)	164	125.31	+	1.31	3.36E-02
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
cell periphery (GO:0071944)	98	43.47	+	2.25	4.58E-12
plasma membrane (GO:0005886)	86	37.35	+	2.3	1.38E-10
membrane (GO:0016020)	109	61.06	+	1.79	2.69E-07

Supplemental Table 3: Significant, down-regulated GO Terms for severe-cold challenged Arabidopsis.

Biological Process	Gene #	Expected	Over/Under	Fold Enrichment	P-Value
response to stimulus (GO:0050896)	224	128.21	+	1.75	7.89E-20
response to chemical (GO:0042221)	137	69.63	+	1.97	1.85E-12
response to stress (GO:0006950)	138	72.84	+	1.89	3.23E-11
response to abiotic stimulus (GO:0009628)	113	56.69	+	1.99	9.74E-10
response to organic substance (GO:0010033)	97	48.31	+	2.01	6.95E-08
response to endogenous stimulus (GO:0009719)	78	35.03	+	2.23	9.49E-08
response to hormone (GO:0009725)	77	34.56	+	2.23	1.32E-07
cellular response to stimulus (GO:0051716)	98	53.37	+	1.84	6.05E-06
response to lipid (GO:0033993)	60	26.67	+	2.25	2.39E-05
biological regulation (GO:0065007)	167	113.32	+	1.47	2.82E-05
cellular response to chemical stimulus (GO:0070887)	61	28.21	+	2.16	6.37E-05
response to wounding (GO:0009611)	34	11.23	+	3.03	7.24E-05
response to oxygen-containing compound (GO:1901700)	79	42.54	+	1.86	2.44E-04
protein complex oligomerization (GO:0051259)	9	0.76	+	11.87	6.28E-04
cellular process (GO:0009987)	255	204.67	+	1.25	6.96E-04
cellular response to organic substance (GO:0071310)	48	21.36	+	2.25	8.60E-04
response to decreased oxygen levels (GO:0036293)	19	4.56	+	4.17	1.21E-03
cellular response to endogenous stimulus (GO:0071495)	40	16.45	+	2.43	1.28E-03
response to oxygen levels (GO:0070482)	19	4.59	+	4.14	1.32E-03
cellular response to hormone stimulus (GO:0032870)	39	15.96	+	2.44	1.63E-03
regulation of biological process (GO:0050789)	149	103.6	+	1.44	1.65E-03
response to radiation (GO:0009314)	56	28.18	+	1.99	3.54E-03

response to external stimulus (GO:0009605)	73	40.89	+	1.79	3.65E-03
response to hypoxia (GO:0001666)	18	4.45	+	4.04	3.66E-03
response to light stimulus (GO:0009416)	55	27.6	+	1.99	4.37E-03
response to alcohol (GO:0097305)	38	16.45	+	2.31	9.54E-03
response to abscisic acid (GO:0009737)	35	14.79	+	2.37	1.22E-02
negative regulation of response to stimulus (GO:0048585)	15	3.56	+	4.22	1.75E-02
response to osmotic stress (GO:0006970)	31	12.32	+	2.52	2.11E-02
regulation of phenylpropanoid metabolic process (GO:2000762)	6	0.4	+	15.01	2.16E-02
regulation of cellular process (GO:0050794)	124	85.51	+	1.45	2.36E-02
response to oxidative stress (GO:0006979)	24	8.45	+	2.84	2.49E-02
hormone-mediated signaling pathway (GO:0009755)	31	12.82	+	2.42	3.30E-02
response to external biotic stimulus (GO:0043207)	61	34.05	+	1.79	3.75E-02
response to other organism (GO:0051707)	61	34.05	+	1.79	3.75E-02
response to biotic stimulus (GO:0009607)	61	34.08	+	1.79	3.79E-02
biological process involved in interspecies interaction between organisms (GO:0044419)	61	34.27	+	1.78	4.09E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
hormone binding (GO:0042562)	8	0.45	+	17.59	1.25E-04
RNA binding (GO:0003723)	4	22.32	-	0.18	6.95E-03
DNA-binding transcription factor activity (GO:0003700)	47	22.96	+	2.05	7.93E-03
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
plasma membrane (GO:0005886)	89	42.4	+	2.1	1.73E-08
cell periphery (GO:0071944)	98	49.35	+	1.99	2.38E-08
membrane (GO:0016020)	109	69.32	+	1.57	6.25E-04

Supplemental Table 4: Significant, up-regulated GO Terms for severe-cold challenged Arabidopsis.

Biological Process	Gene #	Expected	Over/Under	Fold enrichment	P-value
response to stimulus (GO:0050896)	402	279.15	+	1.44	1.33E-14
response to stress (GO:0006950)	260	158.6	+	1.64	4.08E-13
regulation of biological process (GO:0050789)	335	225.57	+	1.49	2.06E-12
response to chemical (GO:0042221)	248	151.61	+	1.64	3.90E-12
response to wounding (GO:0009611)	73	24.45	+	2.99	5.30E-12
response to oxygen-containing compound (GO:1901700)	171	92.62	+	1.85	3.15E-11
biological regulation (GO:0065007)	353	246.72	+	1.43	4.05E-11
regulation of cellular process (GO:0050794)	283	186.17	+	1.52	1.43E-10
cellular process (GO:0009987)	549	445.61	+	1.23	1.74E-09
cellular response to chemical stimulus (GO:0070887)	121	61.42	+	1.97	1.15E-08
response to external biotic stimulus (GO:0043207)	138	74.14	+	1.86	1.34E-08
response to other organism (GO:0051707)	138	74.14	+	1.86	1.34E-08
response to biotic stimulus (GO:0009607)	138	74.2	+	1.86	1.38E-08
biological process involved in interspecies interaction between organisms (GO:0044419)	138	74.62	+	1.85	2.31E-08
response to external stimulus (GO:0009605)	157	89.02	+	1.76	2.40E-08
response to organic substance (GO:0010033)	176	105.19	+	1.67	5.15E-08
response to endogenous stimulus (GO:0009719)	136	76.27	+	1.78	4.47E-07
cellular response to stimulus (GO:0051716)	186	116.2	+	1.6	4.69E-07
cellular response to organic substance (GO:0071310)	95	46.51	+	2.04	5.71E-07
response to salt stress (GO:0009651)	52	18.66	+	2.79	7.03E-07
regulation of response to stimulus (GO:0048583)	85	40.08	+	2.12	1.12E-06
response to hormone (GO:0009725)	133	75.25	+	1.77	1.16E-06

defense response to other organism (GO:0098542)	117	63.49	+	1.84	1.43E-06
positive regulation of biological process (GO:0048518)	92	45.82	+	2.01	3.06E-06
regulation of metabolic process (GO:0019222)	183	117.64	+	1.56	5.65E-06
cellular response to endogenous stimulus (GO:0071495)	77	35.82	+	2.15	5.82E-06
response to osmotic stress (GO:0006970)	63	26.82	+	2.35	5.93E-06
regulation of biosynthetic process (GO:0009889)	134	78.1	+	1.72	7.04E-06
cellular response to oxygen-containing compound (GO:1901701)	74	34.2	+	2.16	7.60E-06
response to lipid (GO:0033993)	107	58.06	+	1.84	8.92E-06
response to alcohol (GO:0097305)	76	35.82	+	2.12	1.02E-05
defense response (GO:0006952)	127	73.24	+	1.73	1.10E-05
cellular response to hormone stimulus (GO:0032870)	74	34.74	+	2.13	1.71E-05
hormone-mediated signaling pathway (GO:0009755)	63	27.9	+	2.26	3.62E-05
cell communication (GO:0007154)	134	80.65	+	1.66	3.64E-05
response to abscisic acid (GO:0009737)	69	32.19	+	2.14	4.13E-05
regulation of cellular biosynthetic process (GO:0031326)	128	76.42	+	1.67	6.38E-05
response to abiotic stimulus (GO:0009628)	185	123.43	+	1.5	6.85E-05
positive regulation of metabolic process (GO:0009893)	64	29.16	+	2.19	7.55E-05
signal transduction (GO:0007165)	122	72.34	+	1.69	8.72E-05
signaling (GO:0023052)	124	74.23	+	1.67	1.22E-04
regulation of DNA-templated transcription (GO:0006355)	112	65.53	+	1.71	1.88E-04
regulation of nucleic acid-templated transcription (GO:1903506)	112	65.56	+	1.71	1.91E-04
regulation of macromolecule biosynthetic process (GO:0010556)	119	70.99	+	1.68	1.92E-04
regulation of RNA biosynthetic process (GO:2001141)	112	65.62	+	1.71	1.95E-04

response to bacterium (GO:0009617)	73	37.26	+	1.96	4.19E-04
negative regulation of biological process (GO:0048519)	98	55.87	+	1.75	4.40E-04
developmental process (GO:0032502)	238	173.75	+	1.37	6.15E-04
response to organonitrogen compound (GO:0010243)	46	19.44	+	2.37	8.02E-04
positive regulation of cellular process (GO:0048522)	71	36.84	+	1.93	1.34E-03
response to acid chemical (GO:0001101)	66	33.18	+	1.99	1.39E-03
regulation of cellular metabolic process (GO:0031323)	145	95.14	+	1.52	1.53E-03
multicellular organism development (GO:0007275)	187	130.82	+	1.43	1.54E-03
regulation of nitrogen compound metabolic process (GO:0051171)	134	86.08	+	1.56	1.57E-03
response to water (GO:0009415)	64	32.19	+	1.99	1.63E-03
response to organic cyclic compound (GO:0014070)	53	24.51	+	2.16	1.64E-03
regulation of macromolecule metabolic process (GO:0060255)	147	97.36	+	1.51	2.01E-03
response to jasmonic acid (GO:0009753)	43	18.18	+	2.36	2.08E-03
regulation of RNA metabolic process (GO:0051252)	113	69.43	+	1.63	2.16E-03
defense response to bacterium (GO:0042742)	62	30.87	+	2.01	2.35E-03
response to fatty acid (GO:0070542)	43	18.3	+	2.35	2.38E-03
metabolic process (GO:0008152)	394	324.82	+	1.21	4.49E-03
regulation of gene expression (GO:0010468)	129	83.86	+	1.54	5.15E-03
protein ubiquitination (GO:0016567)	47	21.54	+	2.18	5.47E-03
response to nitrogen compound (GO:1901698)	50	23.7	+	2.11	6.08E-03
response to fungus (GO:0009620)	59	29.85	+	1.98	6.60E-03
response to chitin (GO:0010200)	27	9.24	+	2.92	6.77E-03
response to water deprivation (GO:0009414)	59	30	+	1.97	7.11E-03

multicellular organismal process (GO:0032501)	194	140.24	+	1.38	8.00E-03
anatomical structure development (GO:0048856)	215	159.32	+	1.35	1.01E-02
regulation of nucleobase-containing compound metabolic process (GO:0019219)	115	73.48	+	1.57	1.03E-02
regulation of primary metabolic process (GO:0080090)	132	87.49	+	1.51	1.05E-02
regulation of response to stress (GO:0080134)	53	26.4	+	2.01	1.23E-02
protein modification process (GO:0036211)	122	79.78	+	1.53	1.33E-02
organic substance metabolic process (GO:0071704)	359	294.25	+	1.22	1.44E-02
protein modification by small protein conjugation (GO:0032446)	47	22.59	+	2.08	1.75E-02
programmed cell death (GO:0012501)	13	2.67	+	4.87	2.60E-02
positive regulation of macromolecule metabolic process (GO:0010604)	50	25.08	+	1.99	2.94E-02
positive regulation of biosynthetic process (GO:0009891)	41	18.75	+	2.19	3.17E-02
secondary metabolic process (GO:0019748)	49	24.3	+	2.02	3.32E-02
programmed cell death induced by symbiont (GO:0034050)	10	1.56	+	6.41	3.48E-02
response to inorganic substance (GO:0010035)	100	63.34	+	1.58	3.73E-02
regulation of defense response (GO:0031347)	46	22.32	+	2.06	3.82E-02
biological process involved in interaction with symbiont (GO:0051702)	10	1.59	+	6.29	4.03E-02
negative regulation of cellular process (GO:0048523)	62	33.96	+	1.83	4.03E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold enrichment**	**P-value**
protein binding (GO:0005515)	262	171.77	+	1.53	8.53E-10
binding (GO:0005488)	401	309.7	+	1.29	3.47E-07
transferase activity (GO:0016740)	160	104.71	+	1.53	1.54E-04
DNA binding (GO:0003677)	96	55.6	+	1.73	7.34E-04
DNA-binding transcription factor activity (GO:0003700)	82	49.99	+	1.64	4.46E-02

Cellular Component	Gene #	Expected	Over/Under	Fold enrichment	P-value
intracellular anatomical structure (GO:0005622)	709	647.3	+	1.1	4.75E-05
nucleus (GO:0005634)	380	318.01	+	1.19	1.05E-02
cellular anatomical entity (GO:0110165)	772	735.81	+	1.05	1.23E-02

Supplemental Table 5: Significant, down-regulated GO Terms for artificially acidified sorghum

Biological Process	Gene #	Expected	Over/Under	Fold enrichment	P-value
photosynthesis (GO:0015979)	74	8.46	+	8.75	1.28E-36
cellular metabolic process (GO:0044237)	570	355.67	+	1.6	2.50E-35
response to abiotic stimulus (GO:0009628)	336	165.73	+	2.03	3.86E-33
photosynthesis, light reaction (GO:0019684)	60	5.72	+	10.49	9.26E-33
response to light stimulus (GO:0009416)	208	80.69	+	2.58	1.48E-30
response to radiation (GO:0009314)	211	82.38	+	2.56	1.50E-30
metabolic process (GO:0008152)	639	436.12	+	1.47	6.16E-30
cellular process (GO:0009987)	794	598.3	+	1.33	8.43E-29
generation of precursor metabolites and energy (GO:0006091)	82	16.4	+	5	5.00E-26
cellular biosynthetic process (GO:0044249)	246	115.7	+	2.13	6.39E-25
biosynthetic process (GO:0009058)	270	134.03	+	2.01	1.63E-24
organic substance biosynthetic process (GO:1901576)	257	125.65	+	2.05	6.37E-24
tetrapyrrole metabolic process (GO:0033013)	63	10.68	+	5.9	9.42E-23
porphyrin-containing compound metabolic process (GO:0006778)	58	8.9	+	6.51	1.30E-22
response to stimulus (GO:0050896)	538	374.81	+	1.44	1.49E-19
organonitrogen compound biosynthetic process (GO:1901566)	143	57.65	+	2.48	3.27E-18
chlorophyll metabolic process (GO:0015994)	41	5.12	+	8.01	4.25E-18
cellular nitrogen compound biosynthetic process (GO:0044271)	131	52.73	+	2.48	2.44E-16
organic substance metabolic process (GO:0071704)	545	395.07	+	1.38	5.76E-16
plastid organization (GO:0009657)	60	13.78	+	4.36	8.31E-16
small molecule metabolic process (GO:0044281)	217	115.09	+	1.89	2.18E-15
tetrapyrrole biosynthetic process (GO:0033014)	31	3.63	+	8.55	8.01E-14

porphyrin-containing compound biosynthetic process (GO:0006779)	29	3.18	+	9.11	2.14E-13
photosynthetic electron transport chain (GO:0009767)	25	2.14	+	11.71	2.82E-13
response to temperature stimulus (GO:0009266)	94	34.89	+	2.69	5.13E-13
chlorophyll biosynthetic process (GO:0015995)	26	2.5	+	10.41	6.53E-13
protein-containing complex assembly (GO:0065003)	79	26.83	+	2.94	1.75E-12
pigment metabolic process (GO:0042440)	44	9.55	+	4.61	8.82E-12
protein-containing complex organization (GO:0043933)	81	29.81	+	2.72	5.80E-11
cellular component assembly (GO:0022607)	95	39.52	+	2.4	2.80E-10
photosynthesis, light harvesting (GO:0009765)	20	1.73	+	11.55	4.12E-10
organic cyclic compound biosynthetic process (GO:1901362)	91	38.15	+	2.39	1.37E-09
amide biosynthetic process (GO:0043604)	68	24.37	+	2.79	1.99E-09
cellular amide metabolic process (GO:0043603)	77	29.53	+	2.61	2.17E-09
organic cyclic compound metabolic process (GO:1901360)	210	126.01	+	1.67	2.27E-09
peptide metabolic process (GO:0006518)	69	25.06	+	2.75	2.34E-09
organonitrogen compound metabolic process (GO:1901564)	320	218.42	+	1.47	2.59E-09
pigment biosynthetic process (GO:0046148)	33	6.53	+	5.06	2.86E-09
electron transport chain (GO:0022900)	29	5.04	+	5.76	4.31E-09
translation (GO:0006412)	62	21.67	+	2.86	8.12E-09
peptide biosynthetic process (GO:0043043)	62	21.87	+	2.83	1.16E-08
chloroplast organization (GO:0009658)	41	10.59	+	3.87	1.20E-08
carboxylic acid metabolic process (GO:0019752)	122	61.92	+	1.97	2.35E-08
cellular aromatic compound metabolic process (GO:0006725)	200	121.5	+	1.65	2.40E-08
organic acid metabolic process (GO:0006082)	152	84.11	+	1.81	3.13E-08

cellular nitrogen compound metabolic process (GO:0034641)	210	130.52	+	1.61	5.21E-08
aromatic compound biosynthetic process (GO:0019438)	80	33.92	+	2.36	7.42E-08
oxoacid metabolic process (GO:0043436)	143	78.68	+	1.82	8.54E-08
small molecule biosynthetic process (GO:0044283)	83	36.18	+	2.29	9.63E-08
cellular component biogenesis (GO:0044085)	137	75.29	+	1.82	2.61E-07
response to stress (GO:0006950)	304	212.94	+	1.43	2.74E-07
heterocycle biosynthetic process (GO:0018130)	73	30.98	+	2.36	4.56E-07
heterocycle metabolic process (GO:0046483)	188	116.14	+	1.62	5.37E-07
photosynthesis, light harvesting in photosystem I (GO:0009768)	13	0.97	+	13.45	1.88E-06
cellular macromolecule biosynthetic process (GO:0034645)	81	38.15	+	2.12	4.89E-06
protein folding (GO:0006457)	31	8.1	+	3.83	6.26E-06
cellular component organization (GO:0016043)	180	114.69	+	1.57	1.38E-05
response to light intensity (GO:0009642)	43	15.03	+	2.86	1.84E-05
photosynthetic electron transport in photosystem I (GO:0009773)	11	0.77	+	14.37	2.37E-05
monocarboxylic acid metabolic process (GO:0032787)	61	26.39	+	2.31	3.18E-05
response to cold (GO:0009409)	49	19.05	+	2.57	4.16E-05
nitrogen compound metabolic process (GO:0006807)	373	286.02	+	1.3	4.40E-05
cellular component organization or biogenesis (GO:0071840)	214	145.75	+	1.47	6.39E-05
cellular lipid metabolic process (GO:0044255)	79	39.28	+	2.01	6.54E-05
photosystem II assembly (GO:0010207)	12	1.17	+	10.27	9.15E-05
response to oxidative stress (GO:0006979)	57	24.69	+	2.31	1.14E-04
lipid metabolic process (GO:0006629)	95	51.85	+	1.83	1.87E-04
thylakoid membrane organization (GO:0010027)	14	2.09	+	6.68	5.67E-04

NAD(P)H dehydrogenase complex assembly (GO:0010275)	8	0.44	+	18.05	7.79E-04
chloroplast rRNA processing (GO:1901259)	10	0.93	+	10.79	9.14E-04
plastid membrane organization (GO:0009668)	14	2.42	+	5.79	2.50E-03
organic acid biosynthetic process (GO:0016053)	58	28.36	+	2.05	4.03E-03
energy quenching (GO:1990066)	8	0.6	+	13.24	4.40E-03
nonphotochemical quenching (GO:0010196)	8	0.6	+	13.24	4.40E-03
response to heat (GO:0009408)	32	12.05	+	2.66	6.80E-03
lipid biosynthetic process (GO:0008610)	61	30.98	+	1.97	7.09E-03
plastid translation (GO:0032544)	9	0.93	+	9.71	7.27E-03
chaperone-mediated protein folding (GO:0061077)	14	2.78	+	5.04	1.05E-02
response to reactive oxygen species (GO:0000302)	22	6.65	+	3.31	1.16E-02
carboxylic acid biosynthetic process (GO:0046394)	50	24.01	+	2.08	1.22E-02
gene expression (GO:0010467)	110	69.05	+	1.59	1.25E-02
serine family amino acid metabolic process (GO:0009069)	13	2.46	+	5.29	1.46E-02
cellular amino acid metabolic process (GO:0006520)	48	22.8	+	2.11	1.87E-02
fat-soluble vitamin metabolic process (GO:0006775)	8	0.81	+	9.93	2.35E-02
fat-soluble vitamin biosynthetic process (GO:0042362)	8	0.81	+	9.93	2.35E-02
macromolecule biosynthetic process (GO:0009059)	86	51.77	+	1.66	3.29E-02
cell cycle (GO:0007049)	7	27.03	-	0.26	3.46E-02
cellular response to environmental stimulus (GO:0104004)	27	10.19	+	2.65	4.31E-02
cellular response to abiotic stimulus (GO:0071214)	27	10.19	+	2.65	4.31E-02
regulation of photosynthesis (GO:0010109)	11	1.93	+	5.69	4.34E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold enrichment**	**P-value**

oxidoreductase activity (GO:0016491)	138	59.22	+	2.33	2.37E-15
mRNA binding (GO:0003729)	110	42.62	+	2.58	1.04E-14
binding (GO:0005488)	552	415.82	+	1.33	6.26E-13
RNA binding (GO:0003723)	133	65.26	+	2.04	1.47E-10
catalytic activity (GO:0003824)	456	336.29	+	1.36	1.81E-10
structural constituent of ribosome (GO:0003735)	44	13.29	+	3.31	1.19E-07
structural molecule activity (GO:0005198)	50	19.42	+	2.58	1.66E-05
heterocyclic compound binding (GO:1901363)	281	205.85	+	1.37	8.38E-05
organic cyclic compound binding (GO:0097159)	282	207.46	+	1.36	1.14E-04
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (GO:0016616)	25	7.25	+	3.45	7.72E-04
unfolded protein binding (GO:0051082)	19	4.59	+	4.14	1.68E-03
chlorophyll binding (GO:0016168)	10	1.09	+	9.19	1.75E-03
oxidoreductase activity, acting on CH-OH group of donors (GO:0016614)	26	8.62	+	3.02	4.45E-03
protein folding chaperone (GO:0044183)	14	2.74	+	5.11	5.31E-03
tetrapyrrole binding (GO:0046906)	38	16.15	+	2.35	7.70E-03
isomerase activity (GO:0016853)	28	10.11	+	2.77	8.29E-03
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold enrichment**	**P-value**
chloroplast (GO:0009507)	572	204.16	+	2.8	4.09E-127
plastid (GO:0009536)	593	220.92	+	2.68	9.80E-126
plastid envelope (GO:0009526)	243	34.68	+	7.01	1.31E-113
thylakoid (GO:0009579)	194	22.04	+	8.8	1.29E-103
envelope (GO:0031975)	260	51.68	+	5.03	8.26E-94
organelle envelope (GO:0031967)	260	51.68	+	5.03	8.26E-94

plastid stroma (GO:0009532)	206	30.25	+	6.81	5.99E-93
plastid thylakoid (GO:0031976)	168	18.17	+	9.25	1.42E-91
chloroplast thylakoid (GO:0009534)	167	17.93	+	9.32	2.11E-91
chloroplast stroma (GO:0009570)	202	29.61	+	6.82	4.01E-91
photosynthetic membrane (GO:0034357)	146	15.55	+	9.39	1.21E-79
thylakoid membrane (GO:0042651)	145	15.35	+	9.45	2.33E-79
plastid membrane (GO:0042170)	157	19.5	+	8.05	4.59E-78
plastid thylakoid membrane (GO:0055035)	137	14.22	+	9.63	1.27E-75
chloroplast thylakoid membrane (GO:0009535)	134	13.9	+	9.64	6.55E-74
chloroplast envelope (GO:0009941)	161	24.49	+	6.57	2.06E-69
cytoplasm (GO:0005737)	881	597.62	+	1.47	1.61E-65
organelle subcompartment (GO:0031984)	196	44.88	+	4.37	9.46E-60
organelle membrane (GO:0031090)	201	62.52	+	3.21	1.47E-42
membrane (GO:0016020)	361	202.63	+	1.78	6.49E-26
cytosol (GO:0005829)	231	106.67	+	2.17	9.21E-25
intracellular anatomical structure (GO:0005622)	993	869.09	+	1.14	2.71E-19
nucleus (GO:0005634)	284	426.97	-	0.67	4.05E-16
intracellular membrane-bounded organelle (GO:0043231)	914	791.31	+	1.16	2.54E-14
membrane-bounded organelle (GO:0043227)	914	793.6	+	1.15	9.30E-14
thylakoid lumen (GO:0031977)	27	2.74	+	9.86	1.16E-13
intracellular organelle (GO:0043229)	918	800.29	+	1.15	2.52E-13
organelle (GO:0043226)	918	802.26	+	1.14	6.96E-13
chloroplast thylakoid lumen (GO:0009543)	23	1.97	+	11.65	1.17E-12

plastid thylakoid lumen (GO:0031978)	23	2.01	+	11.42	1.64E-12
photosystem (GO:0009521)	22	2.58	+	8.53	7.71E-10
cellular anatomical entity (GO:0110165)	1054	987.93	+	1.07	1.70E-09
plastoglobule (GO:0010287)	19	2.42	+	7.86	8.53E-08
chloroplast thylakoid membrane protein complex (GO:0098807)	13	1.05	+	12.41	8.96E-07
plastid inner membrane (GO:0009528)	18	2.7	+	6.67	2.38E-06
photosystem II (GO:0009523)	14	1.81	+	7.72	2.81E-05
chloroplast inner membrane (GO:0009706)	16	2.54	+	6.3	3.42E-05
NAD(P)H dehydrogenase complex (plastoquinone) (GO:0010598)	9	0.52	+	17.19	4.10E-05
apoplast (GO:0048046)	36	12.29	+	2.93	4.13E-05
chloroplast membrane (GO:0031969)	21	5.32	+	3.95	3.46E-04
nucleoid (GO:0009295)	14	2.54	+	5.52	9.29E-04
photosystem I (GO:0009522)	9	0.89	+	10.16	1.21E-03
ribosome (GO:0005840)	39	15.99	+	2.44	1.26E-03
plastid nucleoid (GO:0042646)	13	2.26	+	5.76	1.45E-03
organelle inner membrane (GO:0019866)	34	13.05	+	2.6	1.56E-03
nuclear protein-containing complex (GO:0140513)	12	36.86	-	0.33	2.49E-03
membrane protein complex (GO:0098796)	50	24.49	+	2.04	5.38E-03
chloroplast nucleoid (GO:0042644)	11	1.97	+	5.57	1.15E-02
organellar ribosome (GO:0000313)	13	3.18	+	4.08	3.68E-02

Supplemental Table 6: Significant, up-regulated GO Terms for artificially acidified sorghum

Biological Process	Gene #	Expected	Over/Under	Fold Enrichment	P-Value
cellular process (GO:0009987)	387	261.52	+	1.48	1.09E-28
cellular metabolic process (GO:0044237)	273	155.47	+	1.76	5.07E-24
metabolic process (GO:0008152)	303	190.63	+	1.59	5.61E-21
organic substance metabolic process (GO:0071704)	280	172.69	+	1.62	2.92E-19
primary metabolic process (GO:0044238)	241	144.23	+	1.67	2.95E-16
cellular biosynthetic process (GO:0044249)	116	50.57	+	2.29	1.20E-13
biosynthetic process (GO:0009058)	125	58.58	+	2.13	1.18E-12
organic substance biosynthetic process (GO:1901576)	119	54.92	+	2.17	3.46E-12
nitrogen compound metabolic process (GO:0006807)	205	125.02	+	1.64	1.64E-11
organonitrogen compound metabolic process (GO:1901564)	165	95.47	+	1.73	7.41E-10
organic cyclic compound metabolic process (GO:1901360)	109	55.08	+	1.98	2.10E-08
cellular nitrogen compound metabolic process (GO:0034641)	111	57.05	+	1.95	2.96E-08
response to abiotic stimulus (GO:0009628)	131	72.44	+	1.81	3.27E-08
post-embryonic development (GO:0009791)	84	40.16	+	2.09	8.53E-07
small molecule biosynthetic process (GO:0044283)	46	15.81	+	2.91	1.03E-06
cellular aromatic compound metabolic process (GO:0006725)	101	53.11	+	1.9	1.56E-06
heterocycle metabolic process (GO:0046483)	97	50.77	+	1.91	2.97E-06
response to stimulus (GO:0050896)	229	163.83	+	1.4	4.72E-06
macromolecule metabolic process (GO:0043170)	164	105.44	+	1.56	5.01E-06
multicellular organism development (GO:0007275)	129	76.77	+	1.68	6.33E-06
reproductive structure development (GO:0048608)	74	35.16	+	2.1	7.29E-06
reproductive system development (GO:0061458)	74	35.2	+	2.1	7.54E-06

cellular amide metabolic process (GO:0043603)	39	12.91	+	3.02	9.09E-06
developmental process involved in reproduction (GO:0003006)	80	39.81	+	2.01	1.19E-05
fruit development (GO:0010154)	56	23.56	+	2.38	1.54E-05
response to stress (GO:0006950)	146	93.08	+	1.57	3.26E-05
cellular nitrogen compound biosynthetic process (GO:0044271)	54	23.05	+	2.34	7.05E-05
multicellular organismal process (GO:0032501)	132	82.3	+	1.6	7.42E-05
reproduction (GO:0000003)	86	46.61	+	1.85	1.37E-04
generation of precursor metabolites and energy (GO:0006091)	26	7.17	+	3.63	1.51E-04
small molecule metabolic process (GO:0044281)	91	50.31	+	1.81	1.60E-04
reproductive process (GO:0022414)	85	46.15	+	1.84	1.85E-04
seed development (GO:0048316)	52	22.66	+	2.29	1.98E-04
phosphorus metabolic process (GO:0006793)	68	33.88	+	2.01	2.66E-04
organonitrogen compound biosynthetic process (GO:1901566)	55	25.2	+	2.18	3.39E-04
plastid organization (GO:0009657)	23	6.02	+	3.82	3.81E-04
phosphate-containing compound metabolic process (GO:0006796)	66	33.09	+	1.99	5.31E-04
carboxylic acid metabolic process (GO:0019752)	57	27.06	+	2.11	8.04E-04
protein metabolic process (GO:0019538)	109	66.91	+	1.63	8.87E-04
embryo development (GO:0009790)	36	13.68	+	2.63	9.79E-04
tRNA aminoacylation for protein translation (GO:0006418)	10	1.06	+	9.47	1.05E-03
lipid metabolic process (GO:0006629)	50	22.66	+	2.21	1.26E-03
tRNA aminoacylation (GO:0043039)	10	1.09	+	9.16	1.38E-03
amino acid activation (GO:0043038)	10	1.09	+	9.16	1.38E-03
peptide metabolic process (GO:0006518)	31	10.95	+	2.83	1.53E-03

embryo development ending in seed dormancy (GO:0009793)	33	12.2	+	2.7	1.73E-03
response to chemical (GO:0042221)	134	88.98	+	1.51	1.94E-03
oxylipin biosynthetic process (GO:0031408)	7	0.4	+	17.28	1.96E-03
organic cyclic compound biosynthetic process (GO:1901362)	40	16.68	+	2.4	2.16E-03
amide biosynthetic process (GO:0043604)	30	10.65	+	2.82	2.59E-03
oxylipin metabolic process (GO:0031407)	7	0.44	+	15.9	3.15E-03
nucleobase-containing compound metabolic process (GO:0006139)	73	40.04	+	1.82	3.67E-03
oxoacid metabolic process (GO:0043436)	65	34.39	+	1.89	3.84E-03
jasmonic acid biosynthetic process (GO:0009695)	7	0.46	+	15.29	3.94E-03
anatomical structure development (GO:0048856)	138	93.5	+	1.48	4.14E-03
monocarboxylic acid metabolic process (GO:0032787)	31	11.53	+	2.69	4.32E-03
gene expression (GO:0010467)	59	30.18	+	1.95	5.36E-03
developmental process (GO:0032502)	147	101.97	+	1.44	6.67E-03
photosynthesis (GO:0015979)	16	3.7	+	4.33	7.14E-03
tRNA metabolic process (GO:0006399)	15	3.35	+	4.48	9.82E-03
response to heat (GO:0009408)	19	5.26	+	3.61	1.04E-02
response to osmotic stress (GO:0006970)	37	15.74	+	2.35	1.23E-02
carboxylic acid biosynthetic process (GO:0046394)	28	10.49	+	2.67	1.60E-02
monocarboxylic acid biosynthetic process (GO:0072330)	17	4.44	+	3.83	1.61E-02
response to temperature stimulus (GO:0009266)	36	15.25	+	2.36	1.67E-02
system development (GO:0048731)	101	64.45	+	1.57	1.77E-02
organic acid metabolic process (GO:0006082)	66	36.77	+	1.8	1.87E-02
jasmonic acid metabolic process (GO:0009694)	8	0.88	+	9.09	2.08E-02

translation (GO:0006412)	26	9.47	+	2.74	2.16E-02
organic acid biosynthetic process (GO:0016053)	31	12.4	+	2.5	2.45E-02
peptide biosynthetic process (GO:0043043)	26	9.56	+	2.72	2.52E-02
response to salt stress (GO:0009651)	28	10.95	+	2.56	3.40E-02
cellular component organization (GO:0016043)	82	50.13	+	1.64	3.70E-02
lipid biosynthetic process (GO:0008610)	32	13.54	+	2.36	3.92E-02
response to oxygen-containing compound (GO:1901700)	87	54.36	+	1.6	3.95E-02
RNA metabolic process (GO:0016070)	48	24.46	+	1.96	4.67E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
catalytic activity (GO:0003824)	271	147	+	1.84	1.70E-27
binding (GO:0005488)	296	181.75	+	1.63	4.26E-22
mRNA binding (GO:0003729)	59	18.63	+	3.17	5.00E-11
protein binding (GO:0005515)	172	100.81	+	1.71	2.52E-10
RNA binding (GO:0003723)	74	28.53	+	2.59	4.34E-10
ATP-dependent activity (GO:0140657)	43	12.82	+	3.35	3.57E-08
organic cyclic compound binding (GO:0097159)	153	90.68	+	1.69	4.07E-08
heterocyclic compound binding (GO:1901363)	151	89.98	+	1.68	8.09E-08
ribonucleoside triphosphate phosphatase activity (GO:0017111)	33	8.36	+	3.95	1.80E-07
pyrophosphatase activity (GO:0016462)	35	9.47	+	3.69	2.58E-07
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides (GO:0016818)	35	9.63	+	3.63	3.92E-07
hydrolase activity, acting on acid anhydrides (GO:0016817)	35	9.7	+	3.61	4.70E-07
hydrolase activity (GO:0016787)	90	47.44	+	1.9	1.02E-05
small molecule binding (GO:0036094)	56	23.86	+	2.35	1.97E-05

ligase activity (GO:0016874)	19	4	+	4.75	1.23E-04
translation factor activity, RNA binding (GO:0008135)	13	1.83	+	7.1	2.30E-04
ATP hydrolysis activity (GO:0016887)	20	4.67	+	4.29	2.68E-04
translation regulator activity, nucleic acid binding (GO:0090079)	13	1.87	+	6.96	2.81E-04
ligase activity, forming carbon-oxygen bonds (GO:0016875)	10	1.06	+	9.47	6.17E-04
aminoacyl-tRNA ligase activity (GO:0004812)	10	1.06	+	9.47	6.17E-04
transferase activity (GO:0016740)	101	61.45	+	1.64	1.17E-03
catalytic activity, acting on a tRNA (GO:0140101)	14	2.52	+	5.56	1.18E-03
ion binding (GO:0043167)	85	49.37	+	1.72	2.00E-03
oxidoreductase activity (GO:0016491)	53	25.88	+	2.05	2.50E-03
catalytic activity, acting on RNA (GO:0140098)	27	9.26	+	2.92	2.91E-03
translation elongation factor activity (GO:0003746)	6	0.3	+	20.04	3.67E-03
translation regulator activity (GO:0045182)	13	2.39	+	5.43	3.76E-03
anion binding (GO:0043168)	48	22.52	+	2.13	3.94E-03
metallopeptidase activity (GO:0008237)	11	1.69	+	6.51	4.64E-03
protein folding chaperone (GO:0044183)	9	1.2	+	7.52	1.32E-02
nucleotide binding (GO:0000166)	42	19.86	+	2.11	1.65E-02
nucleoside phosphate binding (GO:1901265)	42	19.86	+	2.11	1.65E-02
ATP-dependent protein folding chaperone (GO:0140662)	8	0.95	+	8.41	2.03E-02
transferase activity, transferring phosphorus-containing groups (GO:0016772)	51	26.54	+	1.92	2.37E-02
catalytic activity, acting on a protein (GO:0140096)	76	45.85	+	1.66	2.90E-02
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
plastid (GO:0009536)	212	96.56	+	2.2	4.82E-29

chloroplast stroma (GO:0009570)	73	12.94	+	5.64	1.50E-28
plastid stroma (GO:0009532)	73	13.22	+	5.52	5.28E-28
chloroplast (GO:0009507)	198	89.24	+	2.22	5.05E-27
cytoplasm (GO:0005737)	370	261.22	+	1.42	2.77E-21
cytosol (GO:0005829)	114	46.63	+	2.44	5.60E-16
plastid envelope (GO:0009526)	57	15.16	+	3.76	5.09E-14
chloroplast envelope (GO:0009941)	45	10.71	+	4.2	2.78E-12
intracellular anatomical structure (GO:0005622)	442	379.88	+	1.16	3.90E-11
membrane (GO:0016020)	157	88.57	+	1.77	1.25E-10
envelope (GO:0031975)	64	22.59	+	2.83	1.74E-10
organelle envelope (GO:0031967)	64	22.59	+	2.83	1.74E-10
intracellular membrane-bounded organelle (GO:0043231)	414	345.88	+	1.2	3.58E-10
membrane-bounded organelle (GO:0043227)	414	346.89	+	1.19	6.86E-10
thylakoid (GO:0009579)	38	9.63	+	3.95	2.51E-09
intracellular organelle (GO:0043229)	414	349.81	+	1.18	4.70E-09
organelle (GO:0043226)	414	350.67	+	1.18	8.85E-09
cellular anatomical entity (GO:0110165)	470	431.83	+	1.09	6.43E-08
plasma membrane (GO:0005886)	101	54.18	+	1.86	8.35E-07
chloroplast thylakoid (GO:0009534)	30	7.84	+	3.83	9.68E-07
plastid thylakoid (GO:0031976)	30	7.94	+	3.78	1.30E-06
plasmodesma (GO:0009506)	43	15.64	+	2.75	4.43E-06
cell-cell junction (GO:0005911)	43	15.64	+	2.75	4.43E-06
symplast (GO:0055044)	43	15.64	+	2.75	4.43E-06

anchoring junction (GO:0070161)	43	15.64	+	2.75	4.43E-06
cell junction (GO:0030054)	43	15.64	+	2.75	4.43E-06
cell periphery (GO:0071944)	110	63.06	+	1.74	5.90E-06
apoplast (GO:0048046)	23	5.37	+	4.28	1.22E-05
organelle subcompartment (GO:0031984)	46	19.62	+	2.35	1.38E-04
vacuole (GO:0005773)	44	18.52	+	2.38	2.12E-04
plastid membrane (GO:0042170)	27	8.52	+	3.17	2.44E-04
thylakoid membrane (GO:0042651)	22	6.71	+	3.28	1.76E-03
photosynthetic membrane (GO:0034357)	22	6.8	+	3.24	2.14E-03
protein-containing complex (GO:0032991)	88	54.48	+	1.62	6.21E-03
plant-type vacuole (GO:0000325)	33	14.03	+	2.35	6.99E-03
cytoplasmic vesicle (GO:0031410)	31	12.94	+	2.4	9.77E-03
intracellular vesicle (GO:0097708)	31	12.98	+	2.39	1.02E-02
nucleolus (GO:0005730)	24	8.84	+	2.72	1.29E-02
chloroplast thylakoid membrane (GO:0009535)	19	6.07	+	3.13	1.57E-02
plastid thylakoid membrane (GO:0055035)	19	6.22	+	3.06	2.11E-02
Golgi apparatus (GO:0005794)	41	20.76	+	1.97	4.43E-02

Supplemental Table 7: Significant, down-regulated GO Terms for severe-cold-challenged sorghum

Biological Process	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
response to light intensity (GO:0009642)	17	3.94	+	4.31	2.76E-03
electron transport chain (GO:0022900)	10	1.32	+	7.57	5.13E-03
regulation of shoot system development (GO:0048831)	11	2.03	+	5.42	3.23E-02
generation of precursor metabolites and energy (GO:0006091)	16	4.3	+	3.72	3.49E-02
Molecular Function	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
protein binding (GO:0005515)	94	60.53	+	1.55	1.05E-02
electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity (GO:0045156)	4	0.12	+	34.39	2.62E-02
Cellular Component	**Gene #**	**Expected**	**Over/Under**	**Fold Enrichment**	**P-Value**
mitochondrial respirasome (GO:0005746)	13	1.22	+	10.69	6.83E-07
respiratory chain complex (GO:0098803)	13	1.26	+	10.33	1.00E-06
respirasome (GO:0070469)	13	1.32	+	9.84	1.73E-06
inner mitochondrial membrane protein complex (GO:0098800)	15	1.98	+	7.59	2.79E-06
oxidoreductase complex (GO:1990204)	14	1.72	+	8.12	4.12E-06
mitochondrial envelope (GO:0005740)	19	3.57	+	5.32	6.04E-06
envelope (GO:0031975)	38	13.56	+	2.8	1.20E-05
organelle envelope (GO:0031967)	38	13.56	+	2.8	1.20E-05
mitochondrial inner membrane (GO:0005743)	16	2.7	+	5.93	2.21E-05
transmembrane transporter complex (GO:1902495)	11	1.09	+	10.1	2.31E-05
transporter complex (GO:1990351)	11	1.13	+	9.72	3.31E-05
mitochondrial protein-containing complex (GO:0098798)	16	2.83	+	5.65	4.23E-05
membrane protein complex (GO:0098796)	24	6.43	+	3.73	4.57E-05
mitochondrial membrane (GO:0031966)	17	3.39	+	5.01	8.38E-05

integral component of membrane (GO:0016021)	22	5.7	+	3.86	9.48E-05
membrane (GO:0016020)	90	53.18	+	1.69	1.72E-04
cytosol (GO:0005829)	57	28	+	2.04	1.78E-04
cytochrome complex (GO:0070069)	7	0.4	+	17.42	2.83E-04
organelle inner membrane (GO:0019866)	16	3.43	+	4.67	4.80E-04
organelle membrane (GO:0031090)	39	16.41	+	2.38	5.05E-04
intrinsic component of membrane (GO:0031224)	27	9.12	+	2.96	5.94E-04
thylakoid membrane (GO:0042651)	16	4.03	+	3.97	3.54E-03
photosynthetic membrane (GO:0034357)	16	4.08	+	3.92	4.15E-03
chloroplast thylakoid membrane (GO:0009535)	15	3.65	+	4.11	4.71E-03
chloroplast thylakoid (GO:0009534)	17	4.7	+	3.61	5.96E-03
plastid thylakoid membrane (GO:0055035)	15	3.73	+	4.02	6.12E-03
thylakoid (GO:0009579)	19	5.78	+	3.29	6.51E-03
plastid thylakoid (GO:0031976)	17	4.77	+	3.57	7.05E-03
mitochondrial respiratory chain complex I (GO:0005747)	7	0.77	+	9.07	1.45E-02
respiratory chain complex I (GO:0045271)	7	0.8	+	8.71	1.84E-02
NADH dehydrogenase complex (GO:0030964)	7	0.85	+	8.28	2.50E-02

Supplemental Table 8: Significant, up-regulated GO Terms for severe-cold-challenged sorghum