Genetic Variation for Lettuce Seed Thermoinhibition Is Associated with Temperature-Sensitive Expression of Abscisic Acid, Gibberellin, and Ethylene Biosynthesis, Metabolism, and Response Genes

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Lettuce (Lactuca sativa ‘Salinas’) seeds fail to germinate when imbibed at temperatures above 25°C to 30°C (termed thermoinhibition). However, seeds of an accession of Lactuca serriola (UC96US23) do not exhibit thermoinhibition up to 37°C in the light. Comparative genetics, physiology, and gene expression were analyzed in these genotypes to determine the mechanisms governing the regulation of seed germination by temperature. Germination of the two genotypes was differentially sensitive to abscisic acid (ABA) and gibberellin (GA) at elevated temperatures. Quantitative trait loci associated with these phenotypes colocated with a major quantitative trait locus (Htg6.1) from UC96US23 conferring germination thermotolerance. ABA contents were elevated in Salinas seeds that exhibited thermoinhibition, consistent with the ability of fluridone (an ABA biosynthesis inhibitor) to improve germination at high temperatures. Expression of many genes involved in ABA, GA, and ethylene biosynthesis, metabolism, and response was differentially affected by high temperature and light in the two genotypes. In general, ABA-related genes were more highly expressed when germination was inhibited, and GA- and ethylene-related genes were more highly expressed when germination was permitted. In particular, LsNCED4, a gene encoding an enzyme in the ABA biosynthetic pathway, was up-regulated by high temperature only in Salinas seeds and also colocated with Htg6.1. The temperature sensitivity of expression of LsNCED4 may determine the upper temperature limit for lettuce seed germination and may indirectly influence other regulatory pathways via interconnected effects of increased ABA biosynthesis.

The timing of seed germination is associated with the presence or absence of seed dormancy, which is the temporary failure of a viable seed to germinate after a specified length of time in a particular environment that would allow germination if the seed were not dormant (Baskin and Baskin, 2004). Genetic and physiological approaches are revealing insights into the determinants of seed germination and dormancy (Bentsink et al., 2006; Finch-Savage and Leubner-Metzger, 2006; Finch-Savage et al., 2007). Mutation studies, phenotypic screening, and surveys of natural variation, largely in Arabidopsis (Arabidopsis thaliana), have identified dozens of genes that influence the imposition or release of dormancy or the ability of seeds to transition from maturation to germination (Bentsink and Koornneef, 2002; Finkelstein et al., 2002; Bentsink et al., 2007). Many of these genes implicate plant hormones, including GA, abscisic acid (ABA), ethylene, cytokinins, brassinosteroids, and auxin (Alvey and Harberd, 2005; Kucera et al., 2005; Achard et al., 2006; Riefler et al., 2006; Seo et al., 2006; Feurtado and Kermode, 2007), as well as sugars (Dekkers and Smeekens, 2007), light (Oh et al., 2007; Gubler et al., 2008; Sawada et al., 2008), temperature (Tamura et al., 2006; Toh et al., 2008), and other factors in complex regulatory webs that sense internal and external conditions and determine whether germination proceeds to completion. In many seeds, the endosperm and testa play important roles in regulating dormancy (Lefebvre et al., 2006; Muller et al., 2006; Bethke et al., 2007; Debeaujon et al., 2007; Nonogaki et al., 2007), and checkpoints for embryo growth after germination sensu stricto have been described (Lopez-Molina et al., 2002; Kucera et al., 2005).

Seed dormancy is dependent on environmental factors. Dry storage (after-ripening) or moist chilling (stratification) are often required to alleviate dormancy, and the range of environmental conditions in
which germination will occur generally expands as dormancy is reduced (Allen et al., 2007). During the periods when they are most likely to germinate (i.e. are least dormant), some seeds have additional requirements for germination-stimulating signals, such as light, GA, or nitrate, and increasing sensitivity to these signals is associated with dormancy loss (Fennimore and Foley, 1998; Batlla and Benech-Arnold, 2005; Kucera et al., 2005; Hillhorst, 2007). Conversely, decreased sensitivity to ABA, a strong germination inhibitor, is associated with reduced dormancy (Koornneef et al., 1984; Walker-Simmons, 1987; Steinbach et al., 1997).

Lettuce (Lactuca sativa) seeds exhibit thermoinhibition (fail to germinate) at temperatures well below the biological upper limit for seedling growth (Cantliffe et al., 1981). Several factors influence the upper temperature limit for lettuce seed germination. Seeds matured under warm temperatures have higher germination temperature limits than do seeds matured under cooler temperatures (Kozarewa et al., 2006). Higher upper temperature limits are heritable, being increased by selection in high-temperature environments (Guzman et al., 1992). The intact endosperm envelope enclosing the lettuce embryo is required for the imposition of thermoinhibition (Borthwick and Robbins, 1928). Exposure to ethylene, GA, and red light can increase the upper temperature limit of lettuce seed germination, and exposure to ABA can decrease it (Reynolds and Thompson, 1973; Saini et al., 1986, 1989; Dutta and Bradford, 1994; Kristie and Fielding, 1994; Gonai et al., 2004). The sensitivity of lettuce seed germination to inhibition by ABA is enhanced at higher temperatures (Robertson and Berrie, 1977; Roth-Bejerano et al., 1999).

Similar responses of germination to temperature and light have also been documented in Lactuca serriola, the progenitor species of cultivated lettuce (Marks and Prince, 1982; Small and Gutterman, 1992). However, certain accessions of L. serriola have thermonhibitory temperature limits up to 10°C higher compared with both cultivated lettuce and other L. serriola accessions (Argyris et al., 2005; Argyris, 2008). Quantitative trait loci (QTLs) associated with temperature sensitivity of germination were identified in a core-mapping recombinant inbred line (RIL) population from a cross between a thermosensitive cultivated lettuce cultivar, 'Salinas', and thermotolerant L. serriola accession UC96US23 (Argyris et al., 2005). The parental lines of the RIL population showed divergent germination behavior at high temperature, with Salinas seeds exhibiting thermoinhibition above approximately 30°C, while UC96US23 seeds germinated at temperatures up to 37°C in the light (Argyris et al., 2008). QTLs associated with seed dormancy have been identified in populations of Arabidopsis and other species (Foley, 2001; Alonso-Blanco et al., 2003; Gu et al., 2006; Bentsink et al., 2007), and the first gene specifically responsible for a seed dormancy QTL was recently identified (Bentsink et al., 2006).

Considerable physiological, gene expression, and mutant data support a role for the balance of GA and ABA synthesis and catabolism in modulating thermoinhibition (Gonai et al., 2004; Toh et al., 2008). Comprehensive transcriptomic analyses of primary and secondary dormancy of Arabidopsis and Nicotiana plumbaginifolia seeds indicate that genes associated with the synthesis and deactivation of GA and ABA are reciprocally regulated in association with the imposition and release of dormancy, with ABA accumulation favored during dormancy imposition and GA accumulation favored during dormancy release (Bove et al., 2005; Cadman et al., 2006; Finch-Savage et al., 2007). Imbition of Arabidopsis seeds at high temperature promotes the expression of genes involved in ABA synthesis (e.g. AtNCED genes encoding 9-cis-epoxycarotenoid dioxygenases) and inhibits the expression of genes involved in GA synthesis (e.g. AtGA3ox genes encoding GA 3ß-hydroxylases; Toh et al., 2008). Seeds of ABA-deficient and ABA-insensitive mutants (abi1 and abi3) in Arabidopsis germinate at supraoptimal temperatures, suggesting a major role for ABA in the thermoinhibition mechanism (Tamura et al., 2006). Mutant analyses and gene expression studies demonstrated that AtNCED6 and AtNCED9, which catalyze the first biochemical step unique to ABA biosynthesis, are specifically required for the induction of dormancy in Arabidopsis seeds (Tan et al., 2003; Lefebvre et al., 2006), with AtNCED9 playing the major role in thermoinhibition (Toh et al., 2008). ABA catabolism is regulated primarily via ABA-8ß-hydroxylases (e.g. CYP707A1 and CYP707A2), whose expression is limited to seeds and mutation or suppression of which results in increased seed dormancy and higher ABA contents compared with the wild type (Kushiro et al., 2004; Okamoto et al., 2006; Gubler et al., 2008). ABA can suppress GA biosynthesis in Arabidopsis seeds, while red light reduces ABA content by down-regulating the expression of AtNCED6 and enhancing the expression of AICYP707A2 (Seo et al., 2006; Oh et al., 2007). In lettuce seeds, ABA content decreases rapidly when seeds are imbibed at optimal temperatures for germination, but it is maintained at elevated levels when imbibition occurs at high temperatures (Yoshioka et al., 1998; Toh et al., 2008). GA, in turn, promotes ABA catabolism and increases the upper temperature limit for lettuce seed germination (Gonai et al., 2004). Germination at high temperature in lettuce can also be promoted by the application of fluridone, an inhibitor of ABA synthesis, suggesting that continued ABA synthesis is involved in the maintenance of thermoinhibition (Yoshioka et al., 1998; Gonai et al., 2004).

Ethylene is also involved in regulating thermoinhibition in lettuce seeds (Abeles, 1986; Saini et al., 1986, 1989). Ethylene formation in plants occurs via the conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC synthase (ACS) and of ACC to ethylene via ACC oxidase (ACO). The conversion of S-adenosylmethionine to
ACC by ACS is the rate-limiting step of ethylene production, and two ACS genes (LsACS1 and LsACS2) have been reported in lettuce (Takahashi et al., 2003). Exogenously applied ethylene at least partially relieved thermoinhibition in lettuce, and seed ethylene production was correlated positively with the ability to germinate at high temperature (Saini et al., 1986, 1989; Prusinski and Khan, 1990; Dutta and Bradford, 1994; Kozarewa et al., 2006). Mutations in ethylene signaling components altered the sensitivities of germinating seeds to exogenous ABA concentration and enhanced seed dormancy, providing strong evidence for cross talk between ABA and ethylene signaling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Matilla and Matilla-Vázquez, 2008).

The convergence of different signals regulating seed dormancy and germination occurs via the GRAS/DELLA proteins. These proteins, which in Arabidopsis include GAI, REPRESSOR OF GA1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, act as repressors of GA-induced signaling and germination (Silverstone et al., 2001; Tyler et al., 2004; Griffiths et al., 2006; Willige et al., 2007). Of these, RGL2 has the most significant role in seed germination, and along with GAI and RGA, it may serve to integrate the influences of light, GA, ABA, and ethylene in mediating the effects of environmental factors on dormancy and germination (Tyler et al., 2004; Achard et al., 2006, 2007; Oh et al., 2006, 2007; Penfield et al., 2006; Ueguchi-Tanaka et al., 2007).

A combination of genetic, molecular, and physiological approaches, particularly in Arabidopsis, has greatly advanced our understanding of the mechanisms underlying the regulation of seed dormancy and germination (Holdsworth et al., 2008). Here, we utilized natural genetic variation, QTL analyses, gene mapping, chemical genetics, hormonal profiling, and gene expression assays to analyze the mechanism(s) underlying the temperature sensitivity of germination in lettuce genotypes. Our results are broadly consistent with prior work, indicating conservation of regulatory mechanisms in seed germination, and provide specific physiological, genetic, and molecular evidence that the regulation of ABA synthesis may be a critical control point for lettuce seed thermoinhibition.

RESULTS

Germination of Cultivated Lettuce (Salinas) and L. serriola UC96US23 Seeds in Response to Light and Temperature

Seeds of both Salinas and UC96US23 germinated rapidly and uniformly at 20°C in the light, with germination beginning at about 16 h and being complete within 30 h after sowing (Fig. 1A). In contrast, only UC96US23 seeds germinated at 35°C in the light, although germination was delayed and less uniform at this temperature compared with 20°C, beginning after 30 h and being completed by 54 h (Fig. 1A). In the dark, Salinas seeds germinated rapidly and completely at 20°C, with a time course similar to that in the light, compared with only 35% final germination for UC96US23 seeds (Fig. 1B). Seeds of both genotypes were unable to germinate at 35°C in the dark (Fig. 1B). Salinas seeds are less dependent upon light than are UC96US23 seeds for germination at 20°C, and light is required for germination of UC96US23 seeds at 35°C.

Transfer experiments were conducted to determine the times during which the imposition of or escape from thermoinhibition occurred. Seeds were imbibed at high (35°C) or low (20°C) temperature for various times in the light or dark and then transferred to the alternative temperature in the same light or dark condition (Supplemental Fig. S1). These experiments established that the initial 6 to 12 h of imbibition is the critical period for the induction of thermoinhibition in Salinas seeds, confirmed that the thermotolerance of UC96US23 seeds requires light, and showed that exposures to 35°C for up to 24 h of imbibition did not induce secondary dormancy.

Effects of Temperature and Light on Sensitivity of Germination to ABA and GA

Seeds of Salinas and UC96US23 exhibited differential sensitivity to exogenously applied ABA and GA.
with respect to thermoinhibition. Seeds of both genotypes germinated more than 95% at 29°C in the light in the absence of ABA (Fig. 2A). UC96US23 seeds were almost 3-fold less sensitive to ABA in comparison with Salinas seeds, with ABA concentrations reducing germination to 50% (ABA$_{50}$ values) of 7.7 and 2.8 μM, respectively (Fig. 2A). As GA synthesis in lettuce seeds is up-regulated by light via phytochrome (Toyomasu et al., 1998), germination tests for GA response were conducted in the dark to reduce de novo GA synthesis and at a temperature just above the upper limit for Salinas seeds (32°C). Seeds of both lines were completely thermoinhibited at this temperature in the dark, failing to germinate in water (Fig. 2B). Provision of GA resulted in an approximately log-linear increase in germination of UC96US23 seeds, with restoration of 100% germination in 100 μM GA; in contrast, Salinas seeds germinated only 31% in 100 μM GA (Fig. 2B).

To assess the genetic basis of the differential sensitivity of Salinas and UC96US23 seeds to ABA and GA, the same RIL population utilized for QTL mapping of high-temperature germination capacity was screened as described above. ABA$_{50}$ values of RILs ranged from 0 to 82 μM at 29°C in the light, and germination in 100 μM GA ranged from 0% to 98% at 32°C in the dark (data not shown; Argyris, 2008). Composite interval mapping analysis of ABA sensitivity (measured as ABA$_{50}$) and germination in response to 100 μM GA (probit-transformed percentage) revealed one highly significant QTL for each of these traits, termed ABA$_{50}$6.1 and GA6.1, which accounted for 16% and 22% of the phenotypic variation for each trait, respectively (Fig. 3). These QTLs mapped to the same 1-log of the odds (1-LOD) confidence interval as Htg6.1, a major QTL associated with high-temperature germination capacity (Argyris et al., 2005, 2008). ANOVA of RIL genotypes at a marker locus closely associated with ABA$_{50}$6.1 showed that the UC96US23 allele conferred significantly reduced ABA sensitivity ($P < 0.05$). The mean ABA$_{50}$ value for RILs homozygous for UC96US23 alleles was 10.1 μM compared with 5.5 μM for Salinas homozygotes at the same locus. Similarly, RILs homozygous for the UC96US23 allele at GA6.1 showed significantly higher mean germination percentages in 100 μM GA (65%) compared with those homozygous for the Salinas allele (12%). Thus, sensitivity of germination to ABA and GA at elevated temperatures is associated with the same genetic region that confers high-temperature germination capacity in UC96US23 seeds.

Effects of Fluridone on the Sensitivity of Germination to Temperature and GA

While neither Salinas nor UC96US23 seeds were able to germinate when incubated in water at 32°C in the dark, the addition of 30 μM fluridone to inhibit carotenoid and ABA synthesis significantly increased germination to approximately 30% in both lines (Fig. 4). Application of 100 μM fluridone alone was sufficient to restore over 90% germination of Salinas seeds but not of UC96US23 seeds under these conditions (Fig. 4). However, adding even 1 μM GA$_{4+7}$ to 100 μM fluridone allowed 100% germination of UC96US23 seeds (data not shown). Combining 10 or 100 μM GA with 30 μM fluridone also allowed complete germination of UC96US23 seeds, while germination of Salinas seeds did not exceed 60% (Fig. 4). These results suggest that the light requirement for GA biosynthesis limits germination of UC96US23 seeds in the dark, which can be replaced by exogenous GA. In Salinas seeds, on the other hand, the response of germination to fluridone in the dark and the inability of GA to stimulate complete germination indicate that ABA may be the primary factor preventing germination at higher temperatures.

Hormonal Contents of Seeds Imbibed under Different Light and Temperature Conditions

If ABA prevents germination of Salinas seeds at 35°C in the light, the ABA content of Salinas seeds would be expected to be greater than that of UC96US23 seeds when imbibed at high temperatures. The initial ABA content of dry Salinas seeds was significantly higher...
(284 ng g\(^{-1}\)) compared with that of UC96US23 seeds (108 ng g\(^{-1}\)) and remained so until 18 h after sowing at 20°C (Fig. 5A). The ABA content in seeds of both genotypes declined rapidly from 2 to 18 h after sowing, decreasing from 296 to 10.5 ng g\(^{-1}\) and from 95 to 14.9 ng g\(^{-1}\) in Salinas and UC96US23, respectively. In comparison with 20°C, ABA amounts in Salinas seeds declined more slowly at 35°C and were significantly higher than in UC96US23 seeds between 2 and 48 h of imbibition (Fig. 5A). Increased imbibition temperature had little effect on ABA contents of UC96US23 seeds (Fig. 5A). Between 24 and 48 h of imbibition at 35°C, ABA content of Salinas seeds was almost 5-fold greater than that of UC96US23 seeds (Fig. 5A), and the latter had completed germination while the former were thermoinhibited (Fig. 1A).

Phaseic acid (PA) is the major metabolite of ABA deactivation, formed by the action of ABA-8'-hydroxylases that are encoded in Arabidopsis and lettuce by CYP707A (ABA8ox) genes (Kushiro et al., 2004; Saito et al., 2004; Yamaguchi et al., 2007; Sawada et al., 2008). Seed PA contents would be expected to increase as ABA contents decreased following imbibition. PA contents were highest in Salinas seeds imbibed at 20°C, in which the decline in ABA content was quantitatively greatest (Fig. 5A). Increased imbibition temperature had little effect on ABA contents of UC96US23 seeds (Fig. 5A). Between 2 and 48 h of imbibition at 35°C, ABA content of Salinas seeds was almost 5-fold greater than that of UC96US23 seeds (Fig. 5A), and the latter had completed germination while the former were thermoinhibited (Fig. 1A).

Seed GA\(_1\), GA\(_3\), and GA\(_4\) contents were generally below the limits of confident detection with the methods employed (data not shown). GA\(_2\) was detected at low levels (<5 ng g\(^{-1}\)) in most samples and did not differ significantly between the genotypes or imbibition temperatures (data not shown). Indole-3-acetic acid and indole-3-Asp contents increased with time after imbibition at both 20°C and 35°C, but there were no significant differences between the two genotypes.
types (Supplemental Fig. S2, A and B). cis-Zeatin riboside content declined in both genotypes at both 20°C and 35°C for the first 24 h of imbibition, then increased only in Salinas seeds imbibed at 35°C (Supplemental Fig. S2C). A similar pattern was exhibited by dihydrozeatin riboside content, although the increase in Salinas seeds at 35°C was quantitatively less (Supplemental Fig. S2D). cis-Zeatin-\(O\)-glucoside content increased following imbibition in both genotypes and at both temperatures and eventually reached higher levels in UC96US23 seeds than in Salinas seeds imbibed at 35°C (Supplemental Fig. S2E). These patterns were repeated in seeds imbibed in the dark for 12 and 24 h (Supplemental Fig. S2, F–J); it is unknown whether the delayed increase in cis-zeatin riboside content would occur in the dark at longer imbibition times. Levels of other hormones and their metabolites that were assayed were low and were not consistently detected, including ABA-Glc ester, neo-PA, 7’-hydroxy-ABA, indole-3-Glu, GA\(_{39}\), GA\(_{49}\), GA\(_{29}\), dihydrozeatin, isopentenyladenine, isopentenyladenosine, cis-zeatin, trans-zeatin, trans-zeatin riboside, and trans-zeatin-\(O\)-glucoside.

Expression of Genes Involved in ABA Synthesis and Metabolism

Differences in seed upper temperature limits, ABA and GA sensitivities, and ABA content between the lettuce genotypes may be due to differential expression of genes in the hormone synthesis, metabolism, perception, and signaling pathways. Therefore, we assayed expression (relative mRNA levels) of genes related to ABA, GA, and ethylene biosynthesis and response in seeds of both genotypes across time courses of germination at 20°C and 35°C in light and darkness by quantitative reverse transcription-PCR (qRT-PCR) and by multiplexed GeXP (Beckman-Coulter) analysis (genes and primers listed in Supplemental Table S1). Lettuce genes without reported full-length clones will be referred to by the names of their closest Arabidopsis homologs without the \(Ls\) prefix; names with the \(Ls\) prefix refer to genes that have been cloned and sequenced from lettuce.

Several genes in the ABA biosynthetic pathway were assayed, including \(LsZEP1\) (for zeaxanthin epoxidase) and \(LsNCED\) (for 9-cis-epoxycarotenoid dioxygenase) alleles. The former is highly homologous to the Arabidopsis \(AtABA1\) gene catalyzing the conversion of zeaxanthin to violaxanthin (Yamaguchi et al., 2007). Expression was analyzed for two of the four \(NCED\) alleles in lettuce (\(LsNCED1\) and \(LsNCED4\)), which have the highest homology to \(AtNCED2\) and \(AtNCED6\), respectively, among the Arabidopsis \(NCED\) family (Sawada et al., 2008). Initially present at relatively low levels, the abundance of mRNAs for \(LsZEP1\) and \(LsNCED4\) declined rapidly to very low or undetectable levels in seeds of both genotypes during imbibition at 20°C in the light (Fig. 7, A and B). In contrast, transcripts for these genes initially decreased but then increased in Salinas seeds after imbibition for more than 12 h at 35°C. In UC96US23 seeds, transcripts for these genes declined more slowly at 35°C than at 20°C but were much lower than in Salinas seeds imbibed at 35°C. No expression was detected for the \(LsNCED1\) allele (Supplemental Fig. S3W). A pattern very similar to that for \(LsZEP1\) was observed for a
When imibed in the dark at 20°C or 35°C, expression patterns of these genes differed much less between genotypes (Fig. 8, A–C; Supplemental Fig. S4, A–D). Expression of both *LsZEP1* and *LsNCED4* was higher at 35°C than at 20°C. *LsABA8ox4* mRNA decreased with time in the dark at 20°C but showed an increasing trend at 35°C in both genotypes.

**Expression of Genes Involved in GA Synthesis and Metabolism**

GA synthesis in lettuce seeds is induced by light via phytochrome, which differentially affects the expression of at least three genes involved in the synthesis of active GAs: *LsGA20ox1* and *LsGA20ox2*, encoding GA 20-oxidases involved in the penultimate steps, and *LsGA3ox1* (previously termed *Ls3h1*), encoding a GA 3β-hydroxylase that catalyzes the final step in GA1 biosynthesis (Toyomasu et al., 1998; Yamauchi et al., 2004; Yamaguchi et al., 2007; Sawada et al., 2008). In Arabidopsis, *AtGA3ox1* and *AtGA3ox2* act redundantly and are essential for seed germination (Mitchum et al., 2006). *LsGA20ox1* mRNA increased transiently in seeds of both genotypes at 8 to 12 h of imbibition at 20°C in the light (Fig. 7F; Supplemental Fig. S5J). When seeds were imibed at 35°C in the light, *LsGA20ox1* mRNA initially increased in UC96US23 seeds and then declined, but remained relatively low in Salinas seeds (Fig. 7F; Supplemental Fig. S5J). Expression of *LsGA20ox2* and *LsGA3ox1* increased at 20°C in both genotypes just prior to the initiation of germination (Fig. 7, G and H; Supplemental Fig. S5, H and J). When imibed at 35°C in the light, mRNA of neither gene was detected in Salinas seeds, while in UC96US23 seeds an increase was evident after a delay (Fig. 7, G and H; Supplemental Fig. S5, I). The genotypic differences in *LsGA20ox1* expression observed in the light (Fig. 7F; Supplemental Fig. S5J) were much less evident in the dark (Fig. 8F; Supplemental Fig. S6J), suggesting that light is involved in regulating this gene. Amounts of *LsGA20ox2* and *LsGA3ox1* transcripts were higher in Salinas seeds than in UC96US23 seeds imbibed in the dark at 20°C (Fig. 8, G and H; Supplemental Fig. S6, H and K).

Lettuce homologs of *AtABA2* (Supplemental Fig. S3B), a short-chain alcohol dehydrogenase/reductase (SDR1) converting xanthoxin to abscisic aldehyde subsequent to the step catalyzed by NCED (Yamaguchi et al., 2007). A homolog to *ABA3*, encoding a molybdenum cofactor to aldehyde oxidase that converts abscisic aldehyde to ABA (Yamaguchi et al., 2007), showed a low but more complex initial expression pattern, but its mRNA also increased in abundance at longer imbibition times at 35°C (Supplemental Fig. S3C).

In Arabidopsis seeds, *AtCYP707A2* is responsible for the metabolism of ABA following imbibition (Kushiro et al., 2004). Transcript abundance of a lettuce homolog of this gene (termed *LsABA8ox4*; Sawada et al., 2008) decreased in both genotypes following imbibition at 20°C in the light but remained higher when imbibition occurred at 35°C (Fig. 7C; Supplemental Fig. S3D).
pathway described above. On the other hand, expression of a lettuce homolog of a gene encoding ent-kaurenoic acid oxidase that converts ent-kaurenoic acid to GA12-aldehyde showed low and variable expression at 20°C in both genotypes and was not detected at 35°C (Supplemental Fig. S5Q). In seeds imbibed in the dark, abundance of LsCPS1, LsKS1, and KO1 mRNAs decreased with imbibition time at 20°C, but abundance increased at 35°C (Supplemental Fig. S6, C, R, and S). Genotypic differences were small, however, except in the case of LsCPS1, where expression was greater in Salinas seeds at high temperature in the dark (Supplemental Fig. S6C).

GA levels are also regulated by expression of GA 2-oxidase (GA2ox) genes that metabolize active GAs (Yamaguchi et al., 2007). Two alleles of GA2ox genes are known in lettuce, LsGA2ox1 and LsGA2ox2 (Nakaminami et al., 2003). LsGA2ox1 mRNA abundance was high in dry seeds and declined rapidly in both Salinas and UC96US23 seeds imbibed at 20°C in the light (Fig. 7J). When imbibed at 35°C in the light, the initial decline was delayed in UC96US23 seeds and reversed in Salinas seeds, resulting in an increasing abundance in the latter (Fig. 7J). Only very low and transient (Supplemental Figs. S5G and S6G) or undetectable (qRT-PCR; data not shown) levels of LsGA2ox2 mRNA were present in these seeds under all conditions. In the dark, LsGA2ox1 mRNA abundance decreased with time at 20°C and increased with time at 35°C in seeds of both genotypes (Fig. 8J).
Expression of Genes Involved in Ethylene Synthesis

The mRNA abundance of a lettuce gene having high homology to the ethylene biosynthetic enzyme ACS (LsACS1) increased between 8 and 24 h of imbibition in the light at 20°C or 35°C in both genotypes (Fig. 7K). While expression was completely repressed in Salinas seeds at 35°C in the light, expression in UC96US23 seeds was only delayed (Fig. 7K), very similar to the expression pattern for LsGA3ox1 (Fig. 7H). A lettuce gene highly homologous to Arabidopsis ACO (AtACO4 or AtEAT1; termed ACO-A here, as a full-length clone has not been reported in lettuce) was expressed in both genotypes in response to temperature in a pattern similar to that of LsACS1 (Fig. 7L). A second ACO homolog allele (ACO-B) was highly abundant in Salinas seeds at 20°C and somewhat less so in UC96US23; expression in both genotypes was delayed and reduced when imbibed at 35°C (Fig. 7M). LsACS1 and ACO-A had similar expression patterns with respect to temperature when seeds were imbibed in the dark, although expression of LsACS1 was much lower in both genotypes in the dark than in the light (Fig. 8, K and L). Expression of ACO-B in seeds imbibed in the dark was similar to its expression in the light (Fig. 8M). Expression of all three genes was also consistently greater in Salinas seeds than in UC96US23 seeds imbibed at 20°C in the dark (Fig. 8, K–M).
Expression of Genes Involved in ABA, GA, and Ethylene Signal Transduction and Action

High temperature could also influence the perception of or sensitivity to ABA, GA, and ethylene, so the expression of genes that are regulated by these hormones are involved in their signal transduction pathways was also assayed. For example, ABI5 is a bZIP transcription factor whose mutation results in ABA insensitivity (Finkelstein and Lynch, 2000). Its expression is induced by ABA, and it can inhibit the late stages of germination and early seedling growth (Lopez-Molina et al., 2001). mRNA abundance of a lettuce homolog of Arabidopsis AtABI5 declined rapidly after imbibition at 20°C in the light in both Salinas and UC96US23 seeds, but it remained high until 12 to 18 h in both genotypes imbibed at 35°C before increasing in Salinas seeds and decreasing in UC96US23 seeds (Fig. 7D). A similar pattern of expression was also observed for ABI3 and ABI4 homologs (Supplemental Fig. S3, G and H), transcription factors also associated with ABA signaling pathways (Feurtado and Kermode, 2007). A very similar pattern of expression was also evident for a lettuce homolog of SNF4 (Fig. 7E), a GA-repressed and ABA-induced gene encoding a regulatory subunit of the SNF-related kinase complex involved in the regulation of reserve accumulation and mobilization and seed dormancy (Bradford et al., 2003; Radchuk et al., 2006; Bolognese et al., 2007; Rosnolet et al., 2007). Other genes that also exhibited a genotype- and temperature-specific expression pattern similar to this included SDR1, AREB2, ERA1, LEA, PER1, PRL1, COP1, COP8, COP11, FUS5, and PHYA (Supplemental Figs. S3, B, K, O, U, Z, and CC, and S7, C, D, F, O, and T), as well as LsZEP1, LsABA8ox4, LsCP51, KO1, and LsK51, as noted previously. This pattern correlated with seed ABA content (Fig. 5A), suggesting that these genes may be coordinately regulated by ABA.

A somewhat different expression pattern was recorded for genes encoding homologs of GAI or GRAS family proteins containing a DELLA domain that act as repressors of germination (Silverstone et al., 2001; Tyler et al., 2004). In Arabidopsis, AtRGL2 mRNA appears following imbibition and remains high in dormant seeds while disappearing in germinating seeds (Ariizumi and Steber, 2007). In lettuce, mRNA abundance of a GRAS family homolog (termed GRAS-A) decreased in seeds imbibed at 20°C, coincident with the completion of germination, but increased and remained high in Salinas seeds imbibed at 35°C, which exhibit thermomorancy (Fig. 7O). ABA has been reported to stabilize DELLA proteins, and RGL2 (along with DELLA proteins GAI and RGA) is required for ABA inhibition of germination (Achard et al., 2006; Penfield et al., 2006). Somewhat surprisingly, GRAS-A mRNA also remained high in UC96US23 seeds imbibed at 35°C but eventually declined as germination occurred between 24 and 48 h (Fig. 7O). Two other GRAS family members, LsGAI2 and GRAS-B, as well as a homolog of the GA receptor GID1 (Ueguchi-Tanaka et al., 2007), maintained somewhat higher mRNA levels in UC96US23 seeds than in Salinas seeds imbibed at high temperature (Supplemental Fig. S5, M–O).

A lettuce homolog of a gene associated with ethylene signal transduction (CTRI) exhibited generally declining mRNA abundance during imbibition, but mRNA levels remained higher in seeds of both genotypes imbibed at 35°C (Fig. 7N). Other genes associated with ethylene signaling (e.g. EIN2 and LsETRI) showed similar patterns (Supplemental Fig. S5, D and F), although LsERS1 mRNA transiently increased in seeds of both genotypes imbibed at 20°C (Supplemental Fig. S5E).

LsMAN1, which encodes an endo-β-mannanase involved in mobilization of the galactomannan cell wall storage reserves of the lettuce endosperm after radicle emergence, is induced by GA and repressed by ABA (Halmer et al., 1975; Dulson et al., 1988; Wang et al., 2004a). LsMAN1 mRNA abundance was very high immediately after radicle emergence in both genotypes at 20°C but remained much lower in seeds imbibed at 35°C, although a small increase occurred in UC96US23 seeds at 36 and 48 h (Supplemental Fig. S5T).

Expression of these genes was also assayed in seeds imbibed in the dark at 20°C and 35°C (Fig. 8, D, E, N, and O; Supplemental Fig. S6, D–F, M–O, and T). Overall, differences between genotypes were reduced in the dark relative to the light. For example, reductions in mRNA abundance of ABI5 and SNF4 in UC96US23 seeds at 20°C in the dark (Fig. 8, D and E) were much less than those that occurred in the light (Fig. 7, D and E), suggesting that light was required for these declines to occur, but the expression patterns in the dark were still closely correlated with ABA content (Fig. 6A).

Expression of Genes in Relation to Light Perception, Signal Transduction, and Action

A panel of genes associated with light perception and action was assayed, and in general, they exhibited relatively small differences in expression patterns in relation to genotype, temperature, and light (Supplemental Figs. S7 and S8). A notable exception was a PIF3 homolog (Supplemental Figs. S7V and S8V) encoding a protein involved in controlling the level of PHYB protein (Leivar et al., 2008) and directly interacting with DELLA proteins (Feng et al., 2008), which showed an expression pattern very similar to that of LsGA3ox1 or LsACS1 (Figs. 7, H and K, and 8, H and K) and correlated with germination responses (Fig. 1). As noted above, mRNA levels of COP1, COP8, COP11, FUS5, and PHYA were elevated in Salinas seeds after longer imbibition times at 35°C. For two genes, ELF3 and SUB1 (Supplemental Figs. S7, K and Z, and S8, K and Z), mRNA was detected only from Salinas seeds; it is possible that polymorphisms between the genotypes
prevented the amplification of mRNAs from UC96US23 seeds in these cases.

ANOVA and Cluster Analysis of Gene Expression Patterns in Response to High Temperature and Light

Relative expression was compared by ANOVA with respect to light treatment (light or dark), genotype (Salinas or UC96US23), temperature (20°C or 35°C), and time of imbibition (12 and 24 h) as the main effects. A total of 796 light × genotype × temperature × imbibition time × gene treatments had expression above the threshold level of detection and were evaluated using linear contrasts. Of this number, 56 treatments (7.0%) exhibited differential expression (P < 0.0001), and among these, 36 genes were differentially regulated by light. Among the 56 treatments, UC96US23 seeds displayed a higher frequency of differential transcription by light/dark treatments than did Salinas seeds (41 and 15 significant treatments, respectively), and significant effects of light regulation were detected more frequently at 24 h of imbibition than at 12 h (41 and 15 significant treatments, respectively). A highly significant light x genotype interaction was detected for eight genes (P < 0.0001). Of these eight, LsZEP1, SDR1, ABI5, LEA, and KO1 were up-regulated by dark in both genotypes, but more strongly in UC96US23 than in Salinas (Figs. 7A and 8A; Supplemental Figs. S3, B, I, and U, S4, B, I, and U, S5, R, and S6R). This transcriptional pattern is consistent with the higher ABA content and apparently lower GA content in UC96US23 seeds relative to Salinas seeds when imibed in the dark (Figs. 5 and 6). Only a single gene encoding the lipid mobilization gene isocitrate lyase was significantly down-regulated by dark in both genotypes, and more strongly in UC96US23 than in Salinas seeds, probably reflecting the reduced germination in the dark (Supplemental Figs. S3S and S4S). ERA1 and PK1, negative regulators of ABA signaling that target ABI3 and ABI5 (Penfield et al., 2005; Perruc et al., 2007), were down-regulated by dark in Salinas seeds but up-regulated in UC96US23 (Supplemental Figs. S3O, S4O, S5U, and S6U). Eleven genes were identified as highly significant (P < 0.0001) in the three-way genotype × light × temperature interaction: LsABA8ox4, ABI5, LsACS1, ACO-A, AREB2, LsGA3ox2, GRAS-A, HYL1, HY5, KO1, and PIP5K. For these genes, the genotypic differences in expression due to light depended upon the imbibition temperature. This list reinforces the close interactions among ABA-, GA-, and light-related genes and points to relationships that may warrant further investigation.

Hierarchical clustering also was performed on the log2 normalized gene expression values to reveal patterns of gene expression across all light and temperature treatments (Fig. 9). For each genotype, the transcript level of each gene at 0 h (dry seed) was used as the baseline level to which mRNA amounts during imbibition were normalized. To prevent bias, genes that were below the level of detection were removed, as was the very highly expressed gene LsMAN1. The clustering results indicate that the top five major clades, representing the genes that are most strongly up- or down-regulated across the 32 genotype, light, and temperature variables, contain only 10 different genes. The first two distinct clades each contained only a single gene. LsNCED4 was down-regulated (relative to dry seeds) across most light and temperature treatments but increased in Salinas seeds (compared with other treatments) at longer incubation times at 35°C. Conversely, relative to the dry seed transcript levels, LsACS1 was strongly up-regulated in both genotypes at 20°C but only in UC96US23 seeds at 35°C. A group of three genes involved in ABA synthesis and signaling, AREB1, ABA3, and HK1, formed a clade subtending LsACS1 that were strongly up-regulated in Salinas seeds in the dark. The next clade consisted of ACO-A, ACO-B, PIF3, and LsGA3ox1, which were strongly up-regulated following imbibition except in Salinas seeds at 35°C. Two ABA-responsive genes, ABH1 and PIP5K, clustered together and exhibited variable expression in both genotypes at 20°C but higher expression in UC96US23 seeds at 35°C. The next major clade contained a group of 15 genes including ABA synthesis, catabolism, and signaling genes and genes early in the GA biosynthesis pathway. Another large clade of 28 genes (SPT to SPA4) was largely composed of light-responsive genes. Between these two large groups were the GA oxidase genes that are up-regulated when conditions permit germination. Finally, a large clade of 21 genes (LsGA20ox2 to GRAS-A-q) was not strongly regulated or had complex expression patterns. These major clusters across all treatments were consistent with the ANOVA results above using only the 12- and 24-h time points. In addition, assays were conducted for several genes on the same extracts using both qRT-PCR and GeXP. These clustered together in most cases (e.g. LsZEP1 and LsABA8ox4), confirming the consistency of the two methods.

Differential Expression Responses Occur over a Narrow Temperature Range

To confirm that the differential patterns of gene expression between 20°C and 35°C were specifically associated with the temperature sensitivity of germination, gene expression in Salinas and UC96US23 seeds imibed in the light at 30°C, where both genotypes complete germination, was compared with expression in seeds imibed in the light at 33°C, where only UC96US23 seeds germinated. This 3°C increase in imbibition temperature was sufficient to induce contrasting germination phenotypes in seeds of the two genotypes and resulted in marked differences in gene expression (Fig. 10). Genes associated with ABA synthesis and metabolism or induced by ABA (LsNCED4, LsABA8ox4, ABI5, and SNF4) were all up-regulated in Salinas seeds imibed at the thermoinhibitory temperature (33°C), while no increase in expression occurred in UC96US23 seeds under this condition (Fig.
10, A–D). Consistent with this, ABA contents in Salinas seeds were greater at 33°C than at 30°C, while ABA contents of UC96US23 seeds were the same at both temperatures (Fig. 6C). A similar expression pattern was also evident for GRAS-A, which would be expected to be repressed by GA (Fig. 10E). In contrast, genes associated with GA and ethylene synthesis (LsGA20ox1, LsGA3ox1, LsACS1, and ACO-B) were expressed more highly at 30°C than at 33°C and were down-regulated in Salinas seeds imbibed at the higher temperature (Fig. 10, F–I). Expression of LsMAN1 was repressed only in Salinas seeds at 33°C, consistent with its promotion by GA and repression by ABA (Fig. 10J). These contrasting gene expression patterns over only a 3°C temperature range, which also differentially affected germination of the two genotypes, confirm that multiple genes are regulated coordinately and differentially over a narrow range of imbibition temperatures in a manner consistent with their relationship to ABA or to GA and ethylene.

### Candidate Gene Mapping: Colocalization of LsNCED4 with Htg6.1

In related studies to be reported separately, a genomic region containing the QTL Htg6.1 (Fig. 3) was

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**Figure 9.** Heat map of gene expression patterns of ABA-, GA-, ethylene-, and light-related genes in Salinas (Sal) and UC96US23 (UC) seeds imbibed at 20°C or 35°C in the light or dark. The data of Figures 7 and 8 and Supplemental Figures S3 to S8 were analyzed by hierarchical clustering of log2 ratios of expression relative to the dry seed for each genotype. Expression was assayed by GeXP unless noted by –q after the gene name, indicating qRT-PCR. Genes are identified in Supplemental Table S1. HAI, Hours after imbibition.
introgressed into near-isogenic lines in the Salinas background. In BC\textsubscript{3}S\textsubscript{2} progeny lines from multiple introgressed families, those homozygous for the UC96US23 \textit{Htg6.1} allele germinated at temperatures 2°C to 3°C higher than did seeds homozygous for the Salinas allele (i.e. derived from null segregants lacking the introgression), confirming that this locus has a major effect on the upper temperature limit for germination (data not shown; Argyris, 2008). To test whether genes involved in the regulation of germination colocalized with \textit{Htg6.1}, 20 candidate genes were mapped using DNA-based markers in the RIL population de-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10}
\caption{Expression of genes associated with ABA, GA, and ethylene synthesis, metabolism, and response in Salinas (Sal) and UC96US23 (UC) seeds imbibed at 30°C or 33°C in the light for 12 or 22 h, respectively. These times were chosen to correspond to the times of initiation of radicle emergence (only of UC seeds at 33°C). Genes are as described in the legend of Figure 7, with the addition of \textit{LsMAN1} (J), an endo-β-mannanase expressed in the endosperm following radicle emergence. mRNA levels assayed by qRT-PCR are plotted relative to the geometric mean of three control genes assayed in the same samples (see “Materials and Methods”); note the different scales in each panel. Means are plotted ±SE (\textit{n} = 3). [See online article for color version of this figure.]
\end{figure}
developed from Salinas and UC96US23. Several of these mapped within confidence intervals of germination-related QTLs (Argyris et al., 2008), the most intriguing of which is LsNCED4, which mapped exactly with the maximum LOD peak marker identifying Htg6.1, ABA3.6.1, and GA6.1 (Fig. 3). When seeds from near-isogenic progeny lines homozygous for the Salinas or UC96US23 alleles at this locus were imbibed at high temperature, expression of LsNCED4 was consistently greater in the lines with the Salinas allele (Argyris, 2008). LsNCED4, therefore, is a strong candidate to be responsible for the phenotypic effects of these QTLs.

DISCUSSION
Sensitivity of Lettuce Seed Germination to Light, Temperature, GA, and ABA

Light and temperature are critical regulators of seed germination that largely determine the expression of dormancy and the seasonality of germination and emergence in the field (Allen et al., 2007; Donohue et al., 2007). There is considerable evidence that light and temperature interact through the phytochromes in regulating germination (Heschel et al., 2007) and that a major effect of Pfr is to promote GA biosynthesis (Toyomatsu et al., 1998). In lettuce seeds, the Pfr requirement for germination increases as the temperature increases (Fielding et al., 1992; Kristie and Fielding, 1994) and the seeds become more sensitive to inhibition by ABA (Roth-Bejerano et al., 1999; Gonai et al., 2004). In Salinas seeds, there is sufficient Pfr (or GA) to permit germination in the dark at lower temperatures but not at higher temperatures (Fig. 1B), and supplemental GA alone does not restore complete germination (Fig. 2B). In UC96US23, on the other hand, only about 30% of the seeds were capable of germinating in the dark at 20°C, although they could all germinate at temperatures as high as 35°C in the light (Fig. 1) and GA was effective in replacing the light requirement (Fig. 2B). Thus, germination of UC96US23 seeds at both low and high temperatures requires light or GA, while germination of Salinas seeds at high temperature is limited primarily by ABA, as the ABA biosynthesis inhibitor fluridone overcomes thermoinhibition (Fig. 4). This is also reflected in the greater sensitivity of Salinas seeds to ABA and of UC96US23 seeds to GA (Fig. 2). The germination phenotypes of Salinas and UC96US23 seeds in response to light, temperature, GA, and ABA indicate that there is a greater dependence upon light (or GA) in UC96US23 seeds but that ABA blocks germination at high temperature only in Salinas seeds.

ABA and GA Contents and Expression of Their Biosynthesis and Metabolism Genes

As predicted by this hypothesis, the ABA content of Salinas seeds remained 5-fold higher than that of UC96US23 seeds imbibed at 35°C in the light (Fig. 5C). This compares well with the 2.75-fold difference in ABA/C contents measured at 29°C (Fig. 2A). Differences between the genotypes were reduced and total ABA contents were higher in seeds imbibed in the dark (Fig. 6A). However, the ABA content of Salinas seeds declined between 12 and 24 h in the dark at 20°C but remained higher in the dark at 35°C, similar to results reported by Gonai et al. (2004) for ‘Grand Rapids’ lettuce seeds. Light promoted the decline in ABA contents of both Salinas and UC96US23 seeds (Figs. 5A and 6A), as has been reported in lettuce and Arabidopsis seeds (Roth-Bejerano et al., 1999; Seo et al., 2006; Sawada et al., 2008). As light stimulates GA biosynthesis (Toyomatsu et al., 1998) and GA promotes ABA metabolism (Gonai et al., 2004; Sawada et al., 2008), it would be expected that light would cause a decline in ABA content. This is opposite to the effect of light on ABA content and metabolism in barley (Hordeum vulgare) embryos, in which light acts to inhibit rather than promote germination (Gubler et al., 2008).

To account for the greater ABA content in Salinas seeds imbibed at high temperature, the expression of genes involved in ABA biosynthesis or metabolism should differ between the genotypes. This was observed for LsZEP1, LsNCED4, and SDR1 gene expression, where mRNA abundance of these genes in Salinas seeds fell rapidly to low or undetectable levels within 8 to 12 h after imbibition at 20°C, but after an initial decrease relative to the dry seeds, it remained relatively constant or increased at 35°C (Fig. 7, A and B; Supplemental Fig. S3B). mRNA abundance of these genes also decreased rapidly in UC96US23 seeds imbibed at 20°C, but it decreased more slowly before eventually falling to low levels at 35°C, consistent with the 24-h delay in initiation of germination under these conditions. The higher and continued expression of these ABA biosynthetic genes in Salinas seeds imbibed at 35°C was consistent with their elevated ABA content relative to UC96US23 seeds (Fig. 5A). In Arabidopsis, AINCED6 and AINCED9, two of several NCED gene family members controlling the first biochemical steps unique to ABA biosynthesis, are required for the induction of dormancy during seed development (Tan et al., 2003; Lefebvre et al., 2006). mRNA levels of AINCED2, AINCED5, and AINCED9 among the NCED family members are elevated when Arabidopsis seeds are imbibed at high temperatures, with AINCED9 apparently playing the primary role in thermoinhibition (Toh et al., 2008). LsNCED4 shows highest sequence homology to AtNCED6 (Sawada et al., 2008), which in Arabidopsis is expressed specifically in the endosperm of developing seeds while AtNCED9 is expressed in both the embryo and the endosperm (Lefebvre et al., 2006). The expression of LsNCED4 in Salinas lettuce seeds imbibed at 35°C was localized exclusively in the embryo, based upon qRT-PCR results from seeds separated into embryo and endosperm + testa + pericarp samples (data not
shown). A central role for \textit{LsNCED4} in regulating thermoinhibition in lettuce is supported also by its colocation with \textit{Htg6.1}, a QTL accounting for over 60% of the genotypic variance for high-temperature germination in these genotypes (Fig. 3; Argyris, 2008), and its unique expression pattern in relation to genotype and temperature (Figs. 9 and 10).

ABA content can also be controlled by metabolism of ABA, primarily to PA via the action of ABA 8'-hydroxylase enzymes encoded by \textit{AtCYP707A1} and \textit{AtCYP707A2} in Arabidopsis (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006). In some cases, ABA accumulation is enhanced by concerted up-regulation of \textit{NCED} genes and down-regulation of \textit{CYP707A} genes (Seo et al., 2006; Oh et al., 2007; Sawada et al., 2008; Toh et al., 2008). In contrast, we found that expression of a lettuce gene (\textit{LsABA8ox4}) having high homology to \textit{AtCYP707A2} (Sawada et al., 2008) was greater in genotypes and conditions in which \textit{LsNCED4} expression was enhanced and ABA content was higher (Fig. 7C). However, the relative abundance of \textit{LsABA8ox4} mRNA was lower than that of \textit{LsNCED4} at longer imbibition times when differences in ABA content were most apparent (Fig. 7, B and C). Synthesis of ABA evidently exceeded metabolism, as Salinas seeds maintained higher ABA levels at high temperature (Fig. 5A). PA content did not show a consistent relationship with \textit{LsABA8ox4} expression (Fig. 5B). PA content was greatest in Salinas seeds imbibed at 20°C, which also exhibited the greatest absolute decrease in ABA content during imbibition. However, PA levels were also higher in Salinas seeds imbibed at 35°C than in UC96US23 seeds imbibed at either 20°C or 35°C. Thus, the elevated expression of ABA biosynthetic enzymes and the accumulation of ABA are apparently accompanied by higher expression of \textit{LsABA8ox4} and ongoing metabolism of both ABA and PA. A similar situation was reported for a GA-metabolizing gene (\textit{AtGA2ox6}), whose expression was up-regulated by GA in a feed-forward manner (Wang et al., 2004b). As noted previously, ABA contents were higher in the dark than at corresponding times in the light (Figs. 5A and 6A), and in Arabidopsis seeds, expression of \textit{AtCYP707A2} was increased by red light (Oh et al., 2007). However, this was evident in our data only for lettuce seeds imbibed at 20°C (compare Figs. 7C and 8C). Different alleles of ABA synthesis and metabolism genes may be capable of independently integrating and responding to multiple inputs, including light, temperature, GA, and ABA.

In Grand Rapids lettuce seeds, light acted via phytochrome to promote the expression of a GA 3β-hydroxylase (\textit{LsGA3ox1}) catalyzing the final step in the synthesis of active GA, but it either had no effect on (\textit{LsGA20ox1}) or inhibited (\textit{LsGA20ox2}) GA 20-oxidases catalyzing earlier steps in the biosynthetic pathway (Toyomatsu et al., 1998). We also found that \textit{LsGA20ox1} and \textit{LsGA20ox2} exhibited different expression patterns in response to temperature in the light (Fig. 7, F and G). \textit{LsGA20ox1} mRNA increased transiently at 8 h of imbibition and then declined in seeds of both genotypes imbibed at 20°C, while \textit{LsGA20ox2} expression increased somewhat later. At 35°C, expression of both \textit{GA20ox} genes was greater in UC96US23 seeds than in Salinas seeds. Thus, while \textit{LsGA20ox1} and \textit{LsGA20ox2} had differing and relatively complex expression patterns, their expression tended to be greater in genotypes and under conditions where germination will occur. \textit{GA20ox} family members also exhibited differing expression patterns in Arabidopsis seeds, but expression of all alleles was repressed at high temperature, as was expression of \textit{AtGA3ox1} and \textit{AtGA3ox2} genes (Toh et al., 2008). In both Salinas and UC96US23 seeds imbibed in the light at either low or high temperature, expression of \textit{LsGA3ox1} was closely associated with germination (Fig. 7H), while expression of \textit{LsGA3ox2} was not as clearly correlated to the completion of germination (Fig. 7I).

We were unable to confirm whether GA1 contents increased with expression of \textit{LsGA3ox1}, as expected, as the levels were generally below the limits of detection of the method used. Neither Chiwocha et al. (2003) nor Gonai et al. (2004) reported consistent effects of imbibition temperature on lettuce seed GA content, but both of those experiments were conducted in the dark, where expression of \textit{LsGA3ox1} and \textit{LsGA3ox2} was much lower than in the light, particularly at high temperatures (Fig. 8, H and I).

Interestingly, genes encoding enzymes active in the early steps of the GA biosynthetic pathway, including \textit{LsCP51}, \textit{KO1}, and \textit{LsKS1}, were up-regulated in Salinas seeds imbibed at high temperature (Supplemental Fig. S5, C, R, and S). In fact, their expression patterns most closely resembled those of ABA-inducible genes such as \textit{ABI5} or \textit{SNF4} (Figs. 7, D and E, and 9). This reciprocal regulation by temperature of genes early and late in the GA biosynthetic pathway (Fig. 11) may reflect feedback relationships regulating GA content. Under high-temperature conditions, when active GA synthesis is repressed due to down-regulation of \textit{LsGA20ox} and \textit{LsGA3ox} alleles, low GA levels may derepress the expression of genes earlier in the pathway. Alternatively, these genes may be responding to the increased ABA levels resulting from up-regulation of the ABA biosynthetic pathway.

GA metabolism via GA 2-oxidase genes may also be involved in regulating GA content and action during germination (Wang et al., 2004b; Yamaguchi et al., 2007). Two \textit{GA2ox} genes have been identified in lettuce, one of which (\textit{LsGA2ox2}) was expressed primarily in seeds and showed some inhibition by red light (Nakaminami et al., 2003). However, we were unable to detect the expression of \textit{LsGA2ox2} consistently via qRT-PCR, and only very low levels were detected transiently by GeXP in our experiments (Supplemental Fig. S5G). \textit{LsGA2ox1} mRNA, however, was abundant in dry seeds and declined during imbibition, except in Salinas seeds imbibed at 35°C (Fig. 7J). In fact, its expression pattern closely matched that of genes early in the GA biosynthetic pathway described above.
Figure 11. Diagram illustrating the effects of high temperature on the expression of genes in the ABA, GA, and ethylene synthesis, metabolism, and response pathways. Arrows indicate promotion and bars indicate inhibition of expression of the indicated genes or of germination. Solid arrows in biochemical pathways indicate single steps, while dashed arrows indicate that multiple steps are involved (not all are shown). The responses of Salinas (cultivated lettuce seeds are shown; in general, UC96US23 seeds had a reduced response to high temperature compared with Salinas seeds. Not shown are reciprocal effects of ABA, GA, or ethylene on the expression of genes in their respective metabolism of the other hormone. For example, ABA enhances the catabolism of GA and can inhibit the expression of GA biosynthesis genes (Gonai et al., 2004; Seo et al., 2006; Zentella et al., 2007), while GA has reciprocal effects to inhibit ABA biosynthesis and promote its catabolism (Oh et al., 2007). Thus, in lettuce seeds imbibed in the light at low temperature, GA biosynthesis is favored, ABA biosynthetic genes are repressed, and ABA content declines rapidly following imbibition. When imbibed at high temperature, expression of LsNCED4 and other ABA biosynthetic genes is stimulated, resulting in an increase in ABA, which may then repress the expression of GA biosynthetic genes even in the light. In UC96US23 seeds, which do not exhibit the promotion of LsNCED4 expression by high temperature (Fig. 10A), expression of GA-related genes is reduced (Fig. 10, F and G), but apparently it is sufficient to allow the completion of germination after a delay.

Expression of ABA- and GA-Responsive Genes in Relation to Thermoinhibition

While the hypothesis proposed above is attractive and consistent with the available data, we do not have data on protein levels or enzyme activities associated with these genes, and as noted earlier, the sensitivity of tissues to phytohormones is equally as important as the hormone levels. Estimating hormone sensitivity from dose-response curves can be confounded by differences in uptake or deactivation of the applied compounds. An additional way to gain insight into endogenous hormonal action is to monitor the expression of genes known to be responsive to the hormones as in vivo reporters of the net hormonal sensitivity and content balance. Therefore, we assayed the expression of a selection of ABA- and GA-responsive genes under the same conditions described above.

In Arabidopsis, ABI5 is a bZIP transcription factor that can inhibit the late stages of germination and early seedling growth and whose expression is induced by ABA (Lopez-Molina et al., 2001). In tomato (Solanum lycopersicum), LeSNF4, encoding an activating subunit of the SnRK1 protein kinase complex, was up-regulated by ABA and down-regulated by GA during germination (Bradford et al., 2003). Thus, high expression of these genes should be indicative of a high effective ABA/GA ratio in the seeds. Lettuce homologs of these and a number of other genes exhibited very similar expression patterns in response to genotype and imbibition temperature (Figs. 7, D and E, and 9). For all of these genes, mRNA abundance declined rapidly following imbibition of either genotype at particularly KO1, and of ABA-inducible genes (Supplemental Fig. S5R; Fig. 9). ABA induced the expression of AtGA2ox6 in Arabidopsis seedlings (Zentella et al., 2007), consistent with higher expression of LsGA2ox1 in Salinas lettuce seeds at high temperature when ABA content is high. ABA-deficient aba2 mutant Arabidopsis seeds also had higher GA4 levels than wild-type seeds, suggesting that endogenous ABA can suppress GA biosynthesis or enhance its metabolism (Seo et al., 2006). On the other hand, mRNA levels of AtGA2ox2 in Arabidopsis seeds were relatively unaffected by imbibition at 22°C or 34°C, despite higher ABA content in the latter (Toh et al., 2008). Specific GA2ox gene family members may be differentially regulated by temperature and ABA.

These contrasting patterns of expression of genes involved in ABA and GA biosynthesis and metabolism are particularly evident when seeds imbeded at 30°C and 33°C in the light are compared (Fig. 10). The high expression of LsNCED4 only in Salinas seeds imbibed at 33°C is particularly striking (Fig. 10A), and LsABA8ox4 shows a similar pattern (Fig. 10B). LsGA20ox1 and LsGA3ox1, on the other hand, show exactly the opposite pattern, with Salinas seeds imbibed at 33°C having the lowest expression (Fig. 10, F and G). These relationships likely reflect the interconnected effects of ABA and GA on the synthesis and metabolism of the other hormone. For example, ABA enhances the catabolism of GA and can inhibit the expression of GA biosynthesis genes (Gonai et al., 2004; Seo et al., 2006; Zentella et al., 2007), while GA has reciprocal effects to inhibit ABA biosynthesis and promote its catabolism (Oh et al., 2007). Thus, in lettuce seeds imbibed in the light at low temperature, GA biosynthesis is favored, ABA biosynthetic genes are repressed, and ABA content declines rapidly following imbibition. When imbibed at high temperature, expression of LsNCED4 and other ABA biosynthetic genes is stimulated, resulting in an increase in ABA, which may then repress the expression of GA biosynthetic genes even in the light. In UC96US23 seeds, which do not exhibit the promotion of LsNCED4 expression by high temperature (Fig. 10A), expression of GA-related genes is reduced (Fig. 10, F and G), but apparently it is sufficient to allow the completion of germination after a delay.
20°C but remained high and increased in Salinas seeds and declined slowly in UC96US23 seeds imbibed at 35°C. Thus, the expression patterns of these genes likely report the net ABA effect, which is consistent with the seed ABA content (Fig. 5A). However, expression of ABA-responsive genes in UC96US23 seeds imbibed at 35°C was elevated longer than would be expected based on ABA content alone (Fig. 5A), suggesting that coincident with the higher ABA content, sensitivity to ABA may have been increased at high temperature.

The GRAS/DELLA proteins act to repress germination, and transcription of their genes is repressed by GA (Lee et al., 2002; Tyler et al., 2004; Arizumi and Steber, 2007). Thus, the levels of their transcripts should report in vivo GA activity, with low levels indicating high GA action. This is consistent with the expression pattern of one lettuce GRAS family gene (GRAS-A), which declined at the time that visible germination began in seeds imbibed at 20°C and remained high in seeds imbibed at 35°C, consistent with a repressor of germination (Fig. 7O). Somewhat surprisingly, however, transcript levels for GRAS-A remained high in UC96US23 seeds imbibed at 35°C, even after germination had occurred. This was also evident for LsGAI2 and GRAS-B, additional GRAS family alleles in lettuce (Supplemental Fig. S5, M and O). ABA has been shown to stabilize other DELLA proteins (Achard et al., 2006; Penfield et al., 2006), but whether transcription of these genes is up-regulated by ABA or high expression is a response to low GA levels is unknown. In Arabidopsis and tomato, regulation of RGL2 transcription and protein stability are complex, and germination can occur in the presence of RGL2 transcript and protein (Bassel et al., 2004; Arizumi and Steber, 2007). In addition, DELLA proteins may up-regulate ABA biosynthesis via expression of XERICO, an H2-type RING E3 protein that promotes ABA accumulation (Ko et al., 2006; Zentella et al., 2007). Thus, GRAS family proteins may be involved in the reciprocal regulation of both GA and ABA biosynthesis and action. Also intriguing in this connection is the expression pattern of PIF3, encoding a phytochrome-interacting protein involved in transcriptional regulation by light and GA (Castillon et al., 2007). Although mutant experiments in Arabidopsis indicated that PIF3 is not involved in regulating germination (Oh et al., 2004), PIF3 mRNA abundance in lettuce seeds correlated with germination and with the expression of LsACO1 and LsGA3ox1, consistent with the promotion of PIF3 transcription by ethylene and of PIF3 action by the degradation of DELLA proteins in the presence of GA (Castillon et al., 2007; Feng et al., 2008).

LsMAN1 encodes an endo-β-mannanase that mobilizes the galactomannan reserves in the cell walls of the lateral endosperm of lettuce (Nonogaki and Morohashi, 1999; Wang et al., 2004a). It is expressed coincident with or immediately after radicle emergence and is induced by GA and repressed by ABA (Halmer et al., 1975; Dulson et al., 1988). This pattern was reflected in the expression pattern of LsMAN1 in response to temperature, with very high expression in seeds imbibed at 20°C but much lower expression in seeds imbibed at 35°C (Supplemental Fig. S5T). However, a low level of expression of LsMAN1 accompanied germination of UC96US23 seeds imbibed at 35°C. This differential response to temperature was particularly clear in the comparison of seeds imbibed at 30°C versus 33°C (Fig. 10J) and in seeds imbibed in the dark (Supplemental Fig. S6T). While the in vivo GA activity appears to be reduced in UC96US23 seeds imbibed in the light at high temperature, it is nonetheless sufficient to result in germination in the light when combined with low ABA content.

Expression of Ethylene Biosynthetic and Signaling Genes in Relation to Thermoinhibition

Ethylene is known to stimulate the germination of seeds of a wide variety of species, particularly under stressful conditions (Kepczynski and Kepczynska, 1997; Matilla and Matilla-Vázquez, 2008). Ethylene can increase the upper temperature limit of lettuce seeds, especially when combined with GA or a cytokinin (Abeles, 1986; Saini et al., 1986; Khan and Prusinski, 1989), and ethylene production is reduced in thermoinhibited seeds (Prusinski and Khan, 1990; Nascimento et al., 2000). While ACS is generally the rate-limiting enzyme in the ethylene biosynthetic pathway, there also is evidence that ACO activity is lower in seeds imbibed at high temperatures (Khan and Prusinski, 1989) and may be important in the transition from dormancy to germination (Calvo et al., 2004). Here, we found distinct expression patterns for LsACS1 and two ACO loci depending upon lettuce genotype and imbibition temperature (Fig. 7, K–M). LsACS1 and ACO-A exhibited expression patterns similar to that of LsGA3ox1 (Fig. 7E), increasing just before visible germination, consistent with the general conclusion that ethylene acts in concert with GA and antagonistically to ABA in seeds (Kucera et al., 2005; Feurtado and Kermode, 2007; Matilla and Matilla-Vázquez, 2008). ACO-B mRNA, however, increased between 2 and 8 h of imbibition at 20°C, then decreased or remained constant; expression was delayed and reduced in both genotypes imbibed at 35°C (Fig. 7M). Assuming that the ACO-B gene is translated and the enzyme is active, ethylene production prior to germination is likely to be dependent upon the activity of ACS, which closely paralleled the time course of germination. Expression of genes associated with ethylene signal transduction and repression of ethylene action (CTR1, EIN2, and LsETR1) was less affected by temperature, although their mRNA levels tended to remain higher at high temperature (Fig. 7N; Supplemental Fig. S5, D and F). There is abundant genetic and transcriptional evidence indicating regulatory interactions among ethylene, ABA, and GA in seed
germination (Matilla and Matilla-Vázquez, 2008). Our data indicate that in lettuce seeds, some key GA and ethylene biosynthetic genes are coordinately regulated by temperature in a reciprocal pattern to that of ABA biosynthetic genes (Figs. 10 and 11). The regulation of the synthesis and action of all three of these hormones is so tightly integrated that environmental influences may cause coordinated adjustments throughout the regulatory network (Oh et al., 2007; Matilla and Matilla-Vázquez, 2008).

Temperature-Sensitive Expression of \( \text{LsNCED4} \) May Determine Lettuce Seed Thermoinhibition

Genetic analyses of a RIL population derived from Salinas and UC96US23 revealed that QTLs for ABA sensitivity and GA response mapped to the same genomic interval as \( \text{Htg6.1} \), a highly significant QTL for high-temperature germination (Argyris et al., 2005, 2008). \( \text{LsNCED4} \) is a strong candidate to be the gene responsible for the \( \text{Htg6.1} \) phenotype based on its colocation with these QTLs and its elevated expression in response to high temperature only in the thermosensitive genotype (Figs. 3, 7B, and 10A). This is consistent with results of a mutant screen for high-temperature germination capacity in Arabidopsis that identified alleles of \( \text{abi3} \) and \( \text{aba1} \), mutations in genes imparting ABA insensitivity and decreased ABA synthesis (Tamura et al., 2006), and with the role of \( \text{NCED} \) gene family members in thermoinhibition of Arabidopsis seed germination (Toh et al., 2008). Thus, in thermosensitive genotypes of lettuce such as Salinas, imbibition at high temperature results in an increase in ABA biosynthesis and accumulation and a decrease in GA and ethylene biosynthesis and accumulation, resulting in inhibition of germination (Fig. 11). The \( \text{LsNCED4} \) allele from UC96US23, on the other hand, does not increase in expression in response to high temperature, maintaining lower ABA levels and permitting germination in the light (Figs. 1A, 5A, 7B, and 10A). In the absence of elevated ABA levels, light promotes the expression of GA (and possibly ethylene) biosynthetic genes that remove the repression of germination by the GRAS/DELLA system. This hypothesis remains to be confirmed through fine mapping of \( \text{Htg6.1} \) in advanced NIL generations and functional analyses of \( \text{LsNCED4} \) alleles in Salinas and UC96US23 to determine the basis of their differential expression. If \( \text{LsNCED4} \) is the primary gene underlying \( \text{Htg6.1} \), it will be of further interest to understand how a change in expression of this gene is propagated through biochemical and regulatory networks to result in the diverse transcriptional changes observed in response to high temperature.

MATERIALS AND METHODS

Seeds of cultivated lettuce (\( \text{Lactuca sativa (Salinas)} \) and \( \text{Lactuca serriola (accession UC96US23)} \) were grown in the field in the summers of 2002 and 2005 at Davis, California. Seeds of RILs were produced in 2002. Details of seed production and storage were described previously (Argyris et al., 2005).

Seed Germination Tests

Germination tests for seeds from Salinas and UC96US23 were conducted with three replications of 25 seeds sown onto two layers of absorbent blotter paper discs (VWR Scientific Products) in 4.7-cm petri dishes moistened with 4 ml of deionized water. Germination was scored when the radicle had emerged from the enclosing tissues (endosperm membrane and fused testa/pericarp). For hormonal and temperature sensitivity assays, Salinas and UC96US23 seeds were germinated in \( \text{GA}_{3} \) (Abbott Labs), ABA (Gibco-Invitrogen), or \( \text{GA}_{3} \) + fluridone (Elanco) at the indicated concentrations and temperatures.

For temperature transfer experiments to determine the times of induction and escape from thermoinhibition, seeds were transferred to 35°C after 4, 6, 8, 10, 12, and 14 h of imbibition at 20°C and transferred to 20°C after 4, 8, 12, 18, and 24 h of imbibition at 35°C. In each case, imbibition was in either light or dark conditions. Germination was scored until 96 h of imbibition.

To assay ABA sensitivity, seeds were imbibed in water and in 1, 3, 10, and 30 \( \mu \text{M} \) ABA in the light at a temperature below the thermoinhibitory threshold for most RILs (29°C). The percentage of germinated seeds was scored at 96 h after the start of incubation. To determine the \( \text{ABA}_{50} \), probit-transformed germination percentages were plotted against log ABA concentration and regression analysis was performed. The intersection of regression lines with probit = 0 (50% of the total seed population) determined \( \text{ABA}_{50} \) values.

For the germination tests of GA alone or \( \text{GA}_{3} + \text{fluridone} \), seeds were sown in a darkroom under a green light-emitting diode (557 nm) safelight (LEDtronics), wrapped in aluminum foil, and transferred to an incubator. Seeds were incubated in darkness at 32°C and scored under the safelight at 24, 48, 72, and 96 h. Percentage germination values were transformed to probits to normalize variances in germination percentages for statistical analyses. Seeds of the RIL population were screened for germination percentage in 100 \( \mu \text{M} \) \( \text{GA}_{3} \) in the dark at 32°C. Probit-transformed percentage under these conditions was mapped as GA sensitivity.

Mapping Population, QTL Analysis, and Candidate Gene Mapping

Details on the production of the Salinas × UC96US23 F2 population, from which RILs were descended, and the linkage map used for QTL analysis from RILs grown in three different environments have been described (Johnson et al., 2000; Argyris et al., 2005). Map construction, plant material preparation, and DNA extraction were as described previously (Kesseli et al., 1994; Johnson et al., 2000; Truco et al., 2007). QTL analysis of germination data utilizing probit-transformed data was performed using Windows QTL Cartographer version 2.0 (Basten et al., 2001). A subset of 486 markers approximately 2 to 3 cm apart were chosen as the framework map for QTL analysis (http://cgpdb.ucdavis.edu/cgpdb2/lettuce_map/december_2006/). ANOVA was conducted to estimate additive effects of QTLs as described previously (Argyris et al., 2005; Gandhi et al., 2005).

Germination and dormancy candidate genes that were identified and placed on the lettuce consensus map are described by Argyris et al. (2008). For \( \text{LsNCED4} \), amplicons of approximately 1.4 kb from Salinas and UC96US23 were obtained using nondegenerate primers designed to the ends of the full-length coding region found in GenBank (ABI2010). The PCR amplicons were purified with ExoSAP-IT reagent (USB). Sequencing reactions were performed using the Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems), and sequencing analysis was conducted on an ABI Prism-3730 DNA analyzer (Applied Biosystems). PCR products were sequenced to 8× redundancy to ensure accurate single-nucleotide polymorphism (SNP) calls. Nondegenerate primers were then designed around a putative SNP detected between the Salinas and UC96US23 sequences at 843 bp, and PCR was performed on the RIL population described above. Subsequent 330-bp PCR products were first screened on SSCP gels to ensure that SNPs were codominant and scorable and then sequenced using the above methods to confirm the SSCP results.

Hormonal and Metabolomic Profiling

Seeds (100 mg dry weight) representing the same time points and temperatures and from the same seed lots as those used for gene expression analysis were collected, frozen, and lyophilized. Hormonal metabolomic profiling was conducted at the National Research Council Plant Biotechnology Institute in Saskatoon, Saskatchewan, Canada, according to the methods described by Chiwocha et al. (2005).
Gene Expression Assays

Salinas and UC96US23 lettuce seeds (three biological replicates of 0.5 g each) were imbibed at the desired temperatures on two germination blotters in 9-cm petri dishes with 14 mL of water. Total RNA was isolated from dry or imbibed lettuce seeds using a phenol-chloroform method (Cooley et al., 1999) with the following modifications. Following overnight LiCl precipitation of RNA, the carbohydrate contaminants were minimized by adding 0.5 mL of 10 mM Tris, pH 8.0, and 50 μL of 2 x KOAc to the pellet for 15 min on ice. The resulting supernatant collected after centrifugation (11,000g, 10 min at 4°C) was precipitated overnight at −20°C after adding 1.3 mL of 100% ethanol. RNA was dissolved in 50 to 100 μL of diethyl pyrocarbonate water after complete drying. DNA in RNA samples was digested with DNase I following the manufacturer’s protocol (Invitrogen). RNA quality was assessed by gel analysis and 260:280 and 260:230 nm ratios.

Candidate and reference (constitutive) gene sequences corresponding to the top BLAST hit were identified within the Composite Genome Project EST database (http://cgpdb.ucdavis.edu/) through sequence homology to known germination/dormancy-related candidates in Arabidopsis (Arabidopsis thaliana) and from existing lettuce sequence data in GenBank. Primer sequences were designed using Primer Express (Applied Biosystems) to amplify 50- to 150-bp PCR amplicons for qRT-PCR analyses. PCR products for genes of interest were sequenced to confirm their identity. Genes and primers used for qRT-PCR analyses are shown in Supplemental Table S1.

Total RNA (4.8 μg) was reverse transcribed using random hexamers (SuperScript First Strand Synthesis System; Invitrogen). cDNA from housekeeping genes and genes of interest was PCR amplified in an Applied Biosystems 7900 Real-Time PCR System using Sybergreen detection. The change in fluorescence for each sample was analyzed by DART PCR 1.0 (www.gene-quantification.de/peirson-dart-version-1). Analysis of expression data for reference genes by geNorm software (http://medgen.ugent.be/~jvdesomp/genorm) identified homologs of a set of three housekeeping genes and genes of interest was PCR amplified in an Applied Biosystems 7300 Real-Time PCR System using Sybergreen detection. The geometric mean of the expression levels of these genes was used to normalize the expression value for each gene of interest. Gene expression experiments were conducted independently on Salinas and UC96US23 seeds produced in 2002 and 2005 for most of the genes shown in Figure 7 with essentially identical results; data for seeds produced in 2005 are shown.

Expression patterns were also confirmed using northern blotting for LsNCED4, LsABA8x4, LsGA20ox1, LsGA3ox1, and LsMANY. Probes were derived utilizing the same sequence sources as above. PCR primers were designed with the Primer3 program (http://frodo.wi.mit.edu/) to generate approximately 500-bp amplicons that were ligated into the pCRII vector and cloned into competent Escherichia coli cells using the TOPO TA cloning kit (Invitrogen). Cloned fragments were sequenced to determine the orientation of insertion and to confirm their identity, and digoxigenin-labeled RNA probes (Roche) were synthesized by either SP6 or T7 RNA polymerase (Ambion) from the antisense DNA strand. Total RNA (5 μg) corresponding to a single replication of each treatment (32 samples) was loaded onto agarose gels and subsequently transferred to nylon membranes (Amersham Pharmacia Biotech). Northern hybridizations were conducted as described previously (Cooley et al., 1999), and expression patterns were consistent with qRT-PCR results (data not shown).

The GenomeLab GeXP Genetic Analysis System (Beckman-Coulter) was also used to assay mRNA levels in the same samples that were used for qRT-PCR (2005 seed production lots). The GeXP system is a capillary gene expression analysis system in which a total of 94 genes of interest were assayed in three separate multiplexed reactions (Hayashi et al., 2007). Three multiplex panels were designed and designated as ABA, GA/ethylene, and light panels, consisting of 31, 32, and 31 genes of interest, respectively. Each gene and primer sequence is listed in Supplemental Table S1. In addition to the genes of interest, each panel contained an internal control gene (Kar) and three normalization genes (ACT2/7, APT1, and TUB2). For each gene target, Arabidopsis sequences were used to identify lettuce homologs from the Compositae Genome Project Database (http://cgpdb.ucdavis.edu/cgpdb2/). Primers were designed using the GenomeLab eXpress Profiler software, which produced fragment sizes ranging from 122 to 424 nucleotides with a seven-nucleotide minimum separation size between each fragment. All PCR products were sequenced to confirm gene identity. cDNA for GeXP was synthesized from 100 ng of total RNA using the GenomeLab GeXP Start Kit.

PCR and multiplex detection were performed according to the manufacturer’s instructions (Hayashi et al., 2007).

For analysis of GeXP data, all genes of interest were normalized against the geometric mean of the three normalization genes (Vandesompele et al., 2002). Hierarchical gene clustering was performed on both GeXP and qRT-PCR data with Genesis software release 1.7.2 using average linkage clustering and Euclidean distances (Sturn et al., 2002). The clustering data are expressed as log2 ratios using a given gene’s dry seed (0 inhibition time) normalized value as the baseline level. For some genes, no transcripts were detected at 0 h after inhibition but were present at a later sampling time. In those instances, relative expression values of 0.0065 (GeXP) or 0.0002 (qRT-PCR; the estimated minimum detection levels) were used for the dry seed data in order to calculate expression ratios.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Germination responses to temperature transfers between 20°C and 35°C.

Supplemental Figure S2. Seed contents of auxin, cytokinin, and their derivatives.

Supplemental Figure S3. Expression of genes associated with ABA biosynthesis, regulation, or response in seeds imbibed at 20°C or 35°C in the light.

Supplemental Figure S4. Expression of genes associated with ABA biosynthesis, regulation, or response in seeds imbibed at 20°C or 35°C in the dark.

Supplemental Figure S5. Expression of genes associated with GA and ethylene biosynthesis, regulation, or response in seeds imbibed at 20°C or 35°C in the dark.

Supplemental Figure S6. Expression of genes associated with GA and ethylene biosynthesis, regulation, or response in seeds imbibed at 20°C or 35°C in the dark.

Supplemental Figure S7. Expression of genes associated with responses to light in seeds imbibed at 20°C or 35°C in the light.

Supplemental Figure S8. Expression of genes associated with responses to light in seeds imbibed at 20°C or 35°C in the dark.

Supplemental Table S1. Gene descriptions, accessions numbers, and primers used in expression assays.

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