Genetic Variation for Thermotolerance in Lettuce Seed Germination Is Associated with Temperature-Sensitive Regulation of ETHYLENE RESPONSE FACTOR1 (ERF1) \(^1\)[OPEN]

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Seeds of most lettuce (Lactuca sativa) cultivars are susceptible to thermoninhibition, or failure to germinate at temperatures above approximately 28°C, creating problems for crop establishment in the field. Identifying genes controlling thermoninhibition would enable the development of cultivars lacking this trait and, therefore, being less sensitive to high temperatures during planting. Seeds of a primitive accession (PI251246) of lettuce exhibited high-temperature germination capacity up to 33°C. Screening a recombinant inbred line population developed from PI251246 and cv Salinas identified a major quantitative trait locus (Htg9.1) from PI251246 associated with the high-temperature germination phenotype. Further genetic analyses discovered a tight linkage of the Htg9.1 phenotype with a specific DNA marker (NM4182) located on a single genomic sequence scaffold. Expression analyses of the 44 genes encoded in this genomic region revealed that only a homolog of Arabidopsis (Arabidopsis thaliana) ETHYLENE RESPONSE FACTOR1 (termed LsERF1) was differentially expressed between PI251246 and cv Salinas seeds imbibed at high temperature (30°C). LsERF1 belongs to a large family of transcription factors associated with the ethylene-signaling pathway. Physiological assays of ethylene synthesis, response, and action in parental and near-isogenic Htg9.1 genotypes strongly implicate LsERF1 as the gene responsible for the Htg9.1 phenotype, consistent with the established role for ethylene in germination thermotolerance of Compositae seeds. Expression analyses of genes associated with the abscisic acid and gibberellin biosynthetic pathways and results of biosynthetic inhibitor and hormone response experiments also support the hypothesis that differential regulation of LsERF1 expression in PI251246 seeds elevates their upper temperature limit for germination through interactions among pathways regulated by these hormones. Our results support a model in which LsERF1 acts through the promotion of gibberellin biosynthesis to counter the inhibitory effects of abscisic acid and, therefore, promote germination at high temperatures.

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Seed dormancy is the failure of a viable seed to germinate under normally favorable environmental conditions, enabling seeds to delay germination until the environment is most conducive to subsequent seedling survival. Seed dormancy is governed by complex gene regulatory networks interacting with environmental cues such as light (Yamaguchi and Kamiya, 2001; Oh et al., 2004) and temperature (Toh et al., 2008; Huo et al., 2013; Huo and Bradford, 2015) to initiate a series of metabolic steps involving phytohormones that have either inhibitory or stimulatory effects on germination (Finch-Savage and Leubner-Metzger, 2006; Bewley et al., 2013). Interactions among anatomical adaptations, environmental cues, and hormonal cross talk result in the wide diversity of seed dormancy phenotypes observed among plant species (Willis et al., 2014).

Although commonly referred to as a seed, lettuce (Lactuca sativa) produces a dry, one-seeded fruit known...
as an achene. While lettuce seeds exhibit little primary dormancy at maturity, they are subject to thermoinhibition (failure to germinate at high temperatures), leading to nonuniform crop establishment and reduced yields in agricultural plantings during warm seasons (Cantliffe et al., 2000). The thermoinhibition trait of lettuce seeds is attributed to the natural adaptation of its progenitor species to a Mediterranean climate having cool, wet winters and hot, dry summers. Seeds shed in the summer will not germinate if unseasonably cold, but while temperatures are still high; when rainfall coincides with cooler temperatures in the autumn, the seeds will germinate and the plants overwinter as rosettes following by flowering the following spring. The upper temperature limit for lettuce seed germination is genotype dependent and influenced by the seed production environment (Gray et al., 1988; Kozarewa et al., 2006). Seeds of most lettuce cultivars, including cv. Salinas (Sal), are thermosensitive and fail to germinate when imibed at 29°C or greater, while seeds of some thermotolerant genotypes can germinate above 90% at 36°C (Thompson et al., 1979). The thermotolerance phenotype of Lactuca serriola US96UC23 (formerly reported as UC96US23), an accession of the wild progenitor species of lettuce, has been dissected genetically (Argyris et al., 2005; Truco et al., 2007), leading to the identification of a gene encoding an enzyme involved in the biosynthesis of abscisic acid (ABA), 9-CIS-EPOXYCAROTENOID DIOXYGENASE4 (LsNCED4), as the causal gene (Argyris et al., 2008, 2011; Huo et al., 2013). While lettuce seed thermoinhibition can be alleviated by seed prehydration (priming) under low temperatures (Schwember and Bradford, 2010), eliminating seed thermoinhibition in lettuce cultivars by incorporating natural genetic variation would be an effective approach to improve crop establishment and lettuce production and ameliorate the effects of rising global temperatures.

While ABA is well known to be involved in regulating seed dormancy (Finkelstein et al., 2008), ethylene has long been associated with seed dormancy release in species of the Compositae family, including lettuce and sunflower (Helianthus annuus; Corbineau et al., 1988; Nascimento et al., 2000; Huo and Bradford, 2015). The ethylene biosynthetic pathway in plants begins with the conversion of S-adenosyl-Met to 1-aminocyclopropane carboxylate (ACC) via ACC synthase (ACS), followed by conversion of the ACC precursor to ethylene via ACC oxidase (ACO; Adams and Yang, 1979). Ethylene production is regulated by both ACS activity that modulates ACC availability and ACO enzymatic activity that converts ACC to ethylene (Linkies and Leubner-Metzger, 2012). Application of ethylene can alleviate the inhibitory effect of high temperature on lettuce seed germination (Abeles, 1986; Khan and Prusinski, 1989) and of secondary dormancy in sunflower seeds (Corbineau et al., 1990), and ethylene production rates of lettuce seeds are reduced by high temperatures during imbibition (Dunlap and Morgan, 1977; Khan and Prusinski, 1989). The mechanism by which ethylene acts in promoting thermotolerance in lettuce seeds is not fully understood. Some studies have found an increase in the activity of a cell wall-remodeling enzyme, endo-β-mannanase (EBM), in parallel with the increase in ethylene production rates in thermotolerant lettuce genotypes during germination at high temperature (Dutta et al., 1997; Nascimento et al., 2000, 2001, 2004), suggesting a direct stimulatory effect of ethylene action on the enzymatic activity associated with weakening the lettuce endosperm and enabling radicle protrusion. Other studies have suggested that ethylene promotes embryonic cell expansion associated with radicle emergence (Abeles, 1986; Dutta and Bradford, 1994).

Ethylene may also promote germination indirectly through interactions with GA and ABA. GA can promote the expression of ethylene biosynthetic enzymes, while inhibition of ethylene synthesis can reduce the expression of GA biosynthetic enzymes (Ogawa et al., 2003; Calvo et al., 2004a, 2004b; Chiwocha et al., 2005; Matilla and Matilla-Vázquez, 2008). In lettuce seeds, genes encoding ethylene and GA biosynthetic enzymes were largely regulated coordinately, with their transcripts rising or falling in parallel in response to imbibition temperature or other manipulations of germination potential (Argyris et al., 2008). Thus, ethylene could promote GA biosynthesis or action, enabling germination at higher temperatures. Alternatively, several studies have reported that ethylene can reduce the synthesis of ABA, enhance its degradation, or alter sensitivity to its action (Beaudoin et al., 2000; Ghassemian et al., 2000; Chiwocha et al., 2005; Wang et al., 2007; Cheng et al., 2009; Subbiah and Reddy, 2010), indicating that ethylene may promote germination by acting as a negative regulator of ABA synthesis or action (Linkies et al., 2009; Linkies and Leubner-Metzger, 2012).

Identifying additional genes and regulatory pathways associated with genetic variation in the temperature sensitivity of germination could clarify the roles of hormones in regulating seed dormancy. Therefore, we studied the basis of thermotolerance in a primitive accession of lettuce, PI251246 (PI), that exhibits tolerance of high temperature during germination (33°C or less; O'Brien, 2007). Analysis of a recombinant inbred line (RIL) mapping population derived from a cross between PI and Sal (Grube, 2004) identified a major quantitative trait locus (QTL) associated with the high-temperature germination (HTG) trait (O'Brien, 2007). Here, we report further genetic and transcriptomic data that strongly support a role for ethylene signaling, and specifically of a lettuce homolog of ETHYLENE RESPONSE FACTOR1 (LsERF1), in the thermotolerance of PI seeds during high-temperature imbibition. Supporting physiological studies indicate that ethylene and LsERF1 may act through the promotion of GA biosynthesis. These results expand upon prior knowledge of the hormonal basis of seed dormancy and connect ethylene action with GA biosynthesis to counter the inhibitory effect of ABA in regulating the sensitivity of lettuce seed germination to elevated temperatures.


High-Temperature Germination QTL Associated with ERF1
RESULTS
Temperature Responses of Seed Germination of Cultivated Lettuce (Sal) and Lettuce Accession PI

Screening seed germination of the parental Sal and PI lines across a range of temperatures revealed significant differences in their sensitivities to inhibition. Both Sal and PI seeds germinated 100% at temperatures below 28°C, but Sal seeds exhibited thermoinhibition between 29°C and 30°C, above which germination was completely prevented, while PI seeds exhibited 100% germination up to 32°C and were only completely inhibited at 34°C (Fig. 1). At 20°C, the PI seeds germinated 2 to 3 h earlier than those of Sal; at 30°C, only PI seeds germinated and did so at a more rapid rate than at 20°C (Supplemental Fig. S1). These experiments established that PI seeds are more thermostolerant during germination than are Sal seeds and identified the critical temperatures at which germination is inhibited in each genotype.

QTL Analysis of HTG Phenotypes Using a PI × Sal RIL Population

As PI and Sal seeds exhibited distinct upper germination temperature limits, the genetic basis of this difference was dissected using QTL analysis of seeds of an F6 population of 161 RILs grown in three environments: Davis, California, in 2004 and 2009 and San Joaquin Valley, California, in 2004. QTL analysis of germination data at 30°C and 35°C of seeds from these three seed production environments of the RIL population revealed a major QTL (Htg9.1) spanning approximately 10 centimorgans on chromosome 9 (Fig. 2). Htg9.1 explained 56% of the phenotypic variance observed for germination at 30°C of seeds across all three production environments and at 35°C of seeds produced in Davis (germination tests were not performed at 35°C on seeds produced in the San Joaquin Valley). The thermostolerant phenotype was determined by the allele at Htg9.1 from the PI parent (A).

Marker-Assisted Selection to Generate Near-Isogenic Lines for Htg9.1 to Confirm Effects on Seed Germination at High Temperatures

To validate the Htg9.1 effect on the HTG trait without being confounded by other quantitative loci present in the population or statistical bias from QTL analyses, a near-isogenic line (NIL) population containing the Htg9.1 genomic region in the Sal genetic background was generated with marker-assisted selection using codominant foreground (linked to the QTL) and background (unlinked to the QTL) markers based on a genetic linkage map of the RIL mapping population. The NILs were developed with three generations of backcrossing to lettuce Sal as the recurrent parent (RP) and RIL 98 as the donor parent (Supplemental Fig. S2). Prior to the first backcross, 56% of the RIL 98 genome was fixed for homoyzogous Sal alleles (B/B) but had homozygous PI alleles (A/A) at the Htg9.1 region. The expected proportion of the RP genome in BC3 plants increased significantly in three backcrossing generations, along with the introgression of Htg9.1. At the BC3 generation, NILs 3 and 28 that were heterozygous for the Htg9.1 region were selected for selfing to generate BC3S1 plants for BC3S2 seed production to provide segregants with different combinations of background RP alleles. To confirm the effect of Htg9.1 on germination at high temperature in a nearly uniform RP (Sal) genetic background, 88 BC3S2 seed lots from BC3S1 line 3-69 (hereafter designated NIL-P) exhibiting a non-HTG phenotype (0% germination at 32°C) were selected for further tests of hormonal and temperature sensitivity due specifically to the allele present in the Htg9.1 region.

Marker-Assisted Identification of Recombinant Lines from a RIL-Derived F2 Population to Refine the Htg9.1 Interval

To further fine-map the Htg9.1 locus, an F2 population was developed from a cross between RIL 107 (female) and RIL 58 (male) to generate additional recombination...
events within the \textit{Htg9.1} region. RIL 107 is thermotolerant and homozygous for the PI (A/A) allele, while RIL 58 is thermosensitive and homozygous for the Sal (B/B) allele at \textit{Htg9.1}. Both RILs carried thermosensitive alleles for a minor QTL on chromosome 2 (data not shown), thus making \textit{Htg9.1} the primary locus segregating for thermoinhibition in this F2 population. Seeds of these RILs had widely differing germination percentages at 30°C (Supplemental Fig. S3). A total of 1,053 F2 seedlings from the RIL 107 × RIL 58 cross were screened with four codominant single-nucleotide polymorphism (SNP) markers evenly spaced across the \textit{Htg9.1} QTL region. Consistent with Mendelian expectations for a 1:2:1 segregation ratio, 235 plants were homozygous for thermotolerant alleles (A/A), 427 plants were heterozygous (A/B), and 204 plants were homozygous for thermosensitive alleles (B/B) at all four markers. In addition, 87 lines had recombination events, as indicated by heterozygosity for at least one but not all of the four markers. From the 87 recombinants, 48 F3 plants that were homozygous for informative recombination events were allowed to self-pollinate to produce F4 seed for germination assays (Supplemental Fig. S4). When F4 seeds were germinated at temperatures ranging from 29°C to 32°C, a clear correlation between HTG phenotype and markers was observed when the germination value for each F4 line was determined based on the areas under the curves of germination percentage versus temperature graphs (Supplemental Tables S3 and S4). The genomic subregions with markers AMNA and ATBU cosegregated with the HTG phenotype, while the other subregions with markers BGZV and BLHY cosegregated with the non-HTG phenotype (Supplemental Table S3). Based on this information, 41 SNP markers specific to the AMNA and ATBU regions were designed based on lettuce seed transcriptome data from the two parents to genotype the F3 recombinants and the PI Sal mapping population to facilitate candidate gene identification. Reanalysis of the QTL utilizing prior phenotypic data and the additional markers further refined the \textit{Htg9.1} interval and confirmed that the NM4182 marker aligned with the central peak of the \textit{Htg9.1} QTL (Fig. 2). Of the 41 additional SNP markers, only NM4182 showed an obvious association between the thermotolerant haplotype (A/A) and the HTG phenotype of the recombinants (Supplemental Table S4).

Identification of \textit{LsERF1} as a Candidate Gene for \textit{Htg9.1}

The NM4182 marker is located on a single genomic scaffold (no. 243; Lsat\_1\_v4\_g\_9\_243; http://gviewer.genomecenter.ucdavis.edu/cgi-bin/gbrowse/lattuga\_version\_3\_2/) of 1 Mb that contains 44 annotated genes.
To determine whether genes encoded in this genomic region were differentially expressed at high versus low temperatures, we searched transcriptome data generated by RNA sequencing (RNAseq) of complementary DNA libraries from PI and Sal seeds imbibed at different temperatures. Although several poten-tially germination-related genes were present on scaffold 243, such as ERF104, ARA5, BSL3, and emb1441 (Supplemental Table S5), the only gene encoded on this scaffold exhibiting differential expression among genotypes and temperatures was a gene with sequence similarity to ERF1 of Arabidopsis (Arabidopsis thaliana); the candidate LsERF1 shares 65% amino acid sequence similarity with AtERF1, with the greatest homology in the conserved AP2/B3 binding domain. In Arabidopsis, AtERF1 encodes a transcription factor that acts near the end of the ethylene signaling and action pathway (Cheng et al., 2013). At 6 h of imbibition at 20°C, PI seeds had the highest abundance of LsERF1 mRNAs relative to PI seeds at 30°C and Sal seeds at 20°C and 30°C (Fig. 3). Interestingly, LsERF1 mRNA abundance subsequently declined in PI seeds, while it continued to increase in Sal seeds from 12 to 24 h at 20°C. A different pattern was observed at 30°C, where PI seeds showed higher LsERF1 expression compared with Sal seeds at 6, 12, and 24 h of imbibition. Expression of LsERF1 continued to increase in PI seeds after 24 h of imbibition at 30°C but declined in Sal seeds (Fig. 3). Because LsERF1 was the only gene on scaffold 243 that exhibited differential expression associated with genotype and imbibition temperature, it was further investigated as a candidate causal gene of the Htg9.1 phenotype.

Differential expression of the LsERF1 gene between PI and Sal points to possible differences in their promoter sequences, and there were no sequence polymorphisms within the coding regions of the PI and Sal alleles. Sequencing the promoters (2,000 bp upstream of the transcriptional start site) revealed that the PI and Sal alleles differed by three SNPs between −1,210 and −1,160 bp upstream of the start codon (Supplemental Fig. S5); the upstream regions beyond 2,000 bp could not be analyzed due to gaps in the lettuce reference genome. Predicted binding site motifs suggested that these SNPs could have affected root-related and TATABOX3 motifs. Further promoter sequencing, reporter gene experiments, and protein-binding assays are required to investigate the reason(s) for differences in the transcript expression patterns of the two LsERF1 alleles in response to temperature.

**Ethylene and Htg9.1 Allelic Effects on Germination at High Temperature**

Due to sequence similarity to AtERF1, the LsERF1 gene was expected to be involved in the ethylene-signaling pathway. Therefore, we examined the ethylene sensitivities of seeds of the parental and NIL genotypes. To conduct meaningful comparisons of differences in ethylene sensitivities among PI, Sal, NIL-PI, and NIL-Sal lines, seeds were germinated at different normally nonpermissive temperatures based on their genotypes (31°C for Sal and NIL-Sal and 35°C for PI and NIL-PI). These temperatures were selected to be 3°C above each parental genotype’s maximum temperature threshold allowing germination, so that no or only a few seeds germinated at these temperatures in the absence of ethylene (Fig. 4A). Exposure to 10 μL L⁻¹ ethylene had little or no effect on the germination of Sal and NIL-Sal seeds at 31°C. In contrast, 83% of PI seeds and 51% of NIL-PI seeds were able to germinate at 35°C in the presence of the same concentration of ethylene (Fig. 4A). Thus, the PI allele of Htg9.1 confers enhanced sensitivity to ethylene in promoting germination at high temperatures.

The temperature of imbibition can affect ethylene biosynthesis (Dunlap and Morgan, 1977; Khan and Prusinski, 1989), possibly confounding responses to ethylene application. To determine the ethylene sensitivity of germination in the absence of endogenous ethylene synthesis, germination assays at different ethylene concentrations were conducted in the presence of aminoethoxyvinylglycine (AVG), which reduces ethylene production by inhibiting ACS enzymatic activity (Saltveit, 2005). The optimal conditions for AVG inhibitory action on Sal and PI seed germination were identified through assays of the parental seeds across a range of temperatures compared with water-imbibed seeds. Increasing concentrations of AVG progressively lowered maximum germination temperatures for seeds of both genotypes (Supplemental Fig. S6). The maximum germination difference between control and
Figure 4. A, Germination of PI, NIL-PI, Sal, and NIL-Sal seeds imbibed with deionized water in the absence and presence of 10 μL L⁻¹ ethylene. B, Germination of PI, NIL-PI, Sal, and NIL-Sal seeds imbibed with 10 mM AVG in the presence of different ethylene concentrations (0.01, 0.1, 1, and 10 μL L⁻¹). All seeds were pretreated with 10 mM AVG for 24 h prior to ethylene exposure for 72 h at each genotype’s corresponding germination temperature. C, Germination of PI, NIL-PI, Sal, and NIL-Sal seeds
AVG-treated (10 mM) seeds was observed at 32°C for PI and 29°C for Sal seeds. At these temperatures, the AVG-treated seeds were thermo inhibited (approximately 20% germination), while control seeds still germinated maximally. In addition to establishing effective concentrations for AVG action, these experiments also confirmed that endogenous ethylene production increases the upper temperature limit for germination.

To confirm that the inhibitory action of AVG was due to the suppression of endogenous ethylene biosynthesis, rates of ethylene production by seeds of each genotype were measured at their germination temperature thresholds (29°C for Sal and NIL-Sal and 32°C for PI and NIL-PI). The highest rates of ethylene production occurred at 36 h after imbibition with water, with 60 μL mg⁻¹ h⁻¹ for PI seeds and 25 μL mg⁻¹ h⁻¹ for Sal seeds (Supplemental Fig. S7). Both NIL-PI (32°C) and NIL-Sal (29°C) seeds showed the highest rates of ethylene production at 48 h after imbibition with water, with 40 μL mg⁻¹ h⁻¹ for NIL-PI and 25 μL mg⁻¹ h⁻¹ for NIL-Sal. The rates of ethylene production declined after rising to a maximum in all genotypes. The rates of ethylene production remained very low and declined over time for all genotypes when seeds were imbibed in 10 mM AVG at the same threshold temperatures (Supplemental Fig. S7). The inhibitory effect of AVG on ethylene production was observed within 24 h of the initial imbibition. Overall, when imbibed with water for 72 h, PI and NIL-PI seeds had the highest total ethylene production at 2,310 and 1,860 μL g⁻¹ h⁻¹, respectively (Supplemental Table S6). However, when imbibed with 10 mM AVG, the total ethylene production for all genotypes was reduced to approximately 200 μL g⁻¹, similar to that for water-imbibed seeds at thermoinhibitory temperatures (Supplemental Table S6).

These tests showed that AVG-treated seeds of all genotypes would have similar low levels of endogenous ethylene production prior to being exposed to different concentrations of exogenous ethylene to test their sensitivity to ethylene perception and signaling. When exposed to different ethylene concentrations (0.01, 0.1, 1, and 10 μL L⁻¹) after pretreatment with 10 mM AVG for 24 h, both PI and NIL-PI seeds were able to germinate in the presence of 1 μL L⁻¹ ethylene at 32°C (Fig. 4B). In contrast, no germination was observed in Sal and NIL-Sal seeds that were exposed to 1 μL L⁻¹ ethylene at 29°C, and 10 μL L⁻¹ ethylene was required to recover full germination (Fig. 4B). Germination recovery of NIL-PI seeds was intermediate, with 50% germination at 1 μL L⁻¹ ethylene and 83% germination at 10 μL L⁻¹ ethylene. This confirms that the inhibitory effect of AVG on germination can be reversed by exposure to ethylene and indicates that seeds of PI and NIL-PI were more sensitive to ethylene compared with Sal and NIL-Sal seeds in promoting germination at their respective upper temperature thresholds.

In studies of the effects of ethylene on germination, application of ACC is often used as a substitute for gaseous ethylene and is effective in some seeds (Hermann et al., 2007; Lin et al., 2013). However, the lettuce endosperm is known to have limited permeability to various solutes (Salanenka and Taylor, 2011), so we tested whether ACC would be readily absorbed and converted to ethylene by intact, ungerminated lettuce seeds. In fact, this was not the case, and while bisected seeds readily converted ACC to ethylene, intact seeds produced no detectable ethylene prior to radicle emergence in the presence of 10 mM ACC (Supplemental Table S7). The intact lettuce endosperm is apparently not permeable to ACC, and reports of the effectiveness of ACC in promoting lettuce seed germination (Khan and Prusinski, 1989) could be due to embryonic tissues of early-germinating seeds breaking through the endosperm and converting ACC to ethylene, which in closed germination vessels would accumulate in the gas phase and exert effects on other ungerminated seeds (Gallardo et al., 1994).

**ABA and Htg9.1 Allelic Effects on Germination at High Temperature**

ABA is known to be involved in the thermoinhibition of lettuce seed germination (Hu et al., 2013), so Htg9.1 could act by altering ABA sensitivity or action in PI seeds. At 1 and 3 μM ABA, PI seeds exhibited a higher sensitivity to inhibition of germination compared with Sal seeds; at higher ABA concentrations (10 and 30 μM), seeds of both genotypes were unable to germinate (Supplemental Fig. S8). Based on these observations,

![Figure 4.](https://academic.oup.com/plphys/article-lookup/DOID)
10 μM ABA was selected for subsequent germination assays with ethylene.

Exposure to 10 μL L⁻¹ ethylene recovered approximately 60% to 80% germination of 10 μM ABA-pretreated PI, Sal, and NIL-Sal seeds (Fig. 4C). NIL-PI seeds showed less ethylene sensitivity (or higher ABA sensitivity) compared with PI seeds at 32°C, as 10 μL L⁻¹ ethylene could rescue only 10% germination. These results indicate that ethylene can counteract ABA inhibition of germination and that this effect may not be dependent on the allele of Htg9.1 that is present.

ABA biosynthesis is necessary for the induction of thermoinduction in Sal seeds and can be blocked by the carotenoid biosynthesis inhibitor fluridone (FLU), enabling germination at warm temperatures (Argyris et al., 2008; Huo et al., 2013). At a temperature that largely inhibited germination in water (31°C), 30 and 100 μM FLU were able to restore the germination capacity of Sal and NIL-Sal seeds to 100% (Fig. 4D). In contrast, only approximately 45% to 55% of PI and NIL-PI seeds were able to germinate at 35°C in both FLU concentrations (Fig. 4D). This suggests that ABA is not the sole factor limiting germination at high temperatures when the PI allele of Htg9.1 is present.

To examine whether FLU could recover germination phenotypes of each genotype after AVG treatment, seeds of all genotypes were pretreated with 10 mM AVG for 24 h at their germination-permissive temperature thresholds (29°C for Sal and NIL-Sal and 32°C for PI and NIL-PI) followed by the addition of 100 μM FLU at the same temperatures. This concentration of AVG strongly inhibited germination in all genotypes, while FLU alone had no effect or only slightly increased germination at the threshold temperatures (Fig. 4E). In the presence of 10 mM AVG and 100 μM FLU, seeds of all genotypes were unable to germinate fully. PI and NIL-PI seeds germinated only 20%, similar to that in AVG alone, while Sal and NIL-Sal seeds exhibited approximately 60% final germination. This result demonstrates that seeds with the PI allele of Htg9.1 are more dependent upon ethylene biosynthesis for germination at high temperature than are seeds with the Sal allele.

**GA and Htg9.1 Allelic Effects on Germination at High Temperature**

GA is well known to be required for seed germination (Bewley et al., 2013), so the effect of blocking GA biosynthesis using the inhibitor paclobutrazol (PAC) on the germination of lettuce genotypes was tested at their maximum threshold temperatures allowing germination. At 100 μM and higher PAC, both PI and Sal seeds were not able to germinate at their respective germination-permissive temperatures (Supplemental Fig. S9). The inhibitory action of 100 μM PAC on germination could not be reversed with 10 μL L⁻¹ ethylene for Sal and NIL-Sal seeds at 28°C or for PI and NIL-PI seeds at 32°C (Fig. 4F). However, germination could be fully restored with the addition of 100 μM GA₃ solution following the ethylene exposure, indicating no nonspecific effect of PAC on germination (Fig. 4F). This result provides evidence that the gene responsible for the ability of Htg9.1 to increase maximum germination temperature acts upstream of or in conjunction with GA.

**Expression Analyses of Genes Associated with the Hormonal Regulation of Seed Dormancy and Germination**

Gene expression (mRNA abundance) assays were performed to assess the effects of exogenous ethylene on the expression of LsERF1 and other genes controlling biosynthetic and regulatory processes essential for seed germination and dormancy. The expression of genes in the ABA, GA, and ethylene biosynthetic and signaling pathways in the presence of water or 10 mM AVG and with or without ethylene was assessed at each genotype's maximum germination temperature threshold (29°C for Sal and 32°C for PI and NIL-PI). Consistent with prior germination assays, AVG-treated seeds of all genotypes failed to germinate at their corresponding germination temperature thresholds, and 1 μL L⁻¹ ethylene could promote germination recovery only in PI and NIL-PI seeds but not in Sal seeds (Fig. 4B). Thus, the expression of germination-associated genes LsERF1, LsAC51, LsAC1, LsGA3ox1 (GA 3-OXIDASE1; GA biosynthesis), and LsMAN1 (ENDO-β-MANNANASE1; germination-associated cell wall modification) and dormancy-promoting genes LsGA3ox2 (GA inactivation) and LsNCED4 was assessed by quantitative reverse transcription-PCR in seeds imbibed in water, AVG, or AVG plus 1 μL L⁻¹ ethylene. In addition, LsERF104, an ERF-like gene also found on scaffold 243, was assayed to test whether the expression of LsERF1 was unique across temperatures and genotypes. Expression patterns of these genes were similar in PI and NIL-PI seeds in all conditions (water, 10 mM AVG, and 1 μL L⁻¹ ethylene) when imbibed at 32°C, their maximum permissive temperature for germination (Figs. 5, A–F and E–H, and 6, A–D and E–H), confirming that it is the introgressed genomic region from PI that is controlling the responses of gene expression to temperature. Germination-associated genes (LsAC51, LsAC1, LsERF1, LsMAN1, and LsGA3ox1) all had high or increasing transcript levels at 6 and 12 h of imbibition in water. However, the presence of 10 mM AVG significantly lowered the expression levels of these germination-promoting genes in PI and NIL-PI seeds compared with water-imbibed seeds. The lower expression level in AVG-treated seeds was maintained until 24 h of imbibition. However, the subsequent exposure of AVG-treated PI and NIL-PI seeds to 1 μL L⁻¹ ethylene for 6 and 12 h induced large increases in the expression levels of these genes. The expression of LsAC51, LsAC1, and LsGA3ox1 genes was most responsive to ethylene, with approximately 20-fold increases compared with seeds in AVG without ethylene exposure (Figs. 5, A, B, E, and F, and 6, A and E). In contrast, LsERF104 expression was unaffected by the treatments, remained high in all conditions, and was not correlated with germination behavior (Fig. 6, D, H, 24 September 2022).
The expression levels of dormancy-associated genes (LsGA2ox2 and LsNCED4) were equal to or higher in AVG-imbibed PI and NIL-PI seeds than in water-imbibed seeds at 6 and 12 h of imbibition and decreased when seeds were exposed to 1 μL L⁻¹ ethylene (Fig. 6, B, C, F, and G). Thus, exposure to 1 μL L⁻¹ ethylene was clearly related to increases in germination-associated gene expression and decreases in dormancy-associated gene expression in these genotypes, consistent with the induction of germination by this ethylene concentration in seeds carrying the PI allele at Htg9.1.

Just below their maximum germination temperature threshold, water-imbibed Sal seeds exhibited relatively high and increasing expression of germination-associated genes (LsACS1, LsACO1, LsERF1, and LsMAN1; Figs. 5, I–L, and 6). However, all these genes showed reduced expression from 6 to 24 h of imbibition when treated with 10 mM AVG. Exposure of AVG-treated Sal seeds to 1 μL L⁻¹ ethylene for 6 h induced slight increases in the expression of all germination-promoting genes except LsGA3ox1, but expression did not increase further at 12 h. Failure of ethylene to induce greater or sustained expression of these genes is consistent with a lack of germination of these seeds in response to 1 μL L⁻¹ ethylene (Fig. 4B). An opposite pattern was observed with dormancy-associated genes such as LsGA2ox2 and LsNCED4, whose expression was higher in AVG-imbibed compared with water-imbibed Sal seeds and remained high or increased over time (Fig. 6, J and K). Exposure to 1 μL L⁻¹ ethylene for 12 h wholly (LsGA2ox2) or partially (LsNCED4) reversed the effect of AVG on Sal seeds for genes associated with dormancy (Fig. 6, J and K). Thus, these patterns of gene expression are consistent with the effects of AVG and ethylene on germination.

Promoter Response Elements of Germination-Associated Genes

Given their consistent expression patterns in response to AVG and ethylene, the promoter regions of

Figure 5. Expression of genes associated with the putative candidate gene of Htg9.1 (LsERF1) and ethylene biosynthesis and signaling pathways in seeds of PI at 32˚C (A–D), NIL-PI at 32˚C (E–H), and Sal at 29˚C (I–L) in various conditions (bars from left): water after 6 h of imbibition; water after 12 h of imbibition; 10 mM AVG after 6 h of imbibition; 10 mM AVG after 12 h of imbibition; 10 mM AVG after 24 h of imbibition; or 24 h in 10 mM AVG before being exposed to 1 μL L⁻¹ ethylene for 6 or 12 h. Results are shown for (from left) LsACO1, LsACS1, LsERF1, and LsMAN1. Error bars indicate s.e. (n = 3 biological replicates).
**LsACS1, LsACO1, LsMAN1, and LsGA3ox1 genes** were examined for potential transcription factor binding sites based on cis-element prediction using PLACE software (Supplemental Table S8). Among the identified binding motifs commonly shared by these genes were the following: (1) the W-box originally identified in the promoter region of the tobacco (*Nicotiana tabacum*) ERF3 gene (Nishiuchi et al., 2004); (2) ethylene-responsive elements (ERELEE4; Audran-Delalande et al., 2012); and (3) ABA-responsive elements recognized by RELATED TO ABI3/VP1 transcription factor (Kagaya et al., 1999). All genes except *LsACS1* contain the GARE2OSREP1 motif, a GA-responsive element in their promoter regions. Only promoter regions of *LsMAN1* and *LsGA3ox1* genes contain a heat shock protein-binding element (Supplemental Table S8).

**DISCUSSION**

Temperature is a critical environmental regulator of seed dormancy, signaling seasonal information to seeds to match their germination potential to periods favorable for seedling emergence and survival as well as influencing subsequent phases of the plant life cycle (Footitt et al., 2014; Batlla and Benech-Arnold, 2015; Burghardt et al., 2015; Huo and Bradford, 2015). In many crops, domestication has largely eliminated these natural dormancy mechanisms, but in others, such as lettuce, they persist and can cause problems for germination and synchronous crop establishment when temperatures exceed permissive limits (Cantliffe et al., 2000). Physiological treatments such as seed priming (prehydration and drying) can overcome such temperature-induced inhibition of germination (Schwember and Bradford, 2010), but a more general approach would be to identify the genetic and physiological mechanisms underlying such thermoinhibition and develop cultivars that no longer exhibit the trait. In previous work, we identified a QTL (*Htg6.1*) and its underlying gene (*LsNCED4*) from the progenitor species of lettuce (*L. serriola*) that demonstrated the critical role of ABA in preventing germination at high temperatures (Argyris et al., 2005, 2008, 2011; Huo et al., 2013) and identified natural alleles that can be used by breeders to ameliorate seed thermoinhibition in lettuce.
cultivars. However, GA is also essential for seed germination (Toyomasu et al., 1993; Toh et al., 2008), and ethylene is known to promote seed germination, particularly in the Compositeae family under stressful conditions such as high temperatures (Saini et al., 1986; Khan and Prusinski, 1989; Huo and Bradford, 2015). Thus, natural variation is likely to present multiple genes and mechanisms that could be employed to domesticate lettuce seeds and overcome their natural thermoinhibition mechanisms.

Identification of the QTL Htg9.1 and Its Candidate Gene LsERF1

In this study, a second QTL (Htg9.1) in lettuce associated with the capacity for germination at high temperatures was identified in the primitive lettuce accession PI. A combination of genetic mapping and transcriptomic approaches led to the identification of LsERF1, a transcription factor acting at the end of the ethylene perception and action pathway, as the likely causal gene for the HTG phenotype of seeds carrying the PI allele at this locus. While differential expression alone is not conclusive evidence that LsERF1 is responsible for Htg9.1, it is consistent with that hypothesis, especially in combination with genetic mapping and supporting physiological data.

Seeds of lettuce genotypes with the ability to germinate in warm conditions often have higher ethylene production rates compared with thermoinhibited genotypes (Nascimento et al., 2000), as was also observed here. The greater sensitivity to ethylene of seeds carrying PI rather than Sal alleles at Htg9.1 also supports the hypothesis that LsERF1 is responsible for the higher upper temperature limit in the former genotypes. The absence of polymorphisms in the coding regions of LsERF1 between Sal and PI genotypes implies that differences in their phenotypes could be due to differential expression in response to temperature rather than to altered function of the encoded protein. This was the case also with different LsNCED4 alleles at Htg6.1 that affected thermoinhibition (Huo et al., 2013). The predicted binding motifs of the three SNPs identified in the promoter region of LsERF1 have not previously been associated with germination or thermoinhibition. However, polymorphisms in the promoter regions for temperature-associated enhancer/activator binding to cause differential transcriptional regulation in the two genotypes might be located farther upstream of the examined regions (Sanyal et al., 2012). The identification of an ethylene-associated transcription factor at Htg9.1, rather than a component earlier in the ethylene perception and signaling pathway, is consistent with a relatively specific role in seed germination and a lack of observed phenotypes indicating broad effects on ethylene responses in other aspects of development.

ERF Transcription Factors Are Involved in the Regulation of Seed Germination

ERFs are members of a family of transcription factors specific to plants that are involved in the ethylene-signaling pathway. The Arabidopsis ERF (AtERF) family of transcription factors has approximately 120 members, all of which contain a highly conserved ERF domain (Yang et al., 2009). The mapped LsERF1 gene on scaffold 243 shares the highest sequence similarity with Arabidopsis AtERF1, which encodes a group III ethylene-responsive element-binding protein-type transcription factor (Lee and Kim, 2003). The AtERF1 protein recognizes and binds to a short cis-acting genomic element known as the GCC box (AGCCGCC; Ohme-Takagi and Shinshi, 1990). AtERF1 can also bind to the DRE/CRT (dehydration-responsive element/ C-repeat) cis-acting element in response to specific abiotic stresses (Cheng et al., 2013). To our knowledge, our study is the first to propose a specific role for ERF in relieving thermoinhibition. However, overexpression of AtERF1 enhanced heat shock stress tolerance due to the activation of heat shock-associated genes as well as improved tolerance to salt and drought stress (Cheng et al., 2013). Those authors proposed that the ability of ERF1 to bind to different promoter motifs in response to different stresses enabled it to integrate jasmonate, ethylene, and ABA signals in response to both abiotic and biotic stresses. This could be due to changes in the protein-binding affinity or association with other binding partners rather than through large changes in transcription, consistent with the relatively small differences in the LsERF1 transcript levels observed among PI, NIL-PI, and Sal seeds in response to ethylene, despite their differences in germinability at high temperature. A more specific role of LsERF1 in regulating thermoinhibition is also supported by an elevated LsERF1 transcript level only in lettuce seeds imibed at high temperature (30°C).

Although not previously associated with thermoinhibition, ERF transcription factors have been studied previously in connection with germination and dormancy. The expression of HaERF1 increased by 5-fold in dormant sunflower seeds in response to cyanide, which alleviated dormancy (Oracz et al., 2008). In beechnut (Fagus sylvatica) seeds, the expression of FsERF1 increased as dormancy was progressively released by moist chilling and ethephon (an ethylene-releasing compound) but was very low when dormancy was enhanced by ABA or warm temperatures (Jimenez et al., 2005). Group VII ERFs have been shown to regulate the expression of ABSCISIC ACID INSENSITIVES, which indirectly modulates the mechanism of nitric oxide control in germination (Gibbs et al., 2014). Another ERF (AERF1) enhanced the sensitivity of seed germination to ABA and salt stress (Lee et al., 2015). Suppression of the expression of Arabidopsis ERFs through histone deacetylation also inhibits germination (Wang et al., 2013). Tomato (Solanum lycopersicum) SIERF2 overexpression lines exhibited premature germination, further implicating ERF genes in promoting germination (Pirrello et al., 2006). An interaction between an ERF transcription factor and GA3ox1 expression was recently suggested to be involved in the regulation of tomato seed germination (Martinez-Andujar et al., 2012; Nonogaki, 2014). It was proposed that, in tomato seeds,
GA biosynthesis first occurs to promote growth in the embryonic radicle/hypocotyl, which exerts mechanical pressure on the adjacent endosperm cap to stimulate ethylene-related wounding responses that lead to the induction of the \textit{ERF1} gene, which, in turn, promotes endosperm cap-specific genes that weaken the cap tissue to allow radicle emergence.

Previous studies showed that ethylene induces endosperm weakening for radicle protrusion in association with the activation of EBM (Nascimento et al., 2001, 2004; Pirrello et al., 2006). Here, we observed essentially parallel expression of \textit{LsERF1} and \textit{LsMAN1} in lettuce seeds. The rapid response of \textit{LsMAN1} expression during the first 6 h after ethylene exposure in seeds having PI alleles at \textit{Htg9.1} is consistent with the high levels of EBM activity detected during early phases of lettuce seed germination (Nascimento et al., 2000), as also occurs in tomato (Nonogaki and Morohashi, 2005; Finkelstein, 2013). In lettuce, enhanced expression of alleles of \textit{LsNCED4} in cultivated varieties following imbibition at elevated temperatures results in higher seed ABA contents and inhibition of germination, while the promoter of the US96UC23 allele of \textit{LsNCED4} is not induced by high temperature, ABA content remains low, and germination is not prevented (Huo et al., 2013). In contrast, high temperature results in low levels of active GA due to suppression of the gene encoding GA 3-oxidase, \textit{GA3ox1} (Argyris et al., 2008; Toh et al., 2008). Low abundance of active GA is also promoted through GA catabolism via the expression of \textit{GA2ox2}, encoding GA 2-oxidase (Yamaguchi et al., 2007; Argyris et al., 2008). Elevated levels of GA can alter the expression of ABA-responsive genes and suppress ABA accumulation through the promotion of ABA catabolism (Toyomasu et al., 1998; Ogawa et al., 2003; Finkelstein, 2013). Cross talk between ABA and GA pathways is such that GA widens the permissive temperature window and promotes germination while ABA narrows the temperature window and promotes dormancy (Fig. 7; Argyris et al., 2008; Bewley et al., 2013; Huo and Bradford, 2015).

Some authors have asserted that ethylene is essential for germination (Abeles, 1986; Khan and Prusinski, 1989; Saini et al., 1989), while others have viewed it as a consequence of germination (Fu and Yang, 1983). Endogenous ethylene appears to be important for thermoinhibition release in some species, including lettuce.
as high temperatures decrease ethylene biosynthesis and sensitivity to ethylene action. Previous studies with lettuce seeds have shown that ethylene can only promote germination during imbibition at warm temperature in the presence of light (Saini et al., 1989). When seeds were imbibed in the dark, thermonhibition could be alleviated by exogenous ethylene only when accompanied by GA (Saini et al., 1986). Likewise, germination of L. serriola US96US23 seeds is light dependent, but they can germinate in the dark when provided with exogenous GA (Argyris et al., 2008). These observations are explained by the promotion of GA biosynthesis by red light in imbibed lettuce seeds (Toyomasu et al., 1993). Genes associated with GA and ethylene biosynthesis in lettuce seeds exhibit parallel expression patterns under both germination-permissive and -inhibiting conditions (Figs. 5 and 6; Argyris et al., 2008), suggesting that they may act in concert to promote germination. The full recovery of germination in Sal and NIL-Sal seeds after FLU treatment is consistent with the established role of ABA in regulating thermonhibition in Sal seeds (Argyris et al., 2008, 2011; Huo et al., 2013). However, the only partial germination recovery observed in the presence of FLU for seeds with PI alleles indicates the involvement of other factors in regulating the germination of PI seeds. Seeds with PI alleles at Htg9.1 continue to be inhibited at high temperatures even in the presence of FLU, indicating their greater dependence upon ethylene compared with seeds having Sal alleles at that locus.

GA and ethylene have been described as acting independently to interfere with ABA inhibitory action for dormancy release (Linkies et al., 2009; Linkies and Leubner-Metzger, 2012). This study suggests that, in lettuce, there is a concerted action of ethylene and GA to overcome the inhibitory effect of ABA and promote thermontolerance in seeds having PI alleles at Htg9.1 (Fig. 7). The ability of ethylene to stimulate both Sal and PI seeds to germinate even in the presence of ABA at their germination-permissive temperatures could be due to ethylene altering the GA and ABA hormonal balance to favor GA (Fig. 7). The opposite patterns of transcript abundances of LsNCED4 and LsGA2ox2 versus LsGA3ox1 in seeds with PI alleles at Htg9.1 emphasize the close relationship between ethylene and GA in suppressing ABA inhibitory action. The inability of ethylene to overcome the PAC inhibition of GA synthesis to promote germination indicates that GA acts downstream of the ethylene-signaling pathway. Our genetic, physiological, and gene expression data support a model in which ethylene promotes GA biosynthesis by LsERF1, thereby reducing the effectiveness of ABA in blocking germination at high temperature (Fig. 7). Based on promoter motifs, it is also possible that LsERF1 might directly promote endosperm cap weakening by stimulating the expression of LsMAN1. However, it is still likely that GA would be required for cell expansion in the embryonic axis for radicle emergence to occur, and our gene expression data do not show increases in LsMAN1 expression without corresponding increases in GA3ox1 expression. A model in which ethylene promotes GA biosynthesis and action would be consistent with earlier proposals that ethylene stimulates embryonic cell growth (Dutta and Bradford, 1994), as this is a known consequence of GA action. Thus, we favor a model in which ethylene acts through LsERF1 to promote GA3ox1 expression and subsequent GA action to overcome ABA inhibition and promote germination (Fig. 7).

While gene expression and physiological data indicate a positive relationship between LsERF1 and LsGA3ox1 in promoting thermontolerance in PI seeds, cis-element prediction of the LsGA3ox1 promoter region did not find the GCC box, which is a short cis-acting element recognized by ERF1 transcription factors (Ohme-Takagi and Shinshi, 1990). However, the 2-kb LsGA3ox1 promoter region contained four repeats of ERELE4, an ethylene-responsive element motif (Itzhaki et al., 1994), suggesting that the induction of LsGA3ox1 expression by the LsERF1 transcription factor could occur through the non-GCC ERF-binding sites, as was reported for the tomato EXPANSIN 11 gene (Martinez-Andujar et al., 2012). Further experiments are needed to conclusively confirm the proposed direct regulatory relationship between LsERF1 and LsGA3ox1. Nonetheless, the consensus of evidence from genetic, transcriptomic, and physiological assays strongly support LsERF1 as the causal gene determining Htg9.1 and a model in which ethylene acts through a GA-dependent pathway to alleviate the thermonhibition of lettuce seeds (Fig. 7).

**MATERIALS AND METHODS**

**Seed Production of Parental Lines and the RIL Mapping Population**

Seeds of an F6 RIL lettuce (Lactuca sativa) mapping population along with the parental lines, Sal and PI, were grown in the field in the summer of 2004 at two locations, at the University of California, Davis, and in the San Joaquin Valley (O’Brien, 2007), and again in 2009 at the University of California, Davis. The RIL population seed production in the San Joaquin Valley was carried out by Progeny Advanced Genetics seed company. The RIL population was developed by R.S. at the U.S. Department of Agriculture, Agricultural Research Service Research Center in Salinas, California (Grube, 2004). The RIL mapping population is composed of 161 F6 families derived by single-seed descent from an F2 population following a cross between PI (female) and Sal (male). The RILs requiring the longest time to reach maturity were planted first, whereas the more rapidly flowering lines were planted later to approximately synchronize flowering so that seed matured in a common environment. Even though lettuce is highly self-pollinating, each plant was bagged at flowering to prevent outcrossing among the RILs. Seeds from all RILs were harvested within 1 month (from mid-August to mid-September). Since flowering is indeterminate, a plot would be harvested when at least two-thirds of the plants had completed seed maturation. Seeds were harvested, cleaned via air screen machine, and stored in paper envelopes at 9°C and 30% relative humidity.

**Map Construction and Genotyping**

An Illumina SNP array, OPA4, was generated as part of the Compositae Genome Project to detect SNPs in expressed genes and evenly distributed markers in lettuce using Illumina Golden Gate technology (http://csgdb.ucdavis.edu/database). This array was used to genotype each RIL from the PI × Sal mapping population for 192 polymorphic SNPs. In addition, 233 co-dominant amplified fragment length polymorphism markers were scored by Keygene from 13 primers based on EcoRI/MseI templates for the same mapping population (O’Brien, 2007). An integrated genetic linkage map consisting of...
these amplified fragment length polymorphism and SNP markers across all nine chromosomal linkage groups was generated using JOINMAP version 3.0. This genetic map was used in the initial QTL analysis of the HTG trait, which led to the identification of a major QTL (Htg9.1) on chromosome 9 (O’Brien, 2007).

Several strategies were utilized to increase marker density within the Htg9.1 region, including a lettuce GeneChip microarray from the analysis of a different RIL population, Sal × US96UC23 (Truco et al., 2013). An RNASeq data set from lettuce seeds and seedlings also identified SNPs within the Htg9.1 interval. SNPs that were detected in the parental lines in both SNP mining approaches were used by LGC Genomics (http://www.lgcgenomics.com/) to genotype each RIL in the PI × Sal mapping population at each locus using the KASP genotyping platform for allele-specific PCR. The haplotype data of the PI × Sal RIL population from the newly added markers were integrated into a new genetic map of linkage group 9 with JOINMAP version 3.0.

Phenotypic Analyses
Germination tests were conducted with three replications of 25 seeds sown onto one layer of absorbent blotter paper discs (VWR Scientific Products) in 4.7-cm petri dishes moistened with 4 mL of deionized water, hormone, or hormone solutions. Specimens were placed on a thermostatically controlled platform at constant temperature conditions from 24°C to 40°C with 1°C increments. Alternatively, seeds were placed into a germination incubator (Hoffman Manufacturing) under continuous fluorescent light and constant temperature conditions at 30°C and 35°C or other specific temperatures as needed. Germination was scored as visible radicle emergence at 72 h after imbibition. For the measurement of ethylene biosynthesis, seeds (30 per replicate) were weighed and placed in a 12-mL glass vial and deionized water or 10 mM AVG (300 μL) was added to each vial, which was then sealed with a rubber septum. The ethylene content of the air space in the vials was measured every 12 h for 144 h using a gas chromatograph equipped with a flame-ionization detector that was calibrated with an ethylene standard prior to measurement (Saltveit and Yang, 1987). In each measurement, 1-mL gas samples were withdrawn with a plastic syringe and injected into the gas chromatograph. The vials were flushed and rescaled after each measurement. The total amount of ethylene produced over 72 h was calculated by summing the volumes of ethylene produced in the intervals between measurements.

QTL Analysis for the HTG Phenotype Using the RIL Mapping Population
QTLs associated with HTG were detected in the RIL population using WinQTL version 2.5 (North Carolina State Bioinformatics Research Center). Specifically, composite interval mapping analysis was performed on mean percentage germination by averaging the germination percentages of three replicates of each RIL at a given constant temperature from three different field production environments. The mean germination percentages were transformed by the probit method (PROC PROBIT; SAS Institute) to normalize variances and data distributions. The threshold for a significant (P = 0.05) log of the odds value for QTLs was determined by 1,000 permutations of the phenotypic data. Following the initial QTL analysis, two different genetic maps with additional SNP markers on chromosome 9 were generated. These maps were used to reanalyze the same phenotypic data to refine the confidence interval of the Htg9.1 QTL.

Development of a NIL Population for Htg9.1

QTL Validation
An NIL population was developed by three generations of backcrossing with Sal as the RP using marker-assisted selection (Supplemental Fig. S2). The donor parent of the Htg9.1 interval with PI alleles was RIL 98 from the F6 RIL mapping population. Marker NM4182, which was located in the center of Htg9.1, was used to distinguish BC3S1 plants containing homozygotes and heterozygotes for Htg9.1. Seeds from 88 BC3S1 plants (BC3S2 seeds) were used for germination tests at 32°C (Supplemental Table S1) to validate the effect of Htg9.1 on HTG phenotype. BC3S2 seeds of some NILs were germinated at higher temperatures (33°C and 34°C) to determine the dominant or recessive effect of the candidate allele on seed germination at high temperatures (Supplemental Table S2). Based on both allelic marker and phenotypic data of BC3S2 seeds, NIL-PI and NIL-Sal were selected for downstream germination assays. NIL-PI plants are the result of introgression of the PI allele of Htg9.1 for three backcross generations into the Sal genetic background, and NIL-Sal plants are segregants from this process with the Sal Htg9.1 allele. Seeds were harvested, cleaned by hand, and stored in paper envelopes at 9°C and approximately 30% relative humidity for 1 month before physiological and expression assays.

Development of an F2 Population for HTG-Associated Candidate Gene Mapping
An F2 population was developed from a cross between RIL 107 (female) and RIL 58 (male) from the F6 RIL mapping population to resequence the Htg Interval (Supplemental Fig. S4). The crosses and F2 population were grown separately in a greenhouse at 25°C/18°C in a 12/12-h photoperiod with a light intensity of 400 W m–2. When the daylength was less than 12 h, supplemental light at the same intensity was used to extend the daylength. F1 plants were screened by PCR for heterozygous alleles at each marker locus to confirm cross pollination. F2 plants were genotyped and screened for recombinants using four markers flanking and within the Htg9.1 QTL (Supplemental Table S3). Recombination was detected by the presence of heterozygous alleles for at least one of the four markers. F3 plants were genotyped and screened with all markers to identify lines with homozygous recombinant alleles to immortalize the recombination events from the F2 generation. F4 seeds were harvested and tested for germination at 32°C and also across a temperature range from 29°C to 32°C with 1°C increments. The germination value for each recombinant line was determined as the sum of the areas under the temperature × germination percentage curve across temperatures. The same germination values used for marker-phenotype association with the initial four markers were utilized for comparison with 41 additional SNP markers.

Differential Expression Analysis
Samples of mRNA were isolated from 150 mg of PI and Sal seeds (dry and imbibed for 6, 12, and 24 h) using a high-throughput protocol for Illumina RNAseq library preparation (Kumar et al., 2012). Complementary DNA libraries (three biological replicates) were prepared as in Zhong et al. (2011) with the elimination of the PCR enrichment step prior to sequencing. Approximately 30 million strand-specific, 100-bp paired-end reads were obtained for each library (F.-Y. Young, R.W. Michelmore, and K.J. Bradford, unpublished data). The abundance estimation for each transcript in read count format was obtained using a PLR script (L. Cortes, University of California, Davis) and normalized using the DESeq2 package (Love et al., 2014). Normalized transcript counts from RNAseq transcriptomic data of PI and Sal seeds imbibed for 0 (dry seeds), 6, 12, and 24 h at 20°C and 30°C were used to examine the expression of all genes present in genomic scaffold 243.

Gene Expression Analyses by Quantitative Real-Time PCR
For expression assays of specific genes, seeds of PI and NIL-PI were imbibed at 32°C, and Sal seeds were imbibed at 29°C. Three biological replicates (150 mg each) of PI, NIL-PI, and Sal seeds were collected after imbibition with deionized water or other solutions for 6, 12, or 24 h as indicated in the figure. Total RNA was extracted from these samples using a phenol-chloroform protocol with modifications and assayed by a real-time PCR system (StepOne; Applied Biosystems) as described previously (Argyris et al., 2008). The mRNA abundances of LsERF1, LsERF104, LsACO1, LsACO1, and LsNCED4 genes were analyzed. The abundance of mRNA for all genes was normalized against assays of three constitutively expressed genes (18S1, UBQ5, and ACT7) as described previously (Huo et al., 2013). Primer information for LsGA3ox1, LsGA2ox2, LsNCED4, LsACO1, and LsACO1 genes was as described by Argyris et al. (2008). Primers for LsERF1 and LsERF104 genes were designed based on genomic sequences of the genes on scaffold 243 obtained from the lettuce genome browser (http://genewer.genomecenter.ucdavis.edu/cgi-bin/gbrowse/lettuga_version_3_2/). Primer sequences were designed using Primer Express (Applied Biosystems) to amplify 50- to 150-bp PCR amplicons and were tested for efficiency prior to their usage in expression analyses (Supplemental Table S9).

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Germination time courses of PI and Sal seeds imbibed at 20°C and 30°C.
Suportal Figure S1: Development of an NIL population using marker-assisted selection to validate the effect of Htg9.1 on seed germination at high temperatures.

Supplementary Figure S2: Temperature sensitivity of the germination of seeds of RILs S8 and 107 from the PI × Sal RIL population.

Supplementary Figure S4: Development of an F2 population derived from a cross between two RILs from the PI × Sal RIL mapping population.

Supplementary Figure S5: Comparison of LsERF1 promoter sequences between PI and Sal alleles.

Supplementary Figure S6: Germination assays of PI and Sal seeds at temperatures ranging from 28°C to 35°C in water or AVG.

Supplementary Figure S7: Rates of ethylene production by PI, NIL-PI, and Sal seeds imibed in water or AVG at warm temperatures.

Supplementary Figure S8: Germination of PI seeds at 32°C and Sal seeds at 28°C after 72 h in the presence of different ABA concentrations.

Supplementary Figure S9: Germination of PI and Sal seeds in the presence of different PAC concentrations.

Supplementary Table S1: The association between marker NM4182 allele haplotypes and germination percentages of BC3F5 seeds at 32°C.

Supplementary Table S2: The association between marker NM4182 and germination percentages of BC3F5 seeds for 11 NILs at 32°C, 33°C, and 34°C.

Supplementary Table S3: Phenotypic association of F4 seeds of recombinant lines with four markers located across the Htg9.1 region.

Supplementary Table S4: Marker NM4182 mapped under the peak of the Htg9.1 QTL and was tightly linked to the candidate gene responsible for the primary effect of Htg9.1.

Supplementary Table S5: List of genes on scaffold 243 and the inferred functions of their encoded proteins.

Supplementary Table S6: Total ethylene production of PI, NIL-PI, Sal, and NIL-Sal seeds over 72 h of imbibition in water or AVG.

Supplementary Table S7: Measurement of ethylene production by intact or bisected PI seeds incubated at 32°C for 12 h.

Supplementary Table S8: List of predicted cis-element-binding motifs in promoter regions spanning 2 kb upstream of the predicted transcription start sites of LsACO1, LsACS1, LsGA3ox1, and LsMAN1.

Supplementary Table S9: List of genes and primer combinations used in quantitative reverse transcription-PCR assays of gene expression.

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High-Temperature Germination QTL Associated with ERF1


