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A gene encoding an abscisic acid biosynthetic enzyme (*LsNCED4*) collocates with the high temperature germination locus *Htg6.1* in lettuce (*Lactuca* sp.)

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Abstract Thermoinhibition, or failure of seeds to germinate when imbibed at warm temperatures, can be a significant problem in lettuce (*Lactuca sativa* L.) production. The reliability of stand establishment would be improved by increasing the ability of lettuce seeds to germinate at high temperatures. Genes encoding germination- or dormancy-related proteins were mapped in a recombinant inbred line population derived from a cross between *L. sativa* cv. Salinas and *L. serriola* accession UC96US23. This revealed several candidate genes that are located in the genomic regions containing quantitative trait loci (QTLs) associated with temperature and light requirements for germination. In particular, *LsNCED4*, a temperature-regulated gene in the biosynthetic pathway for abscisic acid (ABA), a germination inhibitor, mapped to the center of a previously detected QTL for high temperature germination (*Htg6.1*) from UC96US23. Three sets of sister BC_3S_2 near-isogenic lines (NILs) that were homozygous for the UC96US23 allele of *LsNCED4* at *Htg6.1* were developed by backcrossing to cv. Salinas and marker-assisted selection followed by selfing. The maximum temperature for germination of NIL seed lots with the UC96US23 allele at *LsNCED4* was increased by 2–3°C when compared with sister NIL seed lots lacking the introgression. In addition, the expression of *LsNCED4* was two- to threefold lower in the former NIL lines as compared to expression in the latter. Together, these data strongly implicate *LsNCED4* as the candidate gene responsible for the *Htg6.1* phenotype and indicate that decreased ABA biosynthesis at high imbibition temperatures is a major factor responsible for the increased germination thermotolerance of UC96US23 seeds.

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Introduction

The genus *Lactuca* L. belongs to the Compositae or Asteraceae family and is represented by approximately 100 species distributed in warm and temperate regions of Europe, Asia, North America, Africa and Australia (Dziechciarkova et al. 2004; Lebeda et al. 2004). Lettuce (*Lactuca sativa* L.) is an important crop species with an annual farm-gate value of over \$2.7 billion in the US in 2007 (NASS 2008). Thermoinhibition (inhibition of seed germination by high temperature) in lettuce can delay or prevent germination in warm season plantings, resulting in reduced field emergence and stand establishment

(Valdes et al. 1985). Thermoinhibitory conditions often occur in major winter lettuce production areas of California and Arizona where temperatures permissive of germination are exceeded during late summer and fall plantings. Delays in germination and asynchronous emergence can diminish yield (Cantliffe et al. 1981), necessitate multiple harvests, and create other management problems through non-uniformity of crop development, resulting in losses in quality and profitability (Benjamin 1990).

The maximum temperatures for lettuce seed germination are genetically heritable (Argyris et al. 2005; Guzman et al. 1992) and are influenced by environmental factors, such as the temperature and light environment during seed maturation and germination (Contreras et al. 2008, 2009; Gray et al. 1988; Harrington and Thompson 1952; Hayashi et al. 2008; Kozarewa et al. 2006; Kristie and Fielding 1994; Saini et al. 1989; Sung et al. 1998). An intact endosperm envelope that encloses the lettuce embryo, but not the outer fused testa and pericarp, is essential for the imposition of thermoinhibition (Borthwick and Robbins 1928; Dunlap et al. 1990; Globerson et al. 1974; Sung et al. 2008).

Physiologically, the maximum germination temperature of lettuce seeds appears to be determined primarily through the antagonistic interactions of gibberellins (GA) and abscisic acid (ABA) (Gonai et al. 2004; Toh et al. 2008) and the regulation of their synthesis and degradation by phytochrome (Sawada et al. 2008a, b; Seo et al. 2006). Seeds of ABA-deficient and -insensitive mutants (*aba1* and *abi3*) in *Arabidopsis thaliana* germinate at supra-optimal temperatures, supporting a major role for ABA in the thermoinhibition mechanism (Tamura et al. 2006). The expression of genes involved in ABA biosynthesis in Arabidopsis (e.g., *AtNCED* genes encoding 9-*cis*-epoxycarotinoid dioxygenases) is up-regulated at high imbibition temperatures, with *AtNCED9* playing the major role in thermoinhibition, while the expression of genes involved in GA synthesis (e.g., *AtGA3ox* genes encoding GA 3β-hydroxylases) is down-regulated (Toh et al. 2008). In cultivated lettuce, ABA content is maintained at elevated levels when seeds are imbibed at high temperatures, but decreases rapidly when imbibition occurs at optimal temperatures for germination (Argyris et al. 2008b; Toh et al. 2008; Yoshioka et al. 1998). Gibberellins promote ABA catabolism and thus increase the maximum temperature of lettuce seed germination (Gonai et al. 2004). Ethylene application also promotes germination at high temperatures, and endogenous ethylene production is greater in seeds capable of germinating at high temperature (Kozarewa et al. 2006; Prusinski and Khan 1990; Saini et al. 1986). Large scale transcriptional profiling of genes involved in these hormonal biosynthesis and deactivation pathways showed that genetic variation in maximum germination temperature was associated with reciprocal changes in expression of ABA versus GA and ethylene biosynthesis, metabolism, and response genes (Argyris et al. 2008b).

As in many other crop species, molecular markers have been utilized in *Lactuca* species for taxonomic and biodiversity studies, germplasm identification, improving disease resistance, dissecting quantitative traits and marker-assisted selection (MAS) for crop improvement (Argyris et al. 2005; Dziechciarkova et al. 2004; Grube et al. 2005; Lebeda et al. 2007; Michelmore et al. 1991). Molecular marker identification has been enhanced through the development of an expressed sequence tag (EST) database of over 135,000 ESTs from multiple *Lactuca* species through the Compositae Genome Project (CGP) (http://cgpdb.ucdavis.edu/). This database has aided the construction of an integrated genetic map using six inter- and intra-specific crosses of *Lactuca* spp. and comprises over 2,700 markers spanning 1,505 cM in nine linkage groups (LGs) corresponding to the nine chromosomes of *Lactuca* (Truco et al. 2007). More recently, marker density of the integrated genetic map has been greatly augmented using a tiled microarray representing ∼29,000 unigenes for massively parallel genetic mapping, allowing over 10,000 polymorphic loci to be mapped (van Leeuwen et al. 2009). These resources have facilitated the identification of quantitative trait loci (QTLs) and the genetic mapping of candidate genes associated with seed germination, dormancy and thermotolerance in a recombinant inbred line (RIL) population derived from *L. sativa* cv. Salinas and *L. serriola* accession UC96US23 (Argyris et al. 2005, 2008a). For example, a major QTL for high temperature germination (*Htg6.1*) collocated with *LsNCED4*, a key regulated gene in the ABA biosynthetic pathway (Argyris et al. 2008b). The expression of this gene and seed ABA content were inversely correlated with the maximum germination temperature.

The dissection of the genetic architecture of quantitative traits is accomplished effectively through backcrossing and the development of near-isogenic lines (NILs). A gene, chromosomal segment, or QTL can be isolated in a different and uniform genetic background (that of the recurrent parent [RP]) and its additive effects validated (MacKay et al. 2009). NILs have broad applications as immortal mapping populations with greater mapping power as compared to RILs (Keurentjes et al. 2007). In lettuce, NILs and back-cross-inbred line populations have been used to study herbicide resistance, to detect markers associated with disease resistance, and to examine potentially beneficial traits derived from wild germplasm (Jeuken and Lindhout 2004; Mallory-Smith et al. 1990; Paran et al. 1991). NILs are particularly critical for studies of seed traits such as dormancy, as the status of an individual seed is scored as either germinated or not (qualitatively), while populations of seeds exhibit quantitative variation in dormancy

characteristics (Bradford 1996). To this end, NILs have been used effectively in validating seed dormancy QTLs in Arabidopsis and rice (*Oryza sativa* L.) (Bentsink et al. 2006; Gu et al. 2006). In lettuce, the *Htg6.1* QTL for high temperature germination conferred significant increases in the maximum germination temperature in advanced backcross (BC) progeny (Argyris et al. 2008a).

The present study builds on the genetic resources developed in *Lactuca* and extends previous work identifying the role of *Htg6.1* and different allelic forms of *LsNCED4* in regulating thermoinhibition in RILs and advanced BC progeny (Argyris et al. 2005, 2008a, b). Here we report on several candidate gene/QTL co-locations associated with seed traits and in detail on the collocation of *LsNCED4* with *Htg6.1*. We also describe the development and analysis of NILs introgressed with the *Htg6.1* QTL associated with germination thermotolerance.

Materials and methods

Genetic mapping of candidate germination/dormancy genes and QTL analyses

Development of the RIL mapping population derived from a cross between *L. sativa* cv. Salinas and *L. serriola* UC96US23 has been described previously (Argyris et al. 2005; Truco et al. 2007). Utilizing an EST-based mapping approach with sequences retrieved from the CGP database (http://www.cgpdb.ucdavis.edu/), unigene contigs with high protein sequence homology to known germination/dormancy-related candidates (reviewed in Bentsink et al. 2007) were identified through keyword or BLASTX searches (Altschul et al. 1997). Identification of polymorphic EST candidate germination and dormancy contigs and primer design followed the methodology of Lai et al. (2005) (Online Resource Table S1). Polymorphisms in polymerase chain reaction (PCR) amplicons were screened in parental lines Salinas and UC96US23 of the RIL population using agarose or single-stranded conformational polymorphism (SSCP) gel electrophoresis according to the methods of Bodenes et al. (1996) with the following modifications: gels were fixed and stained in a single step with a combination 1% acetic acid/0.15% silver nitrate solution for 10 min, rinsed in deionized H_2O, then developed in 1 M NaOH + 0.037% formaldehyde for 8–10 min. Gels were then rinsed thoroughly for 3 min, air-dried and scored. PCR products for genes of interest were sequenced to confirm their identities. The population of 90 RILs in F7 generation were genotyped with these markers and linkage analysis performed using JoinMap v. 2.0 (Stam 1995) to place candidates on the linkage map. A complementary approach used candidate gene sequences representing *L. sativa* deposited in GenBank and primer sets designed at intervals along the coding sequence to generate 250 bp PCR amplicons and subsequent analysis of these amplicons from both parent lines by SSCP for polymorphism detection.

For germination/dormancy genes from *L. sativa* found in GenBank that were either not found in the CGP database, not polymorphic, or not able to be mapped using the above approaches, a sequencing approach was adopted. Oligonucleotide primers were designed to the ends of full length coding regions and PCR amplicons for Salinas and UC96US23 were purified with ExoSAP-IT® reagent (USB, Cleveland, OH). Sequencing reactions were performed using a Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and sequencing analysis conducted by the genomics facility at UC Davis (http://www.cgf.ucdavis.edu/home/) on an ABI Prism-3730 DNA analyzer (Applied Biosystems, Foster City, CA). PCR products were sequenced up to $8\times$ redundancy to ensure accurate single nucleotide polymorphism (SNP) calls. Primer sets were then designed around putative SNPs detected between the Salinas and UC96US23 sequences, and PCR products were screened on SSCP gels to ensure SNPs were co-dominant and scorable. Screening in the RIL mapping population and genetic mapping were then performed as above.

A subset of 473 markers and mapped germination/dormancy candidate genes approximately 5–7 cM apart was chosen as the framework map (Online Resource Fig. S1) for analysis of phenotypic data for germination at 25°C (low temperature germination, LTG) and 35°C (high temperature germination, HTG), as well as germination at 20°C in darkness following exposure to far-red light (GFR). Data from F7:8 RIL populations consisting of 108 individuals grown in three environments (Davis, CA; Yuma, AZ; and De Lier, The Netherlands) in the summer of 2002 (Argyris et al. 2005) were reanalyzed using Windows QTL Cartographer V. 2.5 (Basten et al. 2001). Details of how these traits were measured are described in Argyris et al. (2005). Listwise deletion was employed to eliminate individuals missing phenotypic data. Subsequent QTL analyses were performed with data sets ranging from 94 to 74 RILs. Germination percentages were probit transformed and QTL effects identified by composite interval mapping (CIM) utilizing standard model 6 and parameters outlined previously (Argyris et al. 2005). QTL were verified by 1,000 permutations of phenotypic data and declared significant ($p < 0.05$) above the permuted log of the odds (LOD) threshold value. Multilocus and least square means analyses were conducted to estimate additive and interaction effects of QTL as described previously (Argyris et al. 2005; Gandhi et al. 2005).

Following the initial QTL analysis, markers from the high density Affymetrix GeneChip® mapping microarray

(van Leeuwen et al. 2009) were combined with the framework markers comprising LG6 from the *Lactuca* integrated map (Truco et al. 2007) to obtain a more densely populated genetic map for the refinement of the *Htg6.1* QTL interval. Single position polymorphisms (SPPs) were obtained for unigenes by analysis of 113 RILs hybridized in duplicate to the lettuce Affymetrix gene chip (van Leeuwen et al. 2008). SPPs were ordered using MadMapper (http://cgpdb.ucdavis.edu/XLinkage/MadMapper) and a SPP was selected from each distinct genetic bin (unique recombinations between adjacent markers) covering LG6 (van Leeuwen et al. 2009). The ordered markers without genetic distances were entered into QTL Cartographer to identify relevant sets of SPP genetic markers that were encompassed by the QTL interval. BLASTX (Altschul et al. 1997) was performed for all unigenes in the QTL interval against the Arabidopsis genome ($1 \times e^{-20}$ threshold) to obtain putative candidate gene identities. Markers encompassing the QTL interval plus the framework markers from the integrated map were entered into JoinMap V 4.0 (Stam 1995). The resulting maps with genetic distances were then reanalyzed in QTL Cartographer to determine the newly defined confidence intervals and effects of the *Htg6.1* QTL.

NIL development and testing

To create NILs for QTL *Htg6.1* that contained *LsNCED4*, MAS using foreground (linked to the QTL) and background (unlinked to the QTL) markers was performed during three generations of backcrossing with *L. sativa* cv. Salinas as the RP as outlined in Fig. 1. Foreground and background MAS was performed with PCR-based markers designed from EST sequences in the CGP database
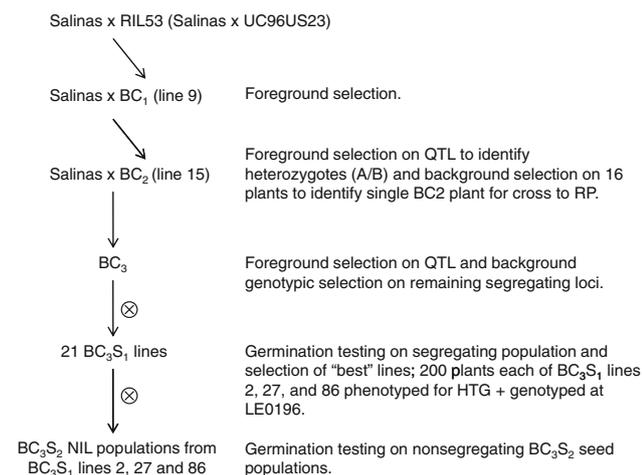
Salinas x RIL53 (Salinas x UC96US23)

Salinas x BC$_1$ (line 9)   Foreground selection.

Salinas x BC$_2$ (line 15)   Foreground selection on QTL to identify heterozygotes (A/B) and background selection on 16 plants to identify single BC2 plant for cross to RP.

BC$_3$ Foreground selection on QTL and background genotypic selection on remaining segregating loci.

⊗

21 BC$_3$S$_1$ lines   Germination testing on segregating population and selection of "best" lines; 200 plants each of BC$_3$S$_1$ lines 2, 27, and 86 phenotyped for HTG + genotyped at LE0196.

⊗

BC$_3$S$_2$ NIL populations from BC$_3$S$_1$ lines 2, 27 and 86   Germination testing on nonsegregating BC$_3$S$_2$ seed populations.

Fig. 1 Pedigree of NILs developed for the introgression of the *Htg6.1* QTL containing *LsNCED4* using backcrossing and marker-assisted selection

(http://www.cgpdb.ucdavis.edu/) (Online Resource Table S2). Markers were selected in areas of the donor parent (DP) genome at the outset of the backcrossing/MAS process to monitor gains in RP genome and QTL introgression based on a genetic linkage map composed of over 1,700 amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and EST markers coalescing into 9 LGs (corresponding to chromosome number) and spanning ∼1,213 cM. Details of map construction were as described previously (Truco et al. 2007). Selected markers were co-dominant and could be easily resolved in backcross populations. To genotype individuals during backcrossing, genomic DNA was extracted as described previously (Kesseli et al. 1994) and PCR-amplified using primer sequences for markers with designations from LE0000-LE3999 (Online Resource Table S2) and an annealing temperature of 57°C. Polymorphisms were visualized using SSCP gels.

The DP of the *Htg6.1* QTL allele was RIL53 of the F$_7$ generation of the RIL population (Fig. 1). While several RILs possessed the UC96US23 chromosomal segment containing the entire confidence interval of *Htg6.1* on LG6, RIL53 possessed the greatest number of RP alleles in comparison to other RILs. The progeny of the cross between Salinas and RIL53 was designated BC$_1$. MAS was performed using six foreground markers within and flanking the QTL interval on LG6. Selection on an additional 25 markers dispersed over the remaining eight LGs was performed concurrently to identify individuals with the highest proportion of the RP. Nine BC$_1$ lines were confirmed as heterozygous at marker LE0196 that was closest to the maximum QTL LOD peak at approximately 65 cM from the end of LG6 (the collocation of *LsNCED4* with *Htg6.1* was not determined until later, so this gene was not used in MAS). LE0196 corresponds to contig CLS_S3_Contig6125 on the high density map which subsequently mapped at 43.0 cM while *LsNCED4* mapped at 43.5 cM (Supplementary Table S3). An individual BC$_1$ plant was backcrossed again to the Salinas RP to yield 120 BC$_2$ plants (Fig. 1). Among these, 16 retained the entire *Htg6.1* QTL confidence interval, of which BC$_2$ NIL15 had the highest percentage of RP alleles. This line was therefore crossed to Salinas to yield 100 BC$_3$ NILs on which foreground and background selections were repeated. All BC$_3$ NILs were allowed to self-pollinate and seed populations segregating at the *Htg6.1* locus were tested for germination as detailed in Argyris et al. (2008a). BC$_3$ NILs 2, 27 and 86 were determined to have the *Htg6.1* introgression (heterozygous), a high percentage of RP genome homozygous, different segregating RP background loci, and their progeny had the greatest HTG percentages among the BC$_3$ NILs tested. Sister NILs derived from each of these three BC$_3$ NILs by one generation of selfing were

used for the evaluation of the effect of *Htg6.1* on increasing the maximum germination temperature.

From each of the BC$_3$ NILs 2, 27 and 86, 400 BC$_3$S$_1$ seeds were sown in Petri dishes at 33°C. From these, germinated seedlings were separated and recorded as displaying a HTG phenotype and the remaining seeds were transferred to 25°C until they germinated. Both HTG and non-HTG seedlings were transplanted to flats in a greenhouse at 25/15°C with ambient light duration and intensity when day length exceeded 14 h. Plants were maintained for 1 week at these temperatures and a 14/10 h light/dark diurnal cycle with a supplemental light intensity of 400 W/m^2 when daylength was <14 h. From the original group of 400 seeds, 200 seedlings (including all those exhibiting a HTG phenotype) of each of the three BC$_3$ NIL lines, as well as Salinas and UC96US23, were transferred to 10-cm pots on 10 November 2006 (Fig. 1). DNA was extracted from all BC$_3$S$_1$ NIL plants. The BC$_3$S$_1$ NILs were allowed to self-pollinate to generate BC$_3$S$_2$ seed. Seeds were hand-harvested beginning 5 April 2007 and continuing until 20 June 2007. Following harvest, seeds of each BC$_3$S$_2$ NIL were cleaned and stored in paper envelopes at 9°C and ∼25% relative humidity (RH) until tested.

Germination testing

Approximately 30 BC$_3$S$_2$ NIL seed lots that had been genotyped at *LsNCED4* (15 homozygous for Salinas alleles [A/A] and 15 homozygous for UC96US23 alleles [B/B] of each the BC$_3$ NILs 2, 27 and 86) as well as parental lines were germinated (two replications of 25 seeds each) on a thermogradient table at 1°C intervals from 27 to 33°C. To test the effect of the *LsNCED4* genotype on germination in larger populations, tests at 30°C were performed on three replicates of 25 seeds. Seeds were distributed on two absorbent blotter paper discs (VWR Scientific Products, West Chester, PA) saturated with 4 mL de-ionized water in 5 cm-diameter Petri dishes (Gelman Sciences, Ann Arbor, MI). Seeds were placed into a germination cabinet (Hoffman Manufacturing, Albany, OR) under continuous fluorescent light and constant temperature conditions and evaluated for germination (radicle emergence) at 72 h.

Gene expression assays

The expression of *LsNCED4* was measured in 100 seeds comprising 20 seeds from each of five BC$_3$S$_2$ NIL seed lots derived from of each of the three BC$_3$ NILs. These combined samples for seeds for each NIL, pooled separately by genotypic class (either A/A or B/B genotypes), were divided into three replications of each genotype and imbibed at 31°C for 24 h. At this time, germinated seedlings were removed, and the remaining seeds were frozen in liquid

nitrogen. RNA was extracted, cDNA synthesized, and quantitative PCR analysis performed as described previously (Argyris et al. 2008b).

Results

QTL and candidate gene mapping

Twenty-three lettuce genes identified in the CGP database and GenBank that could potentially be involved in germination or dormancy were mapped (Table 1). These candidates included genes encoding homologs of proteins involved in hormonal biosynthesis, signaling, or metabolism (9 for ABA, 8 for GA, and 2 for ethylene), a transcription factor involved in seed development (FUSCA3), a protein phosphatase 2C (FsPP2C), and a component of the sucrose non-fermenting (SNF)-related protein kinases (GAL83). At least one candidate gene mapped to each of the nine lettuce chromosomes, with five genes clustering within a 23 cM interval near the bottom of LG2 (Fig. 2). This cluster included two ethylene-related genes encoding ACC SYNTHASE (*LsACS1*) and ETHYLENE INSENSITIVE 2 (*LsEIN2*), two ABA-related genes encoding ENHANCED RESPONSE TO ABA (*LsERA1*) and XANTHINE DEHYDROGENASE (*LsXDH*) as well as *FUSCA3*. A second region of interest occurred on LG4, where 9-*cis*-EPOXYCAROTENOID DIOXYGENASE 1 (*LsNCED1*) and *ent*-KAURENE SYNTHASE (*LsKS* or *GA2*) were separated by only 0.3 cM. *LsNCED4* (GI:84579411) co-segregated with the maximum LOD peak identifying *Htg6.1*, as well as with QTL for germination sensitivity to ABA and GA, as reported previously (Argyris et al. 2008b). Subsequently, segregation analysis of SPP markers using the Affymetrix GeneChip provided an ultra-dense map averaging more than one marker per cM containing the *Htg6.1* QTL on LG6 (Fig. 3). All genes represented on the microarray that were polymorphic for SPPs and mapped within ∼10 cM of *LsNCED4* are listed in Online Resource Table S3. Other than *LsNCED4*, no obvious functional annotations associated with seed germination, plant hormones or temperature sensitivity were evident.

When a new QTL analysis was performed using the ultra high-density genetic map within the QTL region of LG6 and germination data from seeds produced in 2002, the *Htg6.1* QTL was detected in the Salinas × UC96US23 RIL populations grown at Davis and in The Netherlands, as reported previously (Argyris et al. 2005). *Htg6.1* was again detected in the same population from seeds produced in 2006 at Davis, strengthening the evidence for a major genetic component for HTG variation being determined by the *Htg6.1* QTL. The maximum 1-LOD peaks (ranging

Table 1 Candidate genes encoding proteins potentially involved in germination or dormancy mapped in a *Lactuca sativa* cv. Salinas ×
L. serriola UC96US23 RIL population

| Marker[a]	EST[a] or GenBank ID	Gene	Description	LG	Position[b]
ABA related					
LE4128	QGF5H03.yg.ab1	*XDH*	Xanthine dehydrogenase	2	109.6
LE4015	QG CA Contig2867	*ABA1 (ZEP)*	Zeaxanthin epoxidase	3	81.7
LE4019	QGG18L02.yg.ab1	*ABA3*	Molybdenum cofactor sulfurase	7	115.8
LE4010	QG CA Contig7543	*ABI5*	ABA insensitive 5	4	64.5
LE4245	AB120115	*LsAO1*	Abscisic acid aldehyde oxidase	6	21.1
LE4131	QG CA Contig6166	*LsNCED1*	9-*cis*-Epoxycarotenoid dioxygenase 1	4	141.8
LE4250	AB120110	*LsNCED4*	9-*cis*-Epoxycarotenoid dioxygenase 4	6	65.1
LE4129	QGG28D16.yg.ab1	*ERA1*	Enhanced response to ABA	2	100.3
LE4021	QG CA Contig3084	*SnRK2.2*	Protein kinase responsive to ABA	4	23.2
GA related					
LE4209	AB031204	*LsCPS1*	Ent-copalyl diphosphate synthase	9	79.7
LE4022	QGF25D22.yg.ab1	*LsKS*	Ent-kaurene synthase	4	142.1
LE4201	AB031206	*LsGA2ox1*	Gibberellin 2-oxidase 1	7	100.8
LE4251	AB012205	*LsGA3ox1*	Gibberellin 3β-hydroxylase 1	5	153.0
LE4220	AB012206	*LsGA3ox2*	Gibberellin 3β-hydroxylase 2	1	35.7
LE4025	QGF7L19.yg.ab1	*GA20ox*	Gibberellin 20-oxidase	5	41.3
LE4134	QG CA Contig7236	*GAR1*	Suppressor of gibberellin insensitive	8	18.4
LE4130	QG CA Contig5082	*GAI2*	Gibberellin insensitive 2	8	95.1
Ethylene related					
LE4034	QGG10D02.yg.ab1	*LsACS1*	ACC synthase	2	92.0
LE0358	QGC20F11.yg.ab1	*LsEIN2*	Ethylene insensitive 2	2	115.8
Miscellaneous					
LE4132	QG CA Contig5234	*FsPP2C*	Protein phosphatase 2C	9	89.7
LE4133	QGB11D05.yg.ab1	*FUSCA3*	DNA binding/transcription factor	2	102.8
LE4051	QGB28M14.yg.ab1	*GAL83*	Docking protein of SNF1 kinase	4	116.4

[a] http://www.cdgdb.ucdavis.edu

[b] December 2006 map: http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/

from 11 to 23) at 43.5 cM aligned with the map position of *LsNCED4* in all three cases and the 2-LOD *Htg6.1* QTL confidence intervals were approximately 1, 2.5 and 7 cM for seeds grown in Davis 2002, Netherlands 2002, and Davis 2006, respectively (Fig. 3). The proportion of phenotypic variance for HTG explained by the QTL ranged from 44 to 70% while the additive genetic effect of replacement of the Salinas haplotype with the UC96US23 haplotype at *Htg6.1* resulted in 19.6–56% mean increases in germination percentage at 35°C.

In addition to *Htg6.1* on LG6, five QTL for three traits in two environments collocated with candidate genes on three different LGs (Fig. 2). Four QTL were detected for germination in the dark after exposure to far-red light (GFR), two each from Davis- and Yuma-grown populations on LGs 4 and 7, respectively. *Gfr4.1* accounted for 9 and 23% of the phenotypic variation for GFR in the Davis and Yuma environments, respectively, and was localized to a 10 cM 1-LOD confidence interval. The QTL alleles from Salinas

resulted in a 14% mean increase in germination. *Gfr4.1* co-segregated with a homolog of *SnRK2.2*, an ABA-responsive protein kinase in Arabidopsis demonstrated to be involved in ABA signal transduction associated with seed dormancy (Fujii et al. 2007; Nakashima et al. 2009). The second pair of GFR QTL on LG7 (*Gfr7.1*) was of larger magnitude, accounting for 21 and 22% of the phenotypic variation for GFR in the Davis and Yuma environments, respectively, and resulting in a mean additive effect of a 22% increase in germination due to the Salinas haplotype. These QTL co-segregated with a gene potentially encoding GIBBERELLIN 2-OXIDASE 1 (*LsGA2ox1*) that is responsible for the metabolic inactivation of active GAs (Nakaminami et al. 2003; Wang et al. 2004). An analysis of the two-way additive effects of *Gfr*-linked marker loci revealed significant interactions within individual environments ($p < 0.05$ and $p < 0.001$ in Davis and Yuma environments, respectively) but not for data combined across environments. However, the presence of Salinas

Fig. 2 Candidate genes in the lettuce consensus map composed of nine linkage groups and collocations with QTL (LG6 is shown in Fig. 3). Mapped candidate genes are in *bold italics*. Genomic locations of QTL for seed germination traits detected with data reanalyzed for seeds grown in two environments are shown: *solid bars* Davis, CA, *horizontal open bars* Yuma, AZ. *Lengths of bars* denote 1-LOD confidence intervals. Phenotypic variances and additive genetic effects on germination percentage are adjacent to bars (A Salinas, B UC96US23)
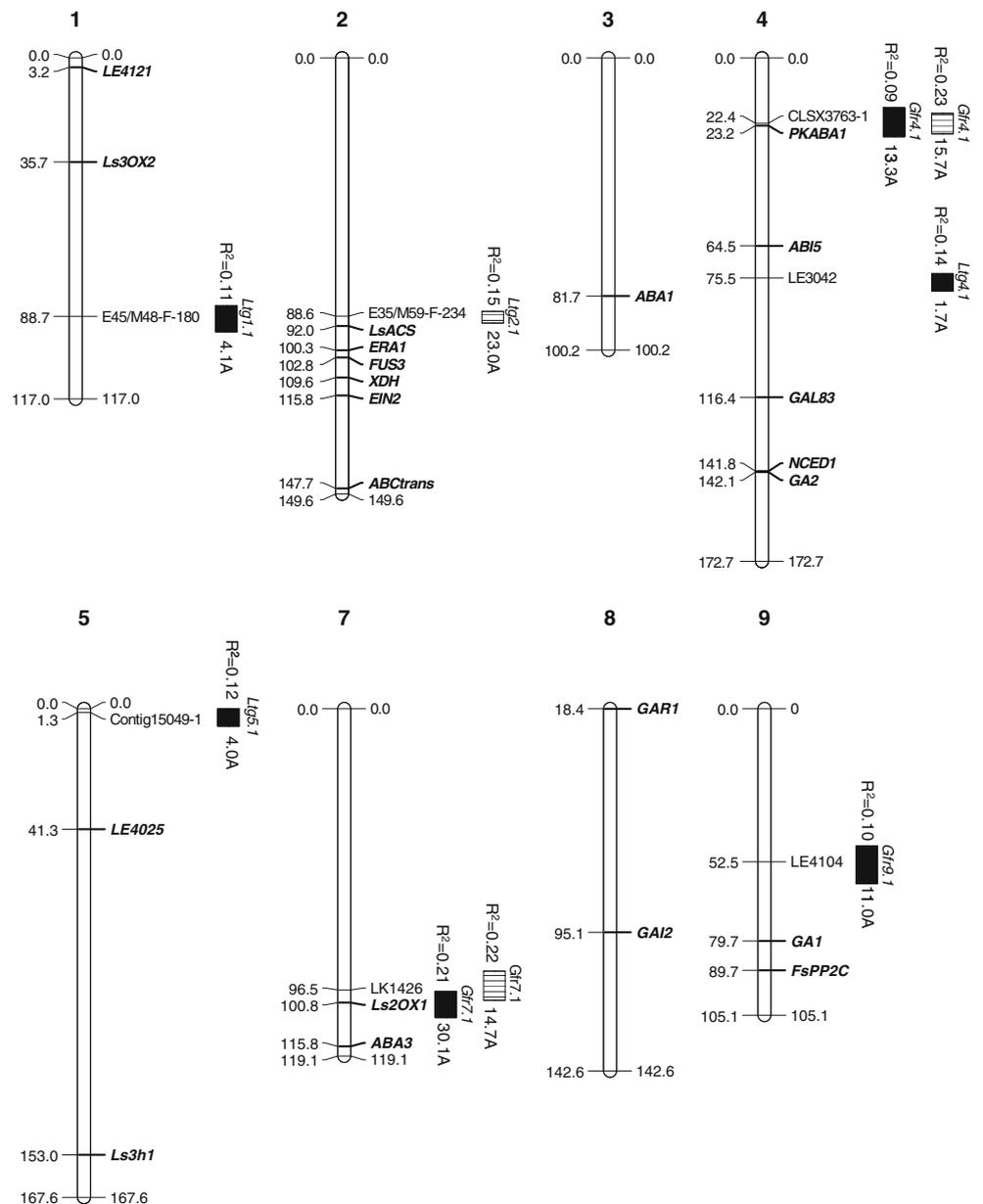
haplotypes at both *Gfr4.1* and *Gfr7.1* resulted in 42.9% mean germination as compared to 6.1% for UC96US23 haplotypes at the same QTL across both production locations, providing some evidence for epistatic interaction between these QTL in stimulating germination following exposure to far-red light.

A single QTL of large magnitude for germination at 25°C (LTG) accounting for 15% of the phenotypic variation was detected on LG2 for seeds grown in the Yuma environment (Fig. 2). The Salinas QTL haplotype resulted in a 23% increase in germination. A gene encoding ACC SYNTHASE (*LsACS1*) regulating the committed step of ethylene biosynthesis (Yang and Hoffman 1984) at 92 cM

mapped just outside the 2-LOD confidence interval for *Ltg2.1* (87–91 cM) (Fig. 2).

Analysis of HTG phenotypes in BC₃S₂ NILs fixed for Salinas or UC96US23 alleles of *LsNCED4* at *Htg6.1*

LsNCED4 was sequenced from BC_3S_2 sister NILs generated from each of the three BC_3 NILs to identify BC_3S_2 seed lots homozygous for each parental allele. Seed lots that were homozygous for the UC96US23 *LsNCED4* allele (B/B) had significantly higher mean germination ($p < 0.001$) over a temperature gradient from 28 to 32°C as compared to sister NIL seed lots homozygous for the

Fig. 3 Genetic map of LG6 and positions, LOD thresholds and genetic effects of *Htg6.1* using markers from the lettuce integrated map and SPP markers from a mapping microarray. Numbers indicate locations (in cM) of genetic markers used in CIM analysis. *Solid bar* Davis, CA 2002, *open bar* De Lier, The Netherlands 2002; *diagonal lined bar* Davis, CA 2006. Phenotypic variances and additive genetic effects are shown *adjacent to bars*. Germination/dormancy candidate genes located on LG6 are in *bold italics*. *Solid, dotted* and *dashed lines* in LOD graph represent Davis 2002, Netherlands 2002 and Davis 2006 populations, respectively. *Solid vertical line* at LOD 5 is average permutated threshold value for declaring QTL significant
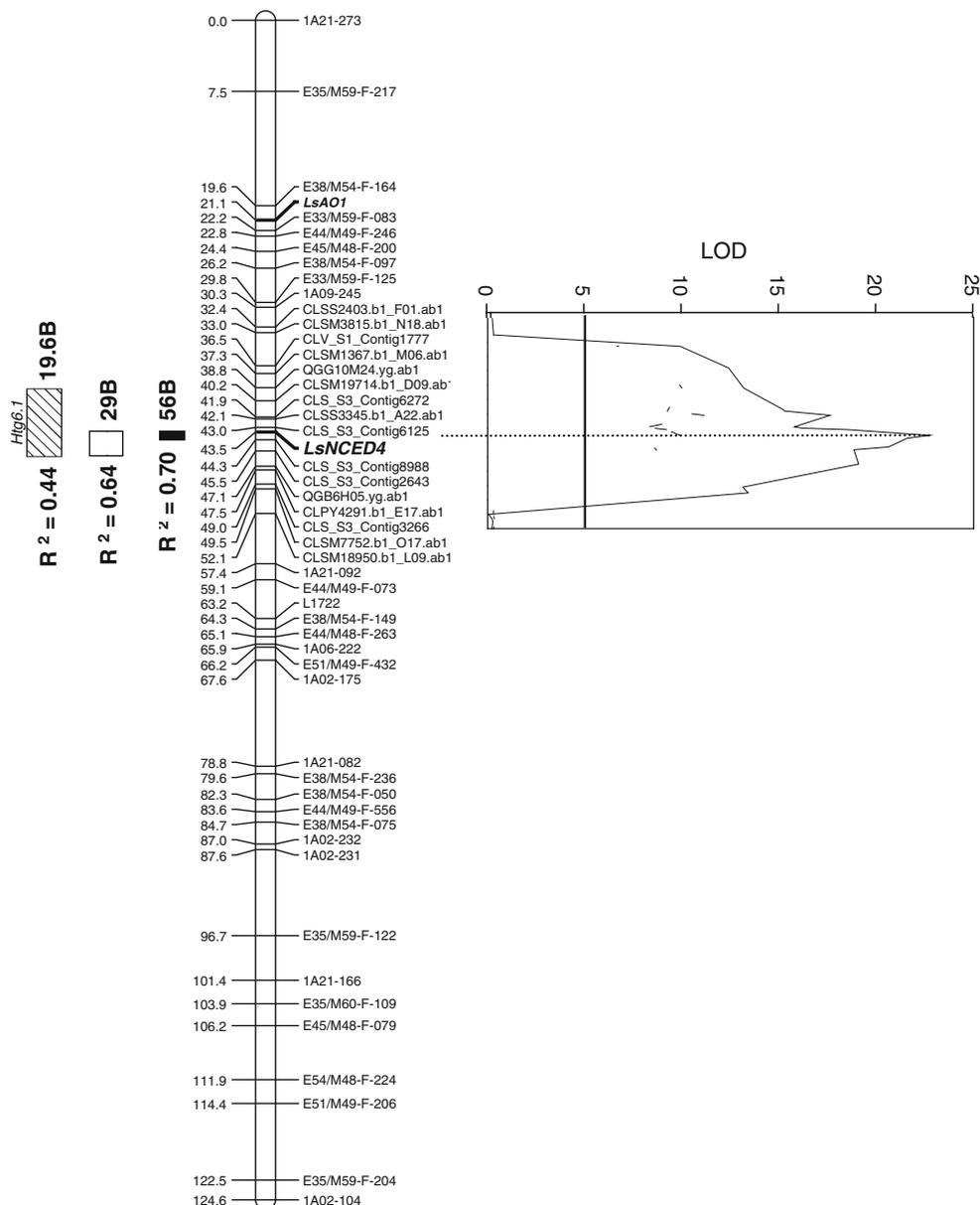
Salinas allele (A/A) (Fig. 4). Combined mean germination of the three sets of BC_3S_2 sister NIL populations of the B/B class was ≥86% up to 29°C before declining to 28% at 32°C. In contrast, the combined mean germination of A/A seeds lots began to decline sharply at 28°C, and was <10% at 30°C and <1% at 32°C due to thermoinhibition. The difference between the two groups was maximal at 30°C, where the combined mean germination of B/B seed lots exceeded that of A/A seeds by 66.2% (71.6% germination in the former as compared to 5.4% germination in the latter). The germination phenotype of A/A seed lots closely paralleled that of the Salinas parental line; however, germination of the UC96US23 parental line was greater than 95% up to 32°C, which was significantly higher than that of the B/B BC_3S_2 NILs (Fig. 4).

Germination of individual BC_3S_2 NIL seed lots homozygous for the B allele at *LsNCED4* varied widely when tested at 30°C, the temperature at which differences in germination between A/A and B/B genotypes were maximized (Fig. 5b, d, f). The overall mean germination for each set of B/B BC_3S_2 NIL populations ranged from 58.3 to 65.5%, and was highest in seeds derived from NIL 86. However, germination ranged from >20 to 100% in the 125 B/B BC_3S_2 NIL seed lots tested from the three sets of sister NILs. In nearly all cases, this was significantly higher than germination of the 70 BC_3S_2 NIL seed lots homozygous for the A allele at *LsNCED4*, which showed less variation and ranged from 0 to <15% germination in most lines (Fig. 5a, c, e). With the exception of four seed lots (2 derived from NIL 2 and 2 derived from NIL 86), there
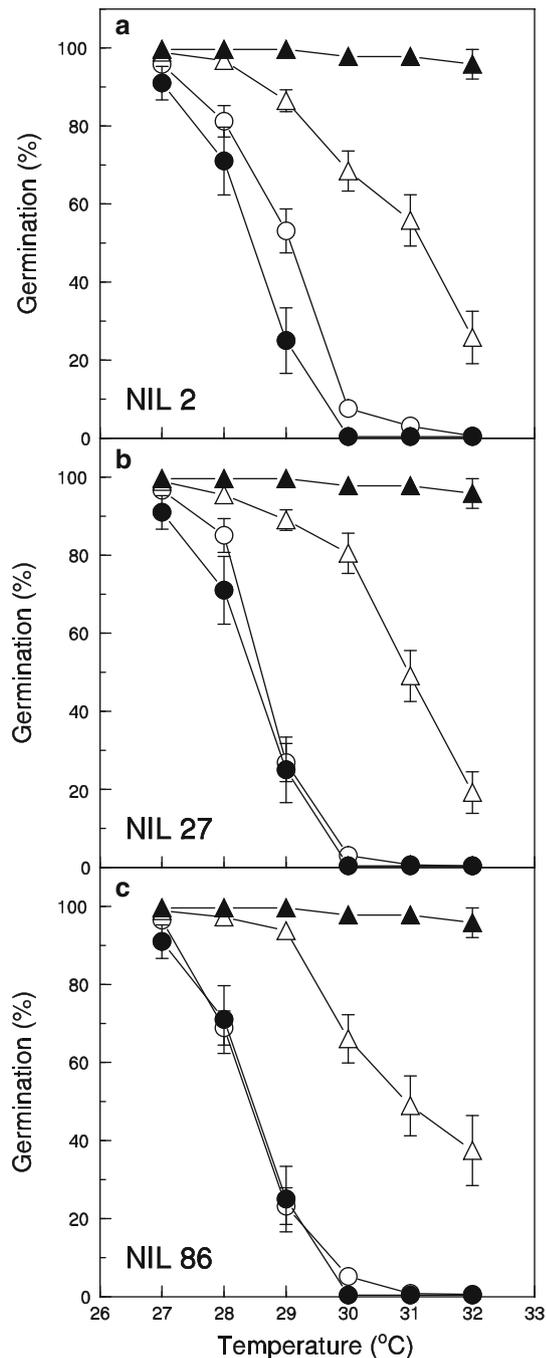
Fig. 4 Germination across a temperature gradient of Salinas (*filled circles*), UC96US23 (*filled triangles*) and 15 BC$_3$S$_2$ sub-NIL seed lots derived from NILs 2 (**a**), 27 (**b**), and 86 (**c**) having either Salinas (*A/A open circles*) or UC96US23 alleles (*B/B open triangles*) at *LsNCED4*. *Error bars* are ±SD. Differences in germination between BC$_3$S$_2$ NIL genotypic classes were significant ($p < 0.001$) between 29 and 32°C

Fig. 5 Germination phenotypes at 30°C of BC$_3$S$_2$ NIL seed lots homozygous at *LsNCED4* for either Salinas (*A/A* **a**, **c**, **e**) or UC96US23 alleles (*B/B* **b**, **d**, **f**) derived from NIL 2 (**a**, **b**), NIL 27 (**c**, **d**) and NIL 86 (**e**, **f**). The sub-NILs are ordered according to increasing germination percentages. The mean germination percentages averaged across sub-NILs were **a**, 5.1% ($n = 17$), **b** 60.7% ($n = 26$), **c** 2.7% ($n = 28$), **d** 58.3% ($n = 46$), **e** 5.4% ($n = 25$), and **f** 65.5% ($n = 53$). *Error bars* indicate ±SD

was a clear separation between the two allelic classes for germination at 30°C.

The expression of *LsNCED4* following imbibition at 30°C in pooled seed lots homozygous for the B allele and exhibiting a HTG phenotype was significantly lower in all

BC$_3$S$_2$ sister NILs when compared with seed lots homozygous for the A allele and exhibiting thermoinhibition (Fig. 6). Mean relative expression levels were similar in all B/B BC$_3$S$_2$ NILs and were 2–2.5-fold lower than in A/A BC$_3$S$_2$ NILs.

Discussion

Mapping of candidate germination/dormancy genes and collocations with QTLs

Genetic mapping of putative candidate genes in lettuce can determine whether they collocate with QTLs influencing germination and dormancy. Those genes that do not collocate can be ruled out as major genetic determinants of the
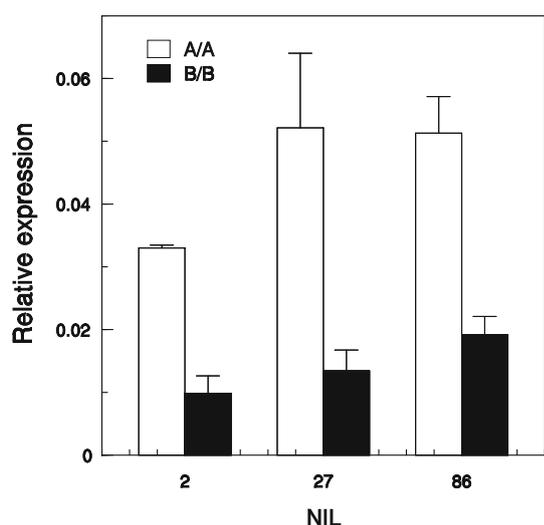
Fig. 6 Expression of *LsNCED4* at 30°C in seed lots pooled according to genotype at LE0196 (*Htg6.1*; 0.5 cM from *LsNCED4*). *A/A* Salinas genotype, *B/B* UC96US23 genotype. *Error bars* show SE of three biological replicates

phenotypes observed, although they may still be under the control of loci within a QTL. Although QTLs typically encompass large genomic intervals and many loci, those candidate genes that do collocate with QTL can suggest identity and function. We mapped 22 candidate genes known to be associated with seed germination and dormancy in a RIL population in which QTLs for germination traits had previously been characterized and identified possible functional associations underlying three QTL.

The *NCED* genes in Arabidopsis, particularly *AtNCED9*, have been shown to play a major role in thermoinhibition of germination (Toh et al. 2008). In lettuce, a major QTL associated with the alleviation of thermoinhibition (*Htg6.1*) was identified on LG6 (Argyris et al. 2005). We previously reported the collocation of *LsNCED4* (which has highest homology to *AtNCED6*) with *Htg6.1*, but the number of markers available at that time was insufficient to fine map the locus (Argyris et al. 2008b). The high-density SPP map developed using a custom Affymetrix GeneChip for lettuce allowed the construction of a high-density linkage map (van Leeuwen et al. 2009) that localized *Htg6.1* to substantially smaller genomic intervals (1–7 cM depending on the seed production environment) than the 15 cM previously reported (Argyris et al. 2005). The proportion of phenotypic variance and additive genetic effects explained by the UC96US23 QTL allele were larger than previously reported; this was largely due to the fact that the QTL analysis was done only with LG6, so all of the phenotypic variance was attributed to this linkage group.

Although not conclusively identifying *LsNCED4* as *Htg6.1*, the QTL interval has been reduced to as little as 1 cM centered on *LsNCED4* (Fig. 3), and no other

polymorphic genes known to map to this region encode proteins with obvious connections to germination (Online Resource Table S3). In addition, several ABA-, GA- and ethylene-related candidate genes can be ruled out as being responsible for the primary effect of the significantly increased maximum germination temperature because they segregate independently of *Htg6.1* (Table 1). These findings strongly point to *LsNCED4* as a key determinant of thermoinhibition in lettuce, consistent with its elevated expression and a higher ABA content in thermoinhibited seeds (Fig. 6; Argyris et al. 2008b) and with similar results for *NCED* genes in Arabidopsis (Gonai et al. 2004; Tamura et al. 2006; Toh et al. 2008). These data also demonstrate the powerful potential of SPP arrays for rapidly fine mapping and identifying candidate genes closely associated with QTL.

Salinas seeds are capable of germinating to high percentages in the dark at 20°C, while UC96US23 seeds require light for germination at all temperatures (Argyris et al. 2008b). Two pairs of QTL for germination in darkness at 20°C following far-red light exposure (GFR) on LGs 4 and 7 were associated with candidate genes for GA catabolism (*LsGA2ox1*) and ABA signaling (*SnRK2.2* or *PKABA1*) (Fig. 2). The QTL were detected in multiple environments and showed evidence of epistatic interactions; therefore, potentially identifying areas of the lettuce genome involved in differentially regulating sensitivity to light in Salinas as compared to UC96US23 seeds. GA metabolism via GA 2-oxidases may be involved in regulating GA content and action during germination (Argyris et al. 2008b; Nakaminami et al. 2003; Wang et al. 2004). Two *GA2ox* genes have been identified in lettuce, one of which (*LsGA2ox2*) was expressed primarily in seeds and showed some responsiveness to red light, although not to GA$_1$ (Nakaminami et al. 2003). The expression (mRNA abundance) of *LsGA2ox1* was higher in both Salinas and UC96US23 seeds imbibed in the dark when compared with seeds imbibed in the light, while *LsGA2ox2* mRNA was detected only briefly following imbibition and at low levels as compared to *LsGA2ox1* in both light and dark (Argyris et al. 2008b). Up-regulation of *LsGA2ox1* in the dark would be expected to reduce active GA levels and suppress germination.

SnRK2.2 mediates ABA signaling during seed germination in Arabidopsis, with *snrk2.2* mutants showing a reduced level of seed dormancy (Fujii et al. 2007; Nakashima et al. 2009). The full length *SnRK2.2* homolog in lettuce has not been cloned nor its expression studied, but up-regulation of *SnRK2.2* would be expected to enhance ABA action and suppress germination. Recently, a putative family of ABA receptor proteins (RCAR or PYR/PYL proteins) have been linked to modification of PP2C phosphatase and SnRK2 kinase activities in the ABA signal

transduction pathway (Cutler et al. 2010; Fujii et al. 2009; Ma et al. 2009; Park et al. 2009), adding significance to this collocation of a lettuce SnRK2.2 homolog with *Gfr4.1*.

Hayashi et al. (2008) identified two significant QTLs for HTG and four for the GFR trait in a cross between lettuce cultivars Diplomat and Margarita. Overlapping QTLs for HTG and GFR phenotypes were associated with a gene for white versus dark brown seed color that Hayashi et al. (2008) termed *br*. However, *br* was identified for brown versus black seed color and is located on chromosome 2 (O'Brien 2007; Waycott et al. 1999), while the *w* locus on chromosome 7 is associated with white versus brown seed color (Kesseli et al. 1994; Waycott et al. 1999). Because the testa color phenotypes in Diplomat and Margarita are dark brown and white, respectively, the locus identified by Hayashi et al. (2008) is likely associated with *w* rather than *br*. As *w* is located on chromosome 7, the HTG and GFR QTLs identified by Hayashi et al. may be associated with the *Gfr7.1* locus reported here. As AFLP markers were used to develop their map, it is not currently possible to compare the locations of these QTLs between the two populations nor to identify candidate genes associated with their QTLs. An additional QTL for HTG was identified on LG9 in a RIL population derived from a cross between Salinas and *L. sativa* accession PI251246 (O'Brien 2007). We are currently developing this population further to fine map this QTL relative to candidate genes. These multiple sources for tolerance of high germination temperatures and reduced light requirements offer the possibility of combining alleles to achieve higher maximum germination temperatures and less dependence on exposure to light in cultivated lettuce seeds.

Phenotypic effects of Htg6.1 in BC$_3$S$_2$ NILs

Introgression of the *Htg6.1* QTL containing *LsNCED4* reduced thermoinhibition and elevated the maximum germination temperature by 2–3°C in BC$_3$S$_2$ NIL seed lots of three sets of sister NILs derived from a single BC$_2$ plant (BC$_2$ NIL15) (Figs. 4, 5). Those lines homozygous for the UC96US23 allele at *LsNCED4*, which mapped at the maximum LOD peak of *Htg6.1* (Fig. 3), showed significantly higher germination at supra-optimal temperatures (28–29°C) and at higher temperatures where Salinas and BC$_2$S$_2$ NIL seed lots with the Salinas allele at *LsNCED4* were completely thermoinhibited (30–32°C). NIL seed lots carrying the UC96US23 allele at *Htg6.1* also exhibited a greater response to seed priming, a hydration/dehydration process that increases the maximum germination temperature of lettuce seeds (Schwember and Bradford 2010). The effect of the UC96US23 allele at *LsNCED4* in significantly increasing germination at high temperature in near-isogenic backgrounds, where effects of other loci influencing

HTG are minimized, supports earlier evidence for the gene as a major genetic determinant of maximum germination temperature in the Salinas × UC96US23 RIL population (Argyris et al. 2008a).

The effect of the *Htg6.1* QTL was maximal at 30°C (Fig. 4), although large variation in germination among individual BC$_3$S$_2$ NILs was observed (Fig. 5). This variance may have resulted from differences in genetic background due to the segregation of the few remaining polymorphic segments. In addition, characteristics of lettuce seed germination are influenced strongly by light and temperature conditions during seed maturation (Contreras et al. 2008, 2009; Hayashi et al. 2008); therefore, much of the variance observed between sister BC$_3$S$_2$ RILs may have been due to maternal environment during seed development. At a minimum, the effect of the UC96US23 QTL haplotype was significant in increasing thermotolerance as compared to BC$_3$S$_2$ NILs lacking the introgression and some NILs may have possessed additional loci that allowed germination at the higher temperatures. Further fine mapping, mutant and transgenic analyses are in progress to conclusively demonstrate the role of *LsNCED4* in determining the maximum germination temperature of lettuce seeds.

Conclusions

Natural variation, as an alternative to mutational approaches, can provide additional genetic resources to identify genes that are involved in the control of seed dormancy (Bentsink et al. 2006; Chiang et al. 2009; Gu et al. 2008, 2006). We have exploited the variation present for thermoinhibition between cultivated lettuce and an accession of its wild progenitor species, *L. serriola*, to map the *Htg6.1* QTL in a RIL population and introgress this QTL into BC$_3$S$_2$ NILs where it was subsequently confirmed to extend the range of germination thermotolerance. The mapping of candidate genes for seed germination and dormancy identified collocation of specific genes with QTL for HTG and germination in darkness and provided evidence that *LsNCED4* may be responsible for the effect of the *Htg6.1* QTL allele from *L. serriola*. The multiple BC NIL populations developed in this work can serve as informative mapping populations to identify other QTLs for HTG, for fine mapping *Htg6.1*, and as advanced germplasm for breeding applications. Thermoinhibition is a common production problem, but breeding efforts have not been focused on the improvement of seed germination characteristics, in part due the lack of tools to select efficiently for improved phenotypes. However, exploitation of the variation present for seed thermoinhibition in natural *Lactuca* populations and the identification of candidate

genes and genetic markers associated with it have provided insight into the physiological factors controlling its imposition and release and should lead to the development of lettuce cultivars with improved high temperature tolerance.

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