Genetic Analysis of Lettuce Seed Thermoinhibition

Jason Argyris[1], Peetambar Dahal[1], María José Truco[1], Oswaldo Ochoa[1], David W. Still[2], Richard W. Michelmore[1] and Kent J. Bradford[1]
[1] Department of Plant Sciences, University of California, Davis, CA 95616-8780, USA
[2] Department of Plant Science, Cal Poly Pomona, Pomona, CA 91768, USA

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Abstract

Seed thermoinhibition (inhibition of germination at warm temperatures) can reduce seedling emergence and stand establishment of cultivated lettuce (*Lactuca sativa* L.) when soil temperatures are warm (>25°C) at planting. Genetic variation for high temperature germination tolerance exists among accessions of lettuce and related wild species. Seeds of a *L. serriola* accession (UC96US23) germinated 100% up to 37°C, while seeds of *L. sativa* 'Salinas' were completely inhibited from germinating at temperatures above 31°C. A recombinant inbred line population, developed from a cross between 'Salinas' and UC96US23, was analyzed for germination capacity at high temperatures. A major quantitative trait loci (QTL) for high temperature germination (*Htg6.1*) and additional QTL having smaller effects were identified. Near-isogenic lines confirmed the effect of *Htg6.1* and are being utilized for further fine-mapping of the locus. Candidate genes associated with seed dormancy have been mapped to test for co-localization with thermoinhibition QTL. Expression patterns of candidate genes, particularly those associated with gibberellin and abscisic acid synthesis and metabolism, have also been analyzed in relation to genotype and germination temperature. Combined genetic and molecular analyses hold promise for elucidating the physiological regulation of thermoinhibition and for developing lettuce cultivars with enhanced stand establishment of warm temperature plantings.

INTRODUCTION

Rapid and uniform germination immediately after planting is essential for optimal stand establishment of annual crop plants. Thermoinhibition (inhibition of seed germination by high temperature) in lettuce *(Lactuca sativa* L.) can delay or prevent germination, resulting in reduced field emergence and stand establishment (Valdes et al., 1985). Thermoinhibitory conditions often occur in major winter lettuce production areas where temperatures permissive of germination are exceeded during late summer and fall plantings. Delays in germination and emergence can diminish yield (Cantliffe et al., 1981) or require multiple harvests and create other management problems through non-uniformity of crop development, resulting in losses in quality and profitability (Benjamin, 1990).

Thermoinhibition is moderated by physiological and environmental factors including light, temperature, hormones, and physical constraints on seed germination (Fielding et al., 1992; Cantliffe et al., 2000). Thermoinhibitory temperature is determined by cultivar and environment, cultivars being divided into thermosensitive types (in general, failure to germinate ≥28°C) and thermotolerant types (germination above 90% at ≤36°C) (Gray, 1975; Thompson et al., 1979; Nascimento et al., 2000). Seed maturation at warmer temperatures has been shown to expand upper temperature limits and increase ethylene production during subsequent seed germination (Sung et al., 1998; Kozarewa et al., 2006). Activities of enzymes responsible for weakening the endosperm membrane enclosing the embryo and allowing radicle emergence at higher temperatures have been shown to be affected by abscisic acid (ABA), gibberellin (GA) and ethylene (Dutta et al., 1994; Nascimento et al., 2000; Gonai et al., 2004). Thermotolerance can be increased by exposure to ethylene, GA, and red light or reduced by ABA (Saini et al., 1986; Dutta and Bradford, 1994). Germination at high temperature can be promoted by the application of

fluridone, an inhibitor of ABA synthesis, suggesting that continued ABA synthesis is involved in the maintenance of thermoinhibition (Yoshioka et al., 1998).

At the molecular level, the control of GA and ABA action in seed dormancy and germination is achieved through a balance of synthesis and catabolism. Light promotes GA synthesis in lettuce seeds by enhancing the expression of *Ls3h1*, a gene encoding a gibberellin 3-β-hydroxylase catalyzing the synthesis of GA_1 (Toyomasu et al., 1998). The nine-cis-epoxycarotenoid dioxygenases (NCEDs) control the first biochemical steps unique to ABA biosynthesis (Tan et al., 2003). Recently, mutant analyses and gene expression studies have demonstrated that AtNCED6 and AtNCED9 activity are specifically required for the induction of dormancy in Arabidopsis seeds (Lefebvre et al., 2006). Abscisic acid catabolism is regulated primarily via ABA-8'-hydroxylases, specifically CYP707A1 and CYP707A2, whose expression is limited to seeds (Okamoto et al., 2006). The *cyp707a1* and *cyp707a2* mutants show increased seed dormancy and higher seed ABA contents compared to wild type, confirming a primary role for ABA in dormancy. Recently, light was shown to decrease AtNCED6 activity and increase CYP707A2 activity, while ABA was involved in the suppression of GA biosynthesis (Seo et al., 2006). Thus, reciprocal regulation of ABA and GA synthesis and deactivation is involved in shifting the balance between seed germination and dormancy (Cadman et al., 2006).

Natural variation in both model and agriculturally important plant species has enabled the identification of quantitative trait loci (QTL) for morphological and physiological traits, including seed dormancy (Li et al., 2003, 2004; Gu et al., 2004, 2005). Eight QTL associated with seed dormancy and temperature sensitivity of germination were identified in a core mapping population from a cross between a cultivated thermosensitive lettuce cultivar ('Salinas') and a thermotolerant *L. serriola* accession UC96US23 (Argyris et al., 2005). A single QTL for high temperature germination (*Htg6.1*) accounted for 25% of the total phenotypic variation for germination at 35°C in seeds from populations of recombinant inbred lines (RILs) produced in two different environments and was associated with the *L. serriola* allele (B/B). Increased thermotolerance is heritable, being improved by selection in high temperature environments (Guzman et al., 1992) and is an alternative to seed priming, which is a seed enhancement procedure that can expand the temperature range of lettuce seed germination while also making it more rapid and uniform (Cantliffe et al., 1981; Valdes and Bradford, 1987).

Near-isogenic lines (NILs) are homozygous lines containing one or several introgressed QTL in an otherwise homozygous, uniform genetic background. These have been utilized to confirm the effects of donor QTL and introgressed genomic segments, while reducing QTL intervals and more accurately defining their effects (Brouwer and St. Clair, 2004). Several QTL for seed dormancy have been validated using NILs, and in one case the gene responsible for the effect has been cloned (Han et al., 1999; Bentsink et al., 2006).

In an ongoing effort to understand the genetic basis of domestication traits, including seed dormancy, the Compositae Genome Project (CGP) consortium has generated over 204,000 expressed sequence tags (ESTs) from five species of *Lactuca* (http://cgpdb.ucdavis.edu/). This repository has facilitated the development of a consensus molecular map in lettuce (Truco et al., 2007) and aided in the genetic mapping of candidate genes based on sequence variation between parental alleles (Michelmore et al., unpublished). Identification and mapping of candidate genes for phenotypes of interest has been employed as an approach to characterize QTL where genes of known function and loci of interest correspond (Barrero et al., 2006).

We have utilized the genetic resources developed in lettuce, including genotyped RILs, the CGP database, and a consensus genetic map, to identify a major QTL controlling thermoinhibition of germination (Argyris et al., 2005). Here we report further characterization of NILs containing the *Htg6.1* QTL introgressed into a 'Salinas' background and mapping of candidate genes to determine whether any collocate with the

QTL. Exploiting the variation present for seed thermoinhibition in natural *Lactuca* populations could provide insight into the physiological factors controlling its imposition and release, identify potential candidate genes responsible for these effects, and lead to the development of cultivars with expanded high temperature tolerance and improved stand establishment.

MATERIALS AND METHODS

Mapping Population, Seed Production, and QTL Analysis

Details on production of the *L. sativa* 'Salinas' × *L. serriola* UC96US23 F2 population, from which RILs were descended, are described in Johnson et al. (2000). A saturated genetic linkage map composed of over 1700 AFLP, SSR, and EST markers coalescing into 9 linkage groups (corresponding to chromosome number) and spanning 1254 cM was utilized for genetic analysis (http://cgpdb.ucdavis.edu/database/genome_viewer/map_data). Details of map construction, plant material preparation and DNA extraction were as described previously (Kesseli et al., 1994; Johnson et al., 2000; Truco et al., 2007). A subset of 165 AFLP markers approximately 10 to 15 cM apart were chosen as the framework map for QTL analysis.

A population of 110 F_8 RILs and parental lines were grown in three separate environments (Davis, California; Yuma, Arizona; and De Lier, The Netherlands) in the summer of 2002 and germination phenotypes were evaluated under different testing regimes including germination in light across a range of temperatures (Argyris et al., 2005).

A QTL analysis of germination data utilizing untransformed and probit transformed data was conducted using Windows QTL Cartographer V. 2.0 (Basten et al., 2001). Following identification of QTL from confidence interval mapping (CIM) analysis, multilocus QTL analysis was performed with data from both single and multiple environments using homozygous RIL genotypes at AFLP marker loci most closely linked to significant QTL. Least squares means and Type III sums of squares and F-tests were estimated for genotypes. Further details of QTL and statistical analysis can be found in Argyris et al. (2005).

Near Isogenic Line (NIL) Development and Testing

A marker-assisted backcrossing strategy was initiated, utilizing as the donor parent RIL 53 (which contains the *Htg6.1* interval in a mixed 'Salinas'/UC96US23 background) and 'Salinas' as the recurrent parent (RP). Plants were grown in the greenhouse and F_1 hybrid crosses were generated, confirmed genotypically, and crossed again to the recurrent parent to yield BC_1 NILs. Five PCR-based markers within and flanking a 50 cM introgressed segment containing the *Htg6.1* interval were used in foreground selection, in which individuals heterozygous for marker genotypes were selected. Background selection on an additional 22 markers was performed concurrently with foreground selection to identify individuals with the highest percentage of RP DNA. A single BC_1 NIL was then crossed again to the RP to yield 110 BC_2 NILs.

Twenty BC_2 NILs genotyped and determined to be heterozygous (A/B) at the same five markers used in BC_1 foreground selection were chosen. These lines were allowed to self-pollinate, generating large pools of BC_2S_1 NIL seeds homozygous for 'Salinas' (A/A, 25%) or UC96US23 (B/B, 25%) alleles or heterozygous (A/B, 50%) at the markers within and flanking *Htg6.1*. Additionally, two BC_2 NILs (51 and 95) homozygous for 'Salinas' alleles (A/A) at the same markers were allowed to self-pollinate to serve as control lines. Two replications of 50 seeds from each line were germinated on a thermogradient table (temperatures 27-34°C) and selected for high temperature germination (HTG) phenotype (germination at 33 or 34°C). Co-segregation of the HTG phenotype with the *L. serriola* genotype (B/B) was assessed by scoring a single EST marker (LE0196) most closely linked to the QTL.

Genetic Mapping

Utilizing an EST-based mapping approach with sequences contained in the CGP database (http://cgpdb.ucdavis.edu/), unigene contigs with the top BLAST hit (≥0.8 expectation value) were identified through keyword searches or sequence homology to known germination/dormancy-related candidates (reviewed in Bentsink et al., 2007). Identification of polymorphic EST candidate germination and dormancy contigs and primer design followed the methodology of Lai et al. (2005). Polymerase chain reaction (PCR) was used to amplify target sequences, and polymorphisms were detected in PCR products of parental lines 'Salinas' and UC96US23 using agarose or single-stranded conformational polymorphism (SSCP) gel electrophoresis. The previously defined RIL population was then genotyped at these markers and linkage analysis was performed using JoinMap v. 2.0 (Stam, 1995) to place candidates onto the linkage map.

RESULTS

Germination of seeds of the parental lines differed significantly across a temperature gradient from 25-37°C, with UC96US23 exhibiting greater tolerance to high temperature during germination than 'Salinas' (Fig. 1). 'Salinas' seeds germinated near 100% at 27°C, but germination declined to 43% at 29°C and was zero at ≥31°C. Germination of UC96US23 seeds remained above 94% at temperatures up to 37°C. This high temperature tolerance was confirmed in seeds produced in field trials during 2005, in which only 5 of 28 accessions of *L. serriola* (including UC96US23) exhibited the ability to germinate ≥80% at 35°C (Argyris et al., unpublished results), suggesting that these accessions may have unique alleles governing germination responses to temperature.

Frequency distributions for HTG in RILs confirmed the large phenotypic variation first observed in the parental lines (Argyris et al., 2005). The QTL analysis performed for HTG revealed several significant QTL, including *Htg6.1*, the most highly significant and largest effect QTL which accounted for nearly 25% of the phenotypic variation for HTG in each of two different seed production environments (Argyris et al., 2005). The least squares mean analyses of germination at the most closely linked genetic marker to *Htg6.1* showed a significant increase in germination when 'Salinas' alleles were substituted by UC96US23 alleles (Fig. 2). Mean seed germination percentages at 35°C for 42 RILs homozygous for 'Salinas' alleles at the closest marker to *Htg6.1* averaged 6%, while those homozygous for UC96US23 alleles (47 RILs) averaged 49%. The mean effect of *Htg6.1* was significant when data for all three seed production environments were combined (P=0.03) and resulted in a 43% increase in germination.

Previous results showed a consistent genetic effect of *Htg6.1* on seed germination at 35°C and a high heritability, indicating promise for the introgression of this QTL into a cultivated background to improve thermotolerance (Argyris et al., 2005). Self-pollinated progeny (BC_2S_1) of BC_2 NILs 2, 27 and 86 demonstrated HTG phenotypes across a range of temperatures at which 'Salinas' was thermoinhibited (Fig. 3). Least-square means for germination of segregating progeny of these NILs were significantly higher ($P \leq 0.05$) from 31-34°C compared to 'Salinas'. At the highest temperatures (33 and 34°C) germination ranged from 25-17 and 23-16%. Randomly selected BC_2S_1 plants with a HTG phenotype (5, 10 and 18 plants from BC_2 NILs 2, 27 and 86, respectively) were all homozygous (B/B) at LE0196, suggesting that this marker is associated with *Htg6.1* (data not shown). The progeny of control NILs 51 and 95, lacking the *Htg6.1* introgression (homozygous for 'Salinas' alleles (A/A) at LE0196), had significantly lower germination over the 31-34°C interval ($P \leq 0.05$) and displayed a similar germination pattern to 'Salinas', being completely thermoinhibited at 33°C (Fig. 3). In contrast, UC96US23 seeds germinated >95% over the entire temperature range. Progeny of the remaining 17 BC_2 NILs segregating for the *Htg6.1* QTL allele showed a range of germination percentages at 31-34°C (data not shown).

Nineteen candidate genes affecting seed germination and seed dormancy (see Bentsink and Koornneef, 2002) have been placed onto the lettuce consensus genetic map (Table 1). Mapped candidates included hormonal biosynthesis, signaling or metabolism

genes (eight involved in ABA biosynthesis or signaling, seven for GA and one for ethylene), and a transcription factor involved in seed development (*FUSCA3*). Several candidate gene/QTL co-locations were detected at sites of previously defined QTL (Argyris et al., 2005); however, no candidate genes were co-located within the *Htg6.1* confidence interval (data not shown). Imbibition at 35°C results in up-regulation of expression of *LsNCED4* (homolog of *AtNCED6*) and inhibition of expression of *Ls3h1* in 'Salinas" seeds relative to UC96US23 seeds (P. Dahal and K.J. Bradford, unpublished results), suggesting that an increase in ABA synthesis and a decrease in GA synthesis is associated with thermoinhibition.

DISCUSSION

A QTL for high temperature germination in lettuce seed (*Htg6.1*) spanning a 16 cM confidence interval on linkage group 6 was previously identified in a F_8 RIL population derived from an interspecific cross between cultivated *L. sativa* 'Salinas' and wild *L. serriola* UC96US23 (Argyris et al., 2005). An approximately 50 cM introgression containing the *Htg6.1* interval has been confirmed to confer the HTG phenotype in segregating seed of self-pollinated BC_2 NILs of three independent lineages (Fig. 3). Germination percentages for BC_2S_1 progeny of BC_2 NILs 2, 27 and 86 satisfied chi-square expectations for a single segregating gene at 33°C ($p = 0.07$, 1.0, and 0.5, respectively). Given that approximately 1/4 of the segregating population will be homozygous (B/B) for the QTL allele imparting the HTG phenotype, resulting in a maximum germination of 25% at high temperature, the *Htg6.1* allele derived from UC96US23 is potentially a recessive gene of large effect. The allele expands the range of thermotolerance by at least 3°C to 34°C, the highest temperature tested in this study. The magnitude of the HTG phenotype at 33 and 34°C for the remaining 17 NILs containing the *Htg6.1* introgression varied, especially at 34°C (14-0.2% germination), possibly due to residual background genetic variation. However, thermotolerance, likely conferred by the *Htg6.1* allele, is apparent in three independent NIL lineages in which the regions and amounts of residual donor parent alleles outside the introgressed region differ, minimizing potential for interacting loci to be consistently present in a manner influencing HTG. The effect of the *Htg6.1* allele on HTG is also supported by the fact that control lines lacking this introgression exhibited significantly lower germination at the highest temperatures (32-34°C) compared to the selected NILs, and followed a similar germination pattern to that of 'Salinas' seeds (Fig. 3).

Genetic mapping of putative candidate genes in lettuce can determine whether they co-localize with QTL influencing germination and dormancy. Those that do co-localize remain as strong candidates; those that do not co-localize can be ruled out as primary determinants of the phenotypes observed, although they may still be under the control of loci within the QTL interval (Wilkinson et al., 2002; Carrari et al., 2003). None of the 22 candidate germination and dormancy genes mapped thus far (Table 1) collocated to the *Htg6.1* interval. Thus, at the present time we can rule out some candidates in the ABA biosynthetic pathway (*ABA1*, *ABA3*) and several important GA-related candidates (*GA20ox*, *GA2ox*, *Ls3ox2*) as being responsible for the effect of *Htg6.1*. Future candidate gene mapping will utilize a Affymetrix GeneChip® genotyping/mapping array developed for lettuce that will greatly increase the number of genes mapped, enhancing our ability to identify those closely associated with germination/dormancy QTL (Caldwell et al., 2007).

Natural variation, as an alternative to a mutational approach, can provide an additional genetic resource to identify genes that are involved in the control of seed dormancy (Bentsink et al., 2006). We have exploited the large degree of variation present for thermoinhibition between cultivated lettuce and an accession of its wild progenitor species, *L. serriola* (Fig. 1) to map the *Htg6.1* QTL in a RIL population. Subsequent NIL development has confirmed the QTL effect, extending the range of thermotolerance by several degrees Celsius. Candidate germination/dormancy genetic mapping has allowed us to rule out specific candidate genes as being responsible for the effect of *Htg6.1* on

upper germination temperature limits. Ongoing work will focus on fine-mapping the QTL to a smaller genomic interval and assessing the effect of *Htg6.1* in non-segregating seed populations. Gene expression studies using both quantitative PCR and microarray approaches will complement this search to identify the physiological mechanisms controlling the temperature sensitivity of lettuce seed germination.

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Tables

Table 1. Candidate germination/dormancy genes mapped in a *Lactuca sativa* 'Salinas' × *L. serriola* UC96US23 RIL population.[1]

Marker	EST or Contig ID[2] or Genbank accession	Gene	Description	Linkage Group
			ABA-related	
LE4128	QGF5H03.yg.ab1	*XDH*	xanthine dehydrogenase	2
LE4129	QGG28D16.yg.ab1	*ERA1*	enhanced response to ABA	2
LE4015	QG CA Contig2867	*ABA1*	zeaxanthin epoxidase	3
LE4010	QG CA Contig7543	*ABI5*	bZIP transcription factor family protein	4
LE4021	QG CA Contig3084	*PKABA1*	protein kinase responsive to ABA1	4
LE4245	AB120115	*LsAO1*	abscisic acid aldehyde oxidase	6
LE4019	QGG18L02.yg.ab1	*ABA3*	molybdenum cofactor sulfurase	7
			GA-related	
LE4220	AB012206	*Ls3ox2*	gibberellic acid 3-hydroxylase 2	1
LE4121	QG CA Contig6347		F-box protein family (GA down-regulated gene)	1
LE4022	QGF25D22.yg.ab1	*GA2*	gibberellin biosynthesis - ent-kaurene synthase	2
LE4051	QGB28M14.yg.ab1	*GAL83*	β-subunit of SNF1 kinase complex	4
LE4025	QGF7L19.yg.ab1	*GA20ox*	gibberellic acid 20-oxidase	5
LE4201	AB031206	*Ls2ox1*	gibberellic acid 2-oxidase 1	7
LE4134	QG CA Contig7236	*GAR1*	suppressor of gibberellic acid insensitive	8
LE4130	QG CA Contig5082	*GAI2*	gibberellic acid insensitive 2	8
			Ethylene-related	
LE4034	QGG10D02.yg.ab1	*ACS*	Aminocyclopropane carboxylate synthase	2
			Miscellaneous	
LE4127	QG CA Contig3515		ABC transporter family protein	2
LE4133	QGB11D05.yg.ab1	*FUSCA3*	DNA binding/transcription factor	2
LE4132	QG CA Contig5234	*FsPP2C*	protein phosphatase 2C	9

[1]June 2005 map: http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/. [2]http://cdgdb.ucdavis.edu.

Figures

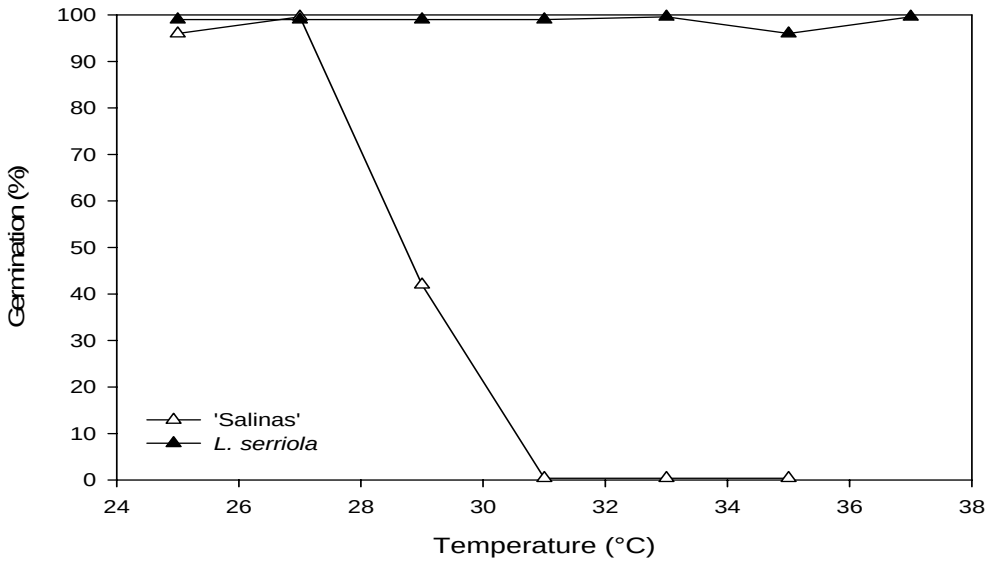

Fig. 1. Germination of seeds of *L. sativa* 'Salinas' (open triangles) and *L. serriola* UC96US23 (filled triangles) parental lines from 25 to 37°C.

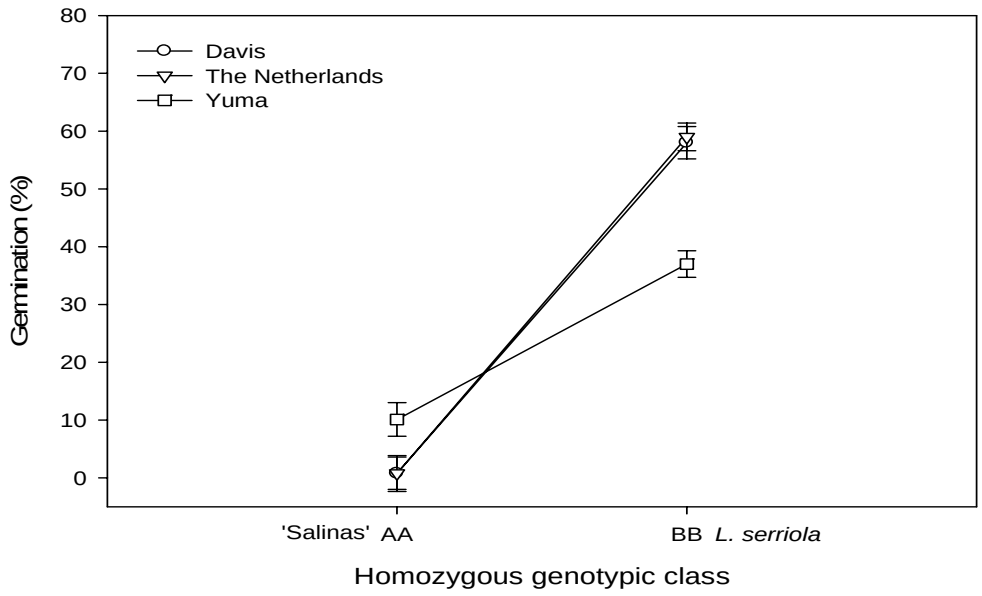

Fig. 2. Effect of *Htg6.1* on mean seed germination at 35°C of RILs homozygous for 'Salinas' (AA) or *L. serriola* UC96US23 (BB) alleles at the QTL. Seeds were produced in Davis, California (circles), De Lier, The Netherlands (inverted triangles) or Yuma, Arizona (squares).

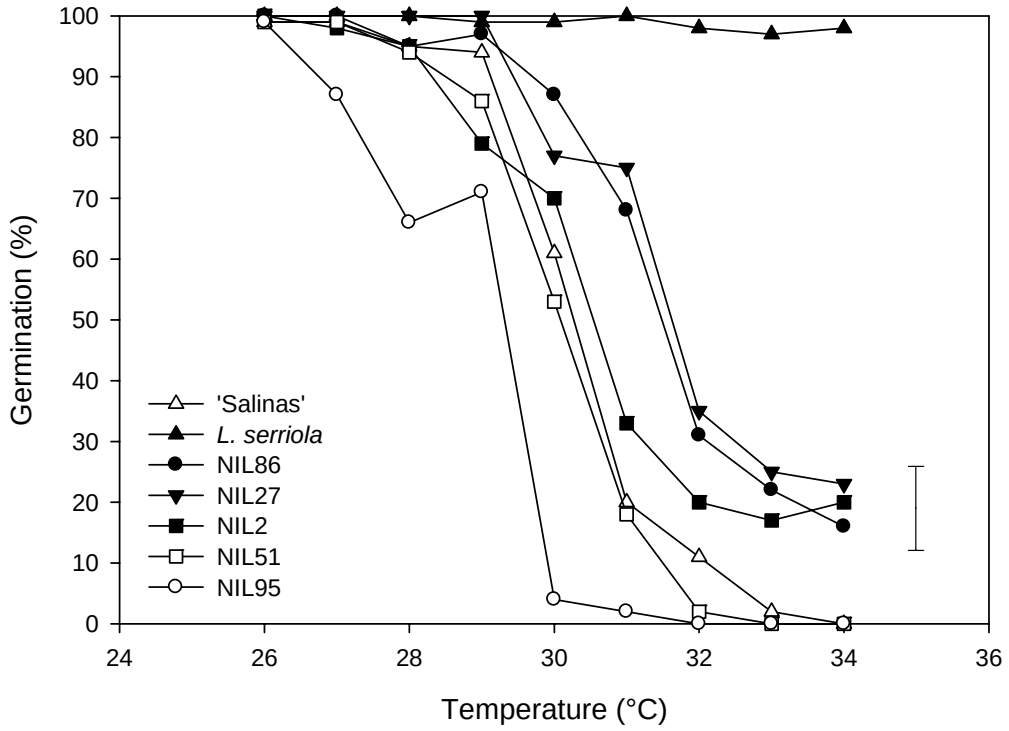

Fig. 3. Mean germination of seeds of parental lines ('Salinas' and *L. serriola*) and of self-pollinated progeny from selected BC_2 NILs segregating for *Htg6.1*. NILs 2, 27 and 86 are homozygous for an introgression containing the *L. serriola Htg6.1* allele; NILs 51 and 95 are homozygous for the 'Salinas' *Htg6.1* allele. Error bar represents experimentwide LSD.