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Abiotic stress QTL in lettuce crop–wild hybrids in greenhouse and field experiments

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Abstract

The development of stress-tolerant crops is an increasingly important goal of current crop breeding. A higher abiotic stress tolerance increases the probability of introgression of genes from crops to wild relatives. This is particularly relevant to the discussion on the risks of novel GM crops engineered to increase abiotic stress resistance. We investigated whether we can predict the fate of crop stress tolerance genes in crop–wild hybrids by applying various forms of stress under controlled conditions. For this, we determined abiotic stress QTL in greenhouse and field experiments in which we subjected recombinant inbred lines from a cross between cultivated *Lactuca sativa* cv. Salinas and its wild relative *L. serriola* to drought, low nutrients and salt stress, and above ground competition. Above ground biomass at the end of the rosette stage was used as a proxy for the performance of plants under a particular stress. A mosaic of abiotic stress QTL were detected over the entire genome with little overlap between QTL from different stresses. Those few QTL clusters that were identified reflected general growth rather than specific stress response and co-located with clusters found in earlier studies for leaf shape and flowering time. Surprisingly, genetic correlations across treatments were often higher among different stress treatments within the same experiment (greenhouse or field), than among the same type of stress applied in different experiments. Moreover, the field stress treatments were more correlated to greenhouse competition treatments than to the other greenhouse stress experiments, suggesting that competition rather than abiotic stress was a major factor in the field. In conclusion, the fate of stress tolerance (trans)genes under field conditions cannot easily be predicted from controlled QTL experiments under abiotic stress and field data are needed to assess potential negative ecological effects of escape of these transgenes into wild relatives.

Introduction

Drought, salinization, and other abiotic stresses are major causes of crop loss and this is expected to increase worldwide due to global warming, leading to a loss of agriculturally available land and reduced yields (Cominelli and Tonelli 2010). An increasing amount of research is focused on developing crops that are resistant to abiotic stresses, such as drought, salinity, heat, cold, flooding, and nutrient limitation (as reviewed in Bhatnagar-Mathur et al. 2008; Collins et al. 2008; Witcombe et al. 2008). The introduction of stress tolerant genetically modified (GM) crops could contribute to higher yields under such conditions. At the same time, public and governmental concern about the consequences of transgene escape has led to stringent policies and elaborate risk assessment strategies (Snow et al. 2005; EFSA 2011). In case a transgene contributes to a higher fitness or competitiveness of the wild relative, it could lead to an increased weediness or the invasion of new habitats (Pilson and Prendeville 2004; Warwick et al. 2009). Such increased weediness has been observed for several wild relatives that received conventional crop alleles through hybridization (Ellstrand 2003), although such negative effects have not yet been observed as a consequence of the escape of herbicide or insect tolerance transgenes (Beckie and Owen 2007; Kwit et al. 2011). It has been argued that especially abiotic tolerance transgenes could have potential unwanted effects. For example, acquisition of drought or salt tolerance could expand the typical habitat range of a wild relative (Andow and Zwahlen 2005; Warwick et al. 2009). This risk might be higher for GM crops in cases where several transgenes are stacked, thus conferring tolerance for different abiotic stresses (Halpin 2005).

Currently, Environmental Risk Assessment (ERA) procedures are performed on a case-by-case basis (EFSA 2011), although several reviews have suggested generalized tiered-approach protocols (Andow and Zwahlen 2005; Craig et al. 2008; Romeis et al. 2009). However,

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it is difficult to generate such general protocols or guidelines, since data available to evaluate the potential of transgenes to increase invasiveness and/or weediness are still scarce (Warwick et al. 2009). Given the large research effort to develop new abiotic stress-tolerant transgenic crops, the question arises whether abiotic stress traits should be given specific attention in risk assessment protocols. Specifically, whether abiotic stress QTL studies can be used to predict the fate of a transgene after a hybridization event.

The fate of a transgene after a hybridization event does not depend on its isolated effect on plant fitness of hybrid individuals only, but it also depends on the fitness effects of the genes that are in close linkage with the transgene (Stewart et al. 2003; Chapman and Burke 2006). Therefore, crop alleles and transgenes situated in genomic regions under positive selection are more likely to introgress into the wild population than genomic regions under negative selection (Gressel 1999; Stewart et al. 2003). Quantitative Trait Loci (QTL) studies allow pinpointing such genomic regions under selection and the traits that may introgress to a wild population (Mauricio 2001). The ability to predict the chances of introgression is tightly linked to the reliability of QTL identification, the heritability of trait(s), and the power of the experimental design (Beavis 1998). For example, there are likely significant differences in the ability to detect life history versus stress-related QTL.

A number of QTL studies have successfully identified genomic regions under selection for life history and fitness traits in the field, usually identifying a few genomic regions of major effect (Baack et al. 2008; Dechaine et al. 2009; Latta et al. 2010; Uwimana 2011; Hartman et al. 2012). However, typically there are many genes, proteins, and metabolic pathways involved in a stress response (Vinocur and Altman 2005; Roy et al. 2011). Consequently, slight differences in experimental set-up can cause variation in the genetic response and, therefore, in the location and effect size of QTL detected (Collins et al. 2008). The result would be a mosaic of many different genomic regions causing small to medium-sized effects that would make predicting the introgression chances of abiotic stress transgenes more difficult compared to a single region of large effect, such as we detected in lettuce for growth related traits in earlier work (Hartman et al. 2012).

On the other hand, abiotic stress QTL can coincide with suites of genes that are similarly up- or down-regulated in response to several stresses, as stress-signaling pathways for different abiotic stresses are connected in regulatory networks with common elements (Knight and Knight 2001). Different stresses may also require the same protective action as, for example under cold, drought, and salt stress plants employ similar mechanisms to prevent dehydration (Knight and Knight 2001; Wang et al. 2003), and regulatory genes that can induce such stress responses are the focus of modern transgenic research (Bhatnagar-Mathur et al. 2008; Cominelli and Tonelli 2010). In turn, this might also imply that genomic regions of major effect can be found for general abiotic stress responses (or various abiotic stress traits).

A further important factor to consider in evaluating the chances for transgene introgression is the competitive ability of crop–wild hybrid individuals (Chapman and Burke 2006). Campbell and Snow (2007) compiled studies on sunflower, oilseed rape, and radish and showed that for the majority of studies crop–wild hybrid fitness was reduced under non-competitive circumstances compared to the wild-type. However, under highly competitive conditions hybrid fitness increased, reducing the difference between hybrid and wild genotypes and thereby increasing the chances of introgression of crop alleles to wild populations (Campbell and Snow 2007; Mercer et al. 2006). Although hybrid fitness in lettuce has been studied (Hooftman et al. 2005, 2007), competition and its interaction with abiotic stresses have received little attention.

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In this study, we used a non-transgenic crop–wild model system: Recombinant Inbred Lines (RILs) from a cross between the cultivated Iceberg lettuce (*L. sativa* cv. Salinas) and its wild relative *L. serriola* (UC96US23) (Johnson et al. 2000; Argyris et al. 2005; Zhang et al. 2007). The two parental species have no barriers for hybridization (Ryder and Whitaker 1976; Kesseli et al. 1991; Koopman et al. 2001). There is no transgenic lettuce cultivar commercially cultivated yet, although research on abiotic stress tolerance using transgenes has been reported (Park et al. 2005). In previous work, we have followed plants during their entire life cycle in the greenhouse (Chapter 2) as well as realistic field environments (Hartman et al. 2012) and identified genomic regions under selection for life history and fitness traits.

For this study, we performed a series of stress experiments subjecting plants to drought, salinity, and nutrient limitation in (i) a controlled, non-competitive greenhouse environment, (ii) a controlled, competitive greenhouse environment, and (iii) a non-competitive field environment. We focused on above ground biomass at the end of the rosette stage similar to the moment the crop is normally harvested and therefore pertinent to yield, as an integrative trait to assess the response of the whole plant to a stress (Witcombe et al. 2008). Specifically, we addressed the following questions: (i) Which genomic locations in lettuce carry QTL for the response to drought, salinity, nutrient limitation, and intra-specific competition? (ii) Can we identify clusters of QTL indicating genomic regions involved in a specific stress or in general abiotic stress tolerance? (iii) How does the QTL pattern of abiotic stresses without competition compare to the QTL pattern under competition stress? Finally, we discuss the implications of our results for ERA procedures of future GM crops.

Material & methods

Plant material

We used an existing Recombinant Inbred Line (RIL) population from a cross between a crop species lettuce (*Lactuca sativa* cv. Salinas) and its wild relative prickly lettuce (*L. serriola* UC96US23) (Johnson et al. 2000; Argyris et al. 2005; Zhang et al. 2007). These two fully interfertile relatives (Ryder and Whitaker 1976; Kesseli et al. 1991; Koopman et al. 2001) show marked differences in phenotype. This accession of *L. serriola* has long serrate leaves that contain white bitter latex. Plants have spines up to 2 mm long on the stem base and leaf midribs. It is drought-tolerant with a long taproot with which it can reach water at deep soil layers (Gallardo et al. 1996). In contrast, *L. sativa* cv. Salinas has broad almost circular leaves, without any spines and a low latex content (de Vries 1997); it develops a shallow root system with a short taproot and many lateral branches in the topsoil layer (Jackson 1995). *L. sativa* is therefore adapted to agricultural systems with high inputs of water and nutrients, probably as a consequence of selection during domestication and subsequent breeding (Jackson 1995).

Lactuca serriola mainly occurs in ephemeral ruderal habitats, including roadsides, railways, and construction sites. It is an annual species that flowers in July–August and survives the winter as seed, but sometimes as small rosettes (Y. Hartman, personal observation). Cultivated and wild lettuce are predominantly selfing species, but 1–5% outcrossing rates via insect pollination have been reported (Prince and Carter 1977; D’Andrea et al. 2008; Giannino et al. 2008). Uwimana et al. (2012a) estimated that about 7% of the European *L. serriola* plants were offspring of hybridization events between *L. serriola* and *L. sativa*.

Experimental design

We performed four different abiotic and competition stress experiments in the greenhouse as

Table 1. Aboveground biomass traits examined in four experiments, greenhouse Salt/nutrient limitation (sn), greenhouse Drought/recovery (dr), greenhouse competition (c), and field stress (f), in a *Lactuca sativa* cv. Salinas × *Lactuca serriola* recombinant inbred lines population.

Experiment	Treatment	Abbreviation
Shoot dry weight (g)		
Salt/nutrient	Control	DCsn
	Nutrient limitation	DNsn
	Salt 100mM	DSsn
Drought/recovery	Control	DCdr
	Drought	DDdr
	Recovery	DRdr
Greenhouse competition	Control	DCc
	Drought	DDc
	Nutrient limitation	DNc
	Salt 100mM	DSc
Field	Control	DCf
	Drought	DDf
	Salt 100mM	DSf
Proportion shoot dry weight (%)		
Salt/nutrient	Control	PDCsn
	Nutrient limitation	PDNsn
	Salt 100mM	PDSsn
Drought/recovery	Control	PDCdr
	Drought	PDDdr
	Recovery	PDRdr

well as in the field. These included two greenhouse stress experiments, Drought/recovery (DR) and Salt/nutrient limitation (SN), one greenhouse competition experiment and one field stress experiment (Table 1).

Greenhouse stress experiments

The DR and SN experiments in the absence of competition, were performed under similar experimental conditions in February–March 2009 and April–May 2009, respectively. The only difference was that in the DR experiment, plants were grown in pots with soil, whereas in the SN experiment plants were grown in vermiculite, allowing flushing the substrate with salt solution and more consistency in nutrient limitation. We assumed that the large DR/SN treatment effects overrule differences in soils. The DR experiment consisted of a control, drought, and recovery treatment and the SN experiment consisted of a control, nutrient limitation, salt 100mM, and salt 300mM treatment (Table 1). From 114 available RILs, we selected 60 lines using MapPop (Vision et al. 2000) that maximizes the number of recombination breakpoints and provides a population with the highest amount of genetic variation. We also included the parent lines and 5 replicates per treatment resulting in a total of 930 plants in the DR experiment and 1240 plants in the SN experiment.

We took several measures to minimize variation within RIL families. First, seeds of the RILs and parent lines were germinated in three separate groups based on the results of a separate germination experiment performed in December 2008 (Chapter 2). On day 1, we started with

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the slowest germinating group (6 lines), on day 2, with the average group (46 lines and the wild parent) and on day 3, with the fastest germinating group (8 lines and the crop parent). In addition, we assessed all individuals of each RIL at the end of the establishment period and eliminated the five largest and five smallest seedlings, keeping 15 intermediates. These measures provided seedlings of similar size at the start of the stress period. In order to minimize position effects, we randomized the seedlings and later the plants twice a week during the entire seven-week period of the experiment. During the germination period, we randomized trays and Petri dishes and during the establishment and stress periods, we randomized pots of entire blocks. Each treatment had five blocks and each block contained one individual of all RILs and the parent lines. We also included 25 empty pots per treatment divided over all blocks to monitor stress levels (Appendix 1). In the DR experiment, empty pots were weighed to record the water capacity, whereas in the SN experiment, the electrical conductivity was measured in the plates underneath the pots after flushing the pots to record salt and nutrient stress levels. In addition, temperature and humidity was measured to monitor the stability of greenhouse conditions.

Seeds were placed in Petri dishes on filter paper and watered with sterilized water to induce germination. We added a small amount of TMTD (tetramethyl-thiuram-disulfide) powder to prevent the formation of fungi on the seeds. The Petri dishes were placed in a germination cabinet under 16h of light at 20°C and 8h dark with 15°C. The germination period lasted 9 days after which seedlings were transplanted to pots of 15 cm diameter with soil (DR) or vermiculite (SN) and grown in the greenhouse under 6h dark and 18h light, a minimum of 18°C, under 600 W SON T-Agro lamps generating on average 160 $\mu\text{mol}/\text{m}^2/\text{sec}$ at plant level. This lasted another 9 days.

In the DR experiment, stress was applied by withholding water for 24 days in the drought and recovery treatment, while the control treatment was watered three times a week to keep the soil at maximum water capacity. After this period, we collected the above ground biomass for the drought treatment (Appendix 2). In the recovery treatment, plants were watered again. After four more days, we collected the above ground biomass of the control and the recovery treatment.

In the SN experiment, the stress period lasted 25 days. Treatments were administered twice a week by flushing the pots containing vermiculite from the top. For the first 4 days, stress levels were built up gradually by flushing the pots twice every day. The control treatment was watered with 1.0 g/l nutrient solution (Scotts, Peters Professional Growth, 20:10:20 NPK). Nutrient limitation was induced by watering without added nutrients. Salt stress was induced with 1.0 g/l nutrient in 100mM NaCl solution. At the end of the stress period, we collected the biomass of the control and salt 100mM treatment, and the biomass of the nutrient limitation treatment one day later (Appendix 2).

After collection of the above ground biomass, we immediately measured the fresh weight and samples were dried for three days at 70°C after which shoot dry weight was measured. We estimated the proportion of shoot dry weight by dividing the dry weight by the fresh weight.

Greenhouse competition experiment

The greenhouse experiment including competition was performed in the summer of 2009. We used 90 RILs and the parent lines, aiming at 17 replicates per line per treatment. However, not all lines produced enough seedlings because of variable germination rates, so on average 15.2 seedlings per line were analyzed (see Appendix 3). Seeds were placed directly in the greenhouse in 4 by 4 cm pots with moist soil to induce germination. At the end of the establishment period, we reduced variation due to differential growth by removing the smallest and largest individuals.

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The experiment with competition consisted of four treatments: control, nutrient limitation, salt, and drought. The germination and establishment period each lasted 9 days during which plants were shuffled once a week to prevent position effects. At the start of the stress period, pots were placed directly adjacent to each other (625 plants/m²) to induce competition effects. Pots were placed completely random on four different tables, one for each treatment and treatments were administered twice a week. The control was watered with 1.0 g/l nutrient solution, whereas the nutrient limitation treatment was watered with water without added nutrients. The salt treatment was watered with 1.0 g/l nutrient in 100mM NaCl solution, while drought was induced by withholding water (water was administered only once, in the second week of the stress period). After 23 days of stress, we again collected above ground biomass and measured shoot dry weight, as described above.

Field stress experiment

The field stress experiment was conducted in the same period as the greenhouse competition experiment. We used the same 90 RILs, with on average 13.5 seedlings per line (Appendix 3). We recorded temperature and humidity levels in the field.

The field site at Sijbekarspel, the Netherlands (N52°42', E04°58'), has a clay soil mimicking agricultural conditions with nutrient rich and high water retention conditions. We transplanted seedlings from the greenhouse to the field 9 days after sowing at the end of July 2009 and used a three-block design with a control, drought, and salt block. Within these blocks plants were placed in a grid of 40 by 40 cm with 30 cm distance between individuals. Each block was subdivided into 17 sub blocks, with 10 by 10 planting positions. Each sub block contained all RILs as well as the parental lines. Positions for lines that did not produce enough seedlings were left empty. At the start of the stress period, stress levels were increased gradually by applying the treatments daily for four days, after which treatments were administered three times a week. The control treatment was watered throughout the experiment. Drought stress was induced by withholding water. The salt treatment was watered with a 100mM NaCl solution. After 21 days of stress, we collected above ground biomass at the end of August 2009. Biomass samples were dried for three days at 70°C after which we measured shoot dry weight.

Statistical analysis

Statistical analyses were performed in PASW Statistics 17.0 (SPSS Inc. 2009). Testing for differences between parent lines within treatments was done with T-tests. Prior to estimation of correlation across treatments, heritability values, and QTL analyses, all traits were transformed. To improve normality of distributions biomass data were log-transformed and proportion of shoot dry weight was arcsine-square-root-transformed. Broad-sense heritability was estimated as the proportion of the total phenotypic variance accounted for by the genetic variation (Lynch and Walsh 1998). In addition, we estimated the genetic correlation across treatments for biomass with the following equation (Lynch and Walsh 1998):

$$r_G = \frac{Cov(X,Y)}{\sqrt{(VarX_{RIL})(VarY_{RIL})}}$$

Where $Cov(X,Y)$ is the covariance of the average values of RILs in treatment X and Y , and $VarX_{RIL}$ and $VarY_{RIL}$ is among RIL genetic variation for treatment X and Y , respectively, extracted with procedure VARCOMP (SPSS Inc. 2009). Note that covariances and variances are estimated independently so that the estimates for the correlations can exceed plus or minus one. High correlations indicate that the rank order of RILs is similar (no Genotype \times Environment

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interaction), whereas a correlation close to minus one indicates that the rank order is reversed, possibly due to a genetic trade-off in the performance of plants under different conditions. Correlations close to zero suggest that the traits in the two environments are independent. The matrix of correlation estimates was used to cluster treatments in R using the `hclust` function with Ward's method (version 2.14.0, R development core team 2011). The correlation matrix was first converted into a distances matrix using the formula:

$$D = \frac{1-r_G}{2}$$

Quantitative trait loci analysis

We performed QTL analysis on dry weight for all experiments and on proportion of dry weight for the DR and SN experiments. Genetic map and marker data used in the QTL analysis were obtained from The Compositae Genome Project website (<http://compgenomics.ucdavis.edu>). The genetic map consisted of 1513 markers distributed over the nine chromosomal linkage groups (<http://cgpdb.ucdavis.edu/GeneticMapView/display/>; map version: RIL_MAR_2007_ratio). All QTL analyses were performed with Composite Interval Mapping (CIM) in QTL Cartographer version 2.5.008 (Wang et al. 2010). Tests for the presence of a QTL were performed at 2 cM intervals using a 10 cM window and five background cofactors that were selected via a forward and backward stepwise regression method. Statistical significance threshold values ($\alpha=0.05$) for declaring the presence of a QTL were estimated from 1000 permutations (Doerge and Churchill 1996). One-LOD support intervals and additive effects were calculated from the CIM results. The linkage map and QTL positions were drawn with MapChart 2.2 (Voorrips 2002).

Results

Environmental conditions

For both the Drought/recovery (DR) experiment and the Salt/nutrient limitation (SN) experiment, treatment conditions were stable throughout the stress period (See Appendix 1). In the DR experiment, temperatures ranged from 17.9°C to 22.5°C, with an average temperature of 19.6°C and relative humidity of 77.8%. In the SN experiment, temperatures ranged from 17.9°C to 25.4°C, with an average of 21.3°C and relative humidity of 61.9%. In the field experiment, the average temperature was 19.8°C and relative humidity was 73.5% during the establishment and stress period in July and August 2009. The maximum temperature reached 36.6°C and a minimum of 7.8°C. During the stress period there was no precipitation, providing good conditions for the drought stress treatment.

Shoot dry weight

We found significant differences in shoot dry weight between the cultivated *L. sativa* cv. Salinas and the wild *L. serriola* parents in most greenhouse treatments, except in the control treatment of the SN experiment and in the nutrient limitation and drought treatments of the greenhouse competition experiment (Table 2). There were no significant differences between the parental lines in all field treatments. As expected, within experiments above ground dry weight values were highest in the control treatments for both parental lines, with the only exception being the control dry weight of the crop parent in the greenhouse competition experiment.

For the RILs, broad-sense heritability values ranged from 17.0% to 65.5% (Table 2),

Table 2. The mean and standard deviation (SD) for the parent lines and the recombinant inbred lines (RILs) population for all environments. T-test results indicate significance of differences between the parent lines. Broad-sense heritability values (H^2) given as the percentage of phenotypic variation among RILs. For abbreviations, see Table 1.

Trait	Crop		Wild		T-test			RILs		H^2 (%)
	Mean	SD	Mean	SD	df	T	P	Mean	SD	
Shoot dry weight (g)										
DCsn	3.56	0.53	4.17	0.83	8	-1.399	0.199	4.17	0.69	54.7
DNsn	1.32	0.24	2.27	0.42	8	-4.405	0.002	1.86	0.30	65.5
DSsn	1.85	0.11	1.21	0.20	8	6.148	0.000	1.49	0.30	58.6
DCdr	4.59	0.47	4.76	0.84	7	-0.351	0.000	4.74	0.99	39.7
DDdr	1.61	0.11	1.31	0.05	9	5.279	0.001	1.43	0.14	41.8
DRdr	2.01	0.11	1.79	0.19	8	2.180	0.000	1.96	0.21	22.3
DCc	0.35	0.15	0.97	0.47	31	-5.118	0.000	0.88	0.33	51.1
DNc	0.48	0.17	0.42	0.09	32	1.243	0.223	0.55	0.15	55.1
DSc	0.29	0.14	0.50	0.24	30	-3.074	0.004	0.62	0.19	55.4
DDc	0.39	0.12	0.32	0.14	31	1.552	0.131	0.41	0.14	32.6
DCf	5.07	1.59	5.02	1.58	28	0.088	0.931	6.04	2.17	20.0
DSf	2.73	1.11	2.61	1.17	20	0.242	0.811	4.10	1.69	19.9
DDf	3.12	1.31	2.60	0.70	22	1.145	0.264	5.13	2.01	17.0
Proportion shoot dry weight (%)										
PDCsn	4.58	0.64	7.72	1.05	8	-5.719	0.000	6.24	0.62	28.5
PDNsn	6.39	0.43	11.41	0.93	8	-10.973	0.000	9.53	0.70	78.3
PDSsn	7.32	0.56	10.49	0.45	8	-9.835	0.000	8.52	0.35	73.3
PDCdr	7.60	0.46	13.08	0.53	7	-16.219	0.736	10.78	0.99	64.9
PDDdr	13.13	1.17	16.71	1.37	9	-4.690	0.001	15.53	1.26	64.9
PDRdr	7.05	0.50	11.15	0.67	8	-11.012	0.061	9.43	0.66	58.3

the three lowest heritability values were found in the field. We detected a total of 26 QTL for shoot dry weight in 13 treatments, distributed over all nine linkage groups (Fig. 1, Table 3). The range of Phenotypic Variation Explained (PVE) per QTL varied between 8.1% to 26.0%. For each trait one to four QTL were detected (mean 2.4), except for controls of the DR and the field experiment. The 1-LOD support intervals were on average 3 cM (range 0.4–9.1 cM). In total, only 3 (out of 26) QTL were of major effect (PVE > 25% as defined by Burke et al. 2002). Two major QTL were found for salt dry weight, one at linkage group (LG) 5 of the field experiment and another at LG9 of the SN experiment. Another major QTL was found at LG3 for control dry weight of the SN experiment. Only two QTL were of minor effect (PVE < 10%) and most QTL were of intermediate effect (PVE between 10–25%).

Proportion dry weight

In all SN and DR treatments, the wild parent had a higher shoot dry to fresh weight ratio (proportion dry weight, only measured in these two experiments) than the cultivated parent. This indicates that the wild parent allocated more resources towards building up biomass and support tissue, whereas the crop parent produced broad leaves that held more water. Compared to the control treatments, the proportion dry weight of the parents increased under stress conditions, only in the recovery treatment did the proportion dry weight return to similar values

Table 3. Quantitative trait loci detected by composite interval mapping in a *Lactuca sativa* cv. Salinas × *Lactuca serriola* recombinant inbred lines population. For abbreviations, see Table 1; nd = no QTL detected; PVE = Percentage Variation Explained. Positive additive effects indicate that the crop-type (*L. sativa*) allele increases trait values and negative additive effects indicate that the wild-type (*L. serriola*) allele increases trait values.

LG	Trait	Position (cM)	1-LOD interval	Additive effect	PVE (%)	LOD	Threshold 0.05
1	PDRdr	34.6	33.1–36.5	−0.006	14.3	4.1	3.4
	DCc	46.3	45.6–48.7	−0.025	8.1	3.6	3.5
	PDNsn	72.4	71.5–72.4	−0.010	15.8	4.9	3.2
	PDCsn	74.8	74.0–79.7	−0.005	19.9	6.1	3.4
	PDDdr	95.6	93.7–96.1	0.010	12.2	4.0	3.4
2	DCsn	62.7	62.3–64.1	−0.035	18.0	4.9	3.5
	DNsn	62.8	62.4–64.1	−0.028	13.8	4.1	3.5
	PDCsn	138.5	137.3–139.9	−0.005	20.6	6.7	3.4
3	DCsn	42.9	41.6–44.0	−0.042	25.4	6.6	3.5
	PDCsn	42.9	41.6–44.3	−0.004	12.8	4.7	3.4
	PDNsn	42.9	41.6–44.0	−0.012	19.0	6.0	3.2
	PDCdr	42.9	41.6–44.4	−0.020	44.9	10.3	3.4
	PDRdr	42.9	41.6–44.0	−0.009	26.5	7.0	3.4
	DDf	85.7	85.1–86.3	0.033	13.0	5.0	3.3
4	DSc	10.9	9.6–12.9	−0.019	8.3	3.6	3.4
	PDNsn	82.7	80.3–83.8	−0.009	9.9	3.5	3.2
5	DDf	42.0	38.4–42.4	0.039	18.5	6.5	3.3
	DSf	45.1	44.5–45.3	0.050	25.3	8.5	3.4
	DRdr	76.4	75.7–76.8	0.011	20.7	5.5	3.5
	DNsn	79.9	78.2–87.3	0.030	17.5	5.2	3.5
	PDSsn	93.5	93.2–93.5	0.005	13.9	4.7	3.4
	DSsn	125.1	119.8–125.9	0.026	11.6	3.5	3.4
	DDc	127.3	126.5–129.2	0.012	11.4	3.5	3.3
	DNc	158.4	157.4–161.0	−0.020	15.2	4.7	3.5
6	PDCdr	20.8	19.9–22.1	0.011	17.0	4.9	3.4
	DRdr	38.3	37.0–40.2	−0.010	15.8	4.7	3.5
	DSf	122.2	121.0–125.8	0.033	10.6	4.0	3.4
7	PDNsn	13.2	12.9–15.5	0.010	14.1	4.6	3.2
	DCc	19.2	18.2–21.6	−0.037	16.6	6.7	3.5
	DDc	19.2	18.5–21.7	−0.016	21.2	6.7	3.3
	DSc	19.2	18.4–20.6	−0.031	21.4	8.4	3.4
	DCc	41.7	40.6–42.9	−0.031	12.4	5.1	3.5
	PDSsn	47.3	46.6–48.2	−0.005	15.7	4.4	3.4
	DCc	50.4	50.3–51.6	−0.029	11.2	4.8	3.5
	PDSsn	57.6	54.6–59.9	−0.006	22.5	6.9	3.4
	8	DDdr	10.6	10.0–11.6	−0.010	14.9	4.3
DDdr		20.7	19.4–22.6	−0.010	14.8	4.3	3.4
PDDdr		24.5	23.6–25.1	−0.010	13.7	4.3	3.4
DSc		75.3	75.1–75.5	−0.027	17.0	7.0	3.4
DRdr		106.6	106.3–108.2	0.011	19.9	4.7	3.5
DSf		117.7	116.2–119.0	0.034	11.2	4.0	3.4
9	DSsn	81.2	78.7–85.8	0.041	26.0	6.7	3.4
	PDSsn	86.1	84.3–90.6	0.005	13.6	4.4	3.4
nd	DCdr						
nd	DCf						

Abiotic stress QTL in lettuce hybrids in greenhouse and field

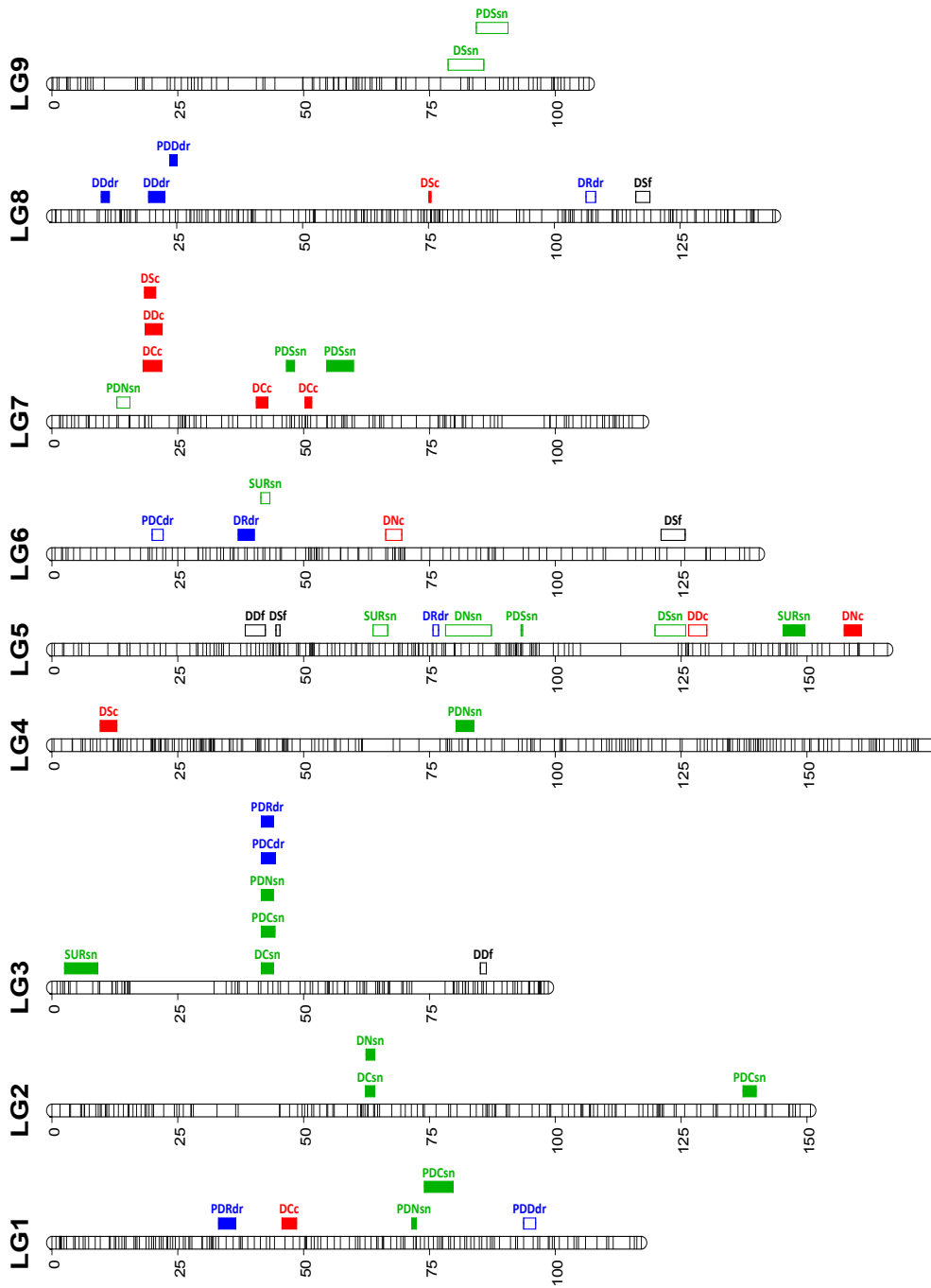


Figure 1. Genomic locations of quantitative trait loci detected in composite interval mapping. Markers are indicated by horizontal lines on the linkage group bars and map distances (cM) are shown on the left side. Bars to the right represent one LOD confidence intervals of QTL; for abbreviations, see Table 1. An open bar indicates that the crop-type (*Lactuca sativa* cv. Salinas) allele increases the trait values, whereas a filled bar indicates that the wild-type (*Lactuca serriola*) allele increases the trait values. Bar colours indicate the experiment: Green = Salt/nutrient limitation, Blue = Drought/recovery, Red = Greenhouse competition, and Black = Field.

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as the control.

For the RILs, the proportion of shoot dry weight showed higher heritability values than shoot dry weight measured in the same treatment, suggesting that the former is a trait related to the growth form of plants and less influenced by the environment than shoot biomass. The only exception was the control proportion shoot dry weight of the SN experiment (28.5%), which was caused by lower variability between lines compared to other treatments, leading to a low RIL variance compared to error variance.

We detected a total of 17 QTL for shoot dry weight in six treatments, distributed over all nine linkage groups (Fig. 1, Table 3). PVE values per QTL varied between 9.9 to 44.9%. For each trait two to four QTL were detected (mean 2.8). The 1-LOD support intervals were on average 2.9 cM (range 0.3–9.1 cM). In total, two (out of 17) QTL were of major effect. These QTL co-localized at LG3 for control and recovery of the DR experiment. Only one QTL was of minor effect and the majority of QTL (14) was of intermediate effect.

Quantitative trait loci clusters

We detected 43 QTL for shoot dry weight and proportion dry weight in total. Three quarters of the QTL (76.7%) had a location that did not overlap with other QTL (Fig. 1). The 10 QTL (23.3%) whose location did overlap with other QTL were located in the centre of LG2 and LG3 and at the top of LG7. On LG2, only two QTL co-localized for the control and nutrient limitation of the SN experiment. On LG3, QTL were located for control shoot dry weight and proportion shoot dry weight of control and nutrient limitation treatments of the SN experiment, as well as control and recovery treatments of the DR experiment. On LG7, all three QTL were detected in the greenhouse competition experiment including shoot dry weight QTL for control, drought, and salt treatments. In all clusters, it was the wild allele (*L. serriola*) that increased above ground biomass and proportion dry weight values. Although we detected a total of 38 QTL in the various greenhouse experiments, not one coincided with any of the five field QTL.

Genetic correlations between treatments

The highest genetic correlations for biomass were invariably between treatments of the same experiment (i.e. the same environment) (Table 4), indicating a low Genotype \times Environment (G \times E) component within experiments. Figure 2 shows that treatments from the same experiment cluster together, the only exception being the control of the DR experiment, which clusters with the treatments of the SN experiment. Furthermore, the greenhouse stress experiments (SN and DR) are placed in one branch, while the greenhouse competition and field experiment are placed in another (Fig. 2). The field treatments showed relatively high genetic correlations with each other (Table 4), suggesting that the specific stress treatments were not the major factors influencing the performance of the RILs.

Discussion

Low clustering of abiotic stress quantitative trait loci

Abiotic stress QTL were detected throughout the genome and generally did not co-localize. The QTL clusters that were detected included control treatment QTL and are more indicative of general growth, rather than an indication for genomic regions specific for a particular stress or for general stress tolerance. Only in two regions did three or more QTL co-localize, one region in the centre of linkage group (LG) 3 and one at the top of LG7. These two regions do coincide with QTL clusters identified in previous genetic analyses of life history and fitness-related traits

Experiment	Greenhouse Salt/nutrient			Greenhouse Drought/recovery			Greenhouse competition			Field		
	Control	Salt 100mM	Nutrient limitation	Control	Drought	Recovery	Control	Drought	Nutrient limitation	Control	Drought	Salt 100mM
Greenhouse Salt/nutrient	Control	1.15	0.77	0.80	0.43	0.38	0.37	0.30	0.48	0.47	0.34	0.31
	Salt 100mM		1.13	0.60	0.34	0.18	0.38	0.22	0.48	0.31	0.24	0.25
	Nutrient limitation		1.09	0.82	0.42	0.08	0.61	0.45	0.61	0.64	0.54	0.51
Greenhouse Drought/recovery	Control			1.28	0.62	0.36	0.53	0.45	0.50	0.56	0.42	0.34
	Drought				1.26	0.63	0.17	0.24	0.22	0.47	0.27	0.40
	Recovery					1.68	-0.40	-0.05	-0.26	0.09	-0.26	-0.16
Greenhouse Competition	Control						1.08	0.69	0.83	0.66	0.64	0.57
	Nutrient limitation							1.05	0.63	0.58	0.46	0.60
	Drought								1.15	0.69	0.68	0.64
	Salt 100mM									1.06	0.73	0.62
Field	Control									1.28	1.04	1.02
	Drought										1.41	1.05
	Salt 100mM											1.37

Table 4. Genetic correlations between treatments within and among environments.

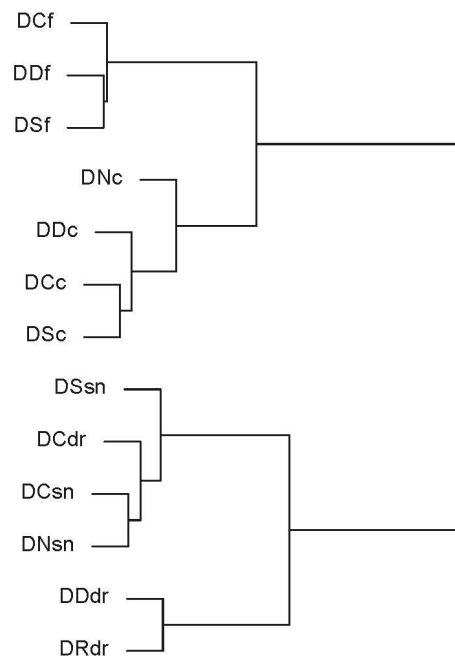


Figure 2. Clustering of treatments based on genetic correlations for biomass; for abbreviations, see Table 1.

(Hartman et al. 2012; Chapter 2).

Within these clusters, five QTL from the greenhouse Salt/nutrient limitation (SN) and Drought/recovery (DR) experiments co-localized on LG3; four of which were QTL for proportion shoot dry weight of SN and DR controls, nutrient limitation, and drought recovery treatments. In earlier studies, we found this region coincided with QTL for leaf shape and seed output traits (Chapter 2). The combination suggests that the transition from narrow wild-type leaves to broad cultivated type leaves has coincided with a reduction of support tissues and an increase in water contents of the leaves of the crop (de Vries 1997), thus affecting the proportion of above ground dry weight in the leaves. Within the cluster on LG7, all QTL were from the greenhouse competition experiment and included a control, drought, and salt QTL. At this same location, several QTL were identified connected to the speed of development most likely governed by a common major gene for earliness of flowering (Hartman et al. 2012), this clustering is further discussed below.

The high number of non-overlapping stress QTL can be due to the fact that indeed many genes are involved, but also to variability in the applied stresses across experiments, and to the statistical power of the experiments (if many small QTL would have escaped detection). First, although we aimed to apply stresses as similar as possible in the different experiments, some differences in design and conditions turned out to be inevitable. As a consequence, the exact amount and timing of applied stress observed by the plants may have differed between the experiments. Indeed, genetic correlations between different abiotic stress treatments within the same experiments were high, whereas there were low correlations between treatments for a specific stress across different experiments, indicating a high Genotype by Environment ($G \times E$) interaction between experiments. This implies that it may be difficult to design and perform a set of experiments that consistently determine the QTL for a particular abiotic stress, because small changes in the set-up, such as differences in plant age and initial growing conditions,

could already cause different expression patterns in response to stress (Collins et al. 2008).

Second, different abiotic stresses probably cause genetic expression patterns involving different genomic regions. This is, for example, supported by the low genetic correlation between the salt and drought treatments of the greenhouse stress experiments. A plant's response to abiotic stresses involves complex signaling pathways, depending on many genes, proteins, and metabolic pathways that may also vary across life stages (Knight and Knight 2001; Roy et al. 2011).

Alternatively, a lack of statistical power caused by small sample sizes and a low number of replicates (necessitated by the scale of the experiment) could lead to low heritability values, leaving QTL undetected (Beavis 1998; Mauricio 2001; Collard et al. 2005). Indeed, field heritability values were lower than in the greenhouse due to a higher environmental variation (Gardner and Latta 2008), even though this was partly countered by a higher number of replicates used in the field. Still, heritability values were high (>50%) in the majority of treatments, indicating a high genetic component underlying the variation and so a good ability to locate QTL (Hyne et al. 1995). Moreover, the two clusters identified are the same as in earlier studies (Hartman et al. 2012; Chapter 2); making it unlikely that other major QTL locations could have gone unnoticed.

Outrunning the competitors

Our results suggest that the wild species, *L. serriola*, might be a better competitor compared to the cultivated varieties. In the greenhouse competition experiments, the wild genomic background induced higher shoot biomass, as seen by the clustering of several competition QTL at the top of LG7. In a selective field experiment, following plants through their entire life cycle, fitness QTL were detected at this same genomic location as well as several QTL connected to the speed of development (Hartman et al. 2012). We found that the wild allele induced early bolting and hence, flowering at this genomic location. This suggests that under competitive circumstances with high plant density, it is selectively advantageous to have a faster development and to bolt earlier to outrun the competitors (Fakheran et al. 2010).

The timing of bolting and flowering influences the ability of crop-wild hybrids to survive and produce biomass. Radish, like lettuce a crop bred for its vegetative parts, also has a delayed flowering time compared to its wild relative. In crop-wild radish hybrids, a decline in white flower color, a dominant crop allele linked to delayed flowering time, was observed after a decade of following crop allele frequencies in experimental competitively selective populations (Campbell and Snow 2007; Campbell et al. 2009; Snow et al. 2010). Both for lettuce and radish it is known that hybridization can produce vigorous crop-wild hybrids that were interpreted to result from new additive genetic combinations leading to increased fitness and (potential) competitiveness (Hooftman et al. 2009, 2011; Snow et al. 2010). The studies on lettuce and radish suggest that, under high population densities, the wild genomic background conferring early flowering at specific genomic locations increases the competing ability of crop-wild hybrids of vegetable crops.

It should be noted that late flowering might be more advantageous if the environment allows an extended flowering period, because increased biomass would result in larger plants and, eventually, more seed output. In addition, plant densities can be highly variable in wild populations, therefore, situations with low plant density and extended flowering period should be tested as well to determine if it is still the wild genomic background that increases the competitive ability of crop-wild hybrids. Nevertheless, in situations with high plant density and a seasonal flowering period, lettuce hybrids with a crop genomic background at LG7 will have a higher likelihood to be outcompeted by their wild relatives and die before reproduction, which has been observed in full life cycle experiments with lettuce (Hooftman et al. 2005, 2007).

Implications for GM Environmental Risk Assessment

Controlled, short-term greenhouse experiments are often used to evaluate the effect of transgenes on the tolerance of crops, rather than observing the effects of stress over the life span of a crop in a range of agricultural conditions (Vinocur and Altman 2005; Bhatnagar-Mathur et al. 2008; Mittler and Blumwald 2010). However, here we indicate that such short-term experiments are not necessarily sufficient or fully informative. We found no overlap in QTL expression between such controlled greenhouse experiments (without competition) and the field situation. The genetic correlations indicated that the greenhouse competition treatments had the highest correlation with the field treatments, suggesting that competition rather than a specific abiotic stress was an important influential factor in the field. In addition, predictions from a particular study only hold as long as selection pressures are similar and $G \times E$ interaction might imply that other genomic regions may come under selection as well, depending on specific conditions in the field (Weinig et al. 2002; Martin et al. 2006).

On a more positive note, several studies have been able to pinpoint specific genomic regions with similar selection effects in various field environments by following plants through their entire life cycle (Baack et al. 2008; Dechaine et al. 2009; Hartman et al. 2012), even though fitness components can have strong $G \times E$ interactions (Mercer et al. 2006, 2007). It has been suggested that these kinds of results can be used in Transgene Mitigation (TM) strategies (Gressel 1999; Stewart et al. 2003), as we explored in earlier work (Hartman et al. 2012; Chapter 2). Techniques for targeted insertion of transgenes into specific regions in the genome are currently being developed (Lombardo et al. 2011; Nandy and Srivastava 2011). A transgene in close linkage with an allele or genomic region that is selected against in the wild is more likely to be purged from the wild population (Stewart et al. 2003). In tests of this strategy, by placing a transgene in linkage with a dwarfing gene, transgenic tobacco and oilseed rape hybrids carrying this gene showed a dramatic reduction in survival capacity of hybrids especially under competition stress (Al-Ahmad et al. 2005; Rose et al. 2009). Our results and those of various studies on radish (Campbell and Snow 2007; Campbell et al. 2009; Snow et al. 2010) indicate that for leafy vegetables selected for a delay in bolting and flowering, genomic regions coding for delay in or even prevention of flowering might be good candidates for such TM strategies (Gressel 1999), especially in situations with high density competition and a seasonal flowering period.

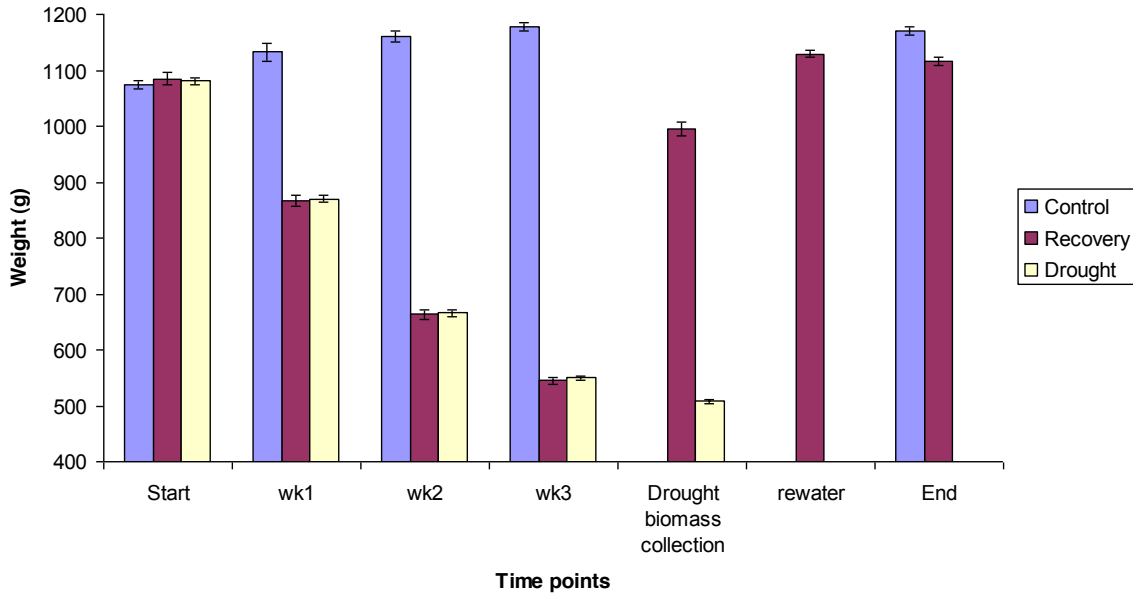
In conclusion, unavoidable differences in experimental set-up can cause a large variation in the QTL results, making predicting genomic selection patterns for specific abiotic stresses challenging, at the least. Therefore, considerably more comprehensive experiments would be required in terms of plants, lines, and manual labor to include specific abiotic stress effects in ERA (Beavis 1998). From our experiments, it appears imperative to include competition, as a selective agent, in such risk assessment experiments and to include multiple plant densities and multi-species environments that reflect the natural ecology of the wild species, as others have also showed (e.g., Mercer et al. 2006; Campbell and Snow 2007). Results of such studies can be used in simulation models, including spatial modeling, designed to predict transgene spread (Hails and Morley 2005), as well as in the design of TM constructs, aiming at mitigating the risk of transgene escape (Stewart et al. 2003).

Acknowledgements

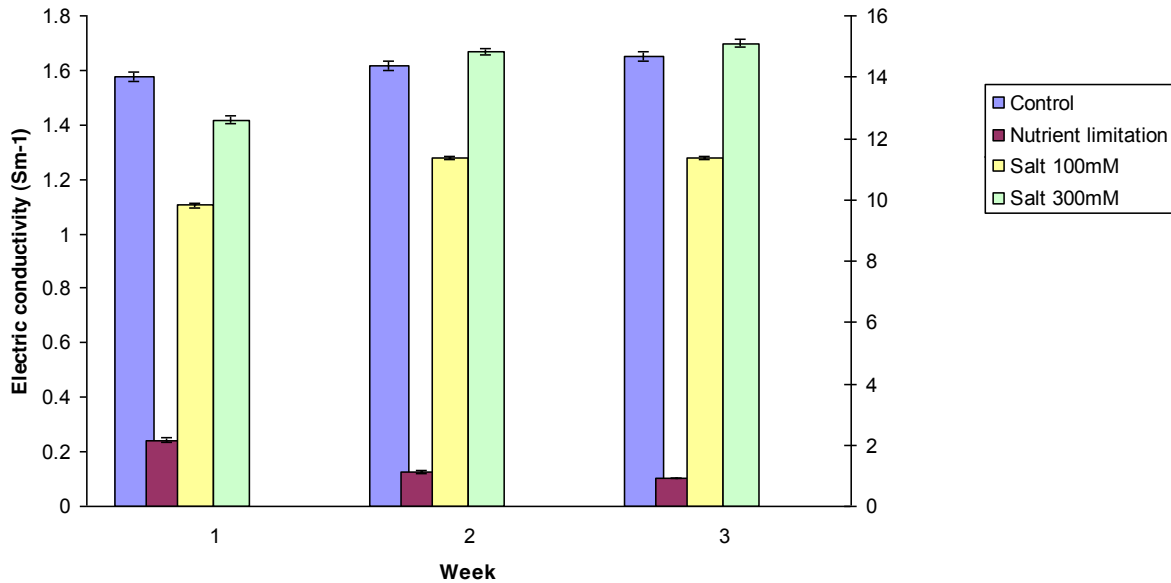
We are grateful to the family Stapel in Sijbekarspel for providing our field plot and to Justus Houthuesen who established and maintained the plot. We like to thank Patrick Meirmans for discussions and his valuable input. We also thank Rob Bregman, Thijs Hendrix, Harold Lemereis, Louis Lie, Ludek Tikovsky, and many others for help in field and greenhouse. RWM is a co-PI on The Compositae Genome Project (<http://compgenomics.ucdavis.edu>) that is supported by the NSF Plant Genome Program award #0820451. This study was funded by the Dutch Organization for Scientific Research (NWO) as part of the ERGO program (838.06.042).

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Appendix 1a. Water capacity of empty pots during the stress period of the Drought/recovery experiment.



Appendix 1b. Electric conductivity of empty pots during the stress period. Control and nutrient limitation treatments are on the left axis and the salt treatments are on the right axis.



Abiotic stress QTL in lettuce hybrids in greenhouse and field

Appendix 2. Schematic representation of experimental design. From left light gray to right dark gray: germination period, establishment period, stress period, and biomass collection. 1 = drought treatment collected; 2 = control and recovery treatment collected (in between plants were rewatered).

Week	1							2							3							4						
Salt/nutrient limitation																												
Drought/recovery																												
Greenhouse competition																												
Field stress																												

Appendix 2. Continued

Week	5							6							7																		
Salt/nutrient limitation																																	
Drought/recovery																									1			2					
Greenhouse competition																																	
Field stress																																	

Appendix 3. Number of replicates per recombinant inbred line (RIL) used in the greenhouse competition and field experiments.

RIL	Greenhouse				Field		
	Control	Nutrient limitation	Drought	Salt 100mM	Control	Drought	Salt 100mM
L. sativa cv. Salinas	17	17	17	17	17	14	10
L. serriola UC96US23	17	17	17	17	13	10	12
114	17	17	17	17	17	14	14
115	17	0	17	17	17	14	13
116	17	17	17	17	15	14	13
119	12	15	13	11	11	10	10
120	17	17	17	17	16	14	14
121	7	17	7	7	17	13	16
122	17	17	17	17	16	16	16
124	14	17	16	15	14	11	7
125	14	17	16	17	17	15	15
126	2	17	3	3	14	12	16
127	17	7	17	17	17	16	13
128	13	8	13	13	9	7	11
129	17	17	17	17	16	15	14
130	17	17	17	17	16	13	12
131	14	17	14	17	7	7	7
132	9	17	10	10	17	15	16
133	10	16	10	10	12	9	8
134	17	17	17	17	16	14	16
135	17	16	17	17	16	14	15
136	15	17	15	16	9	10	14
139	17	17	17	17	17	13	11
140	9	17	10	11	14	13	8
141	9	17	9	9	9	9	6
142	12	17	12	13	17	14	17
143	17	17	15	16	17	15	12
144	17	17	17	17	15	14	16
145	3	13	3	4	10	7	8
146	17	17	17	17	15	16	15
148	17	17	17	17	9	8	9
149	17	17	17	17	17	14	15
150	13	17	14	13	17	11	17
151	17	17	17	17	16	14	14
152	17	17	17	17	16	15	16
154	17	17	17	17	15	12	14
155	16	17	14	16	15	15	13
156	17	17	17	17	15	14	13
158	11	5	12	12	9	7	5
159	14	17	15	15	17	12	14
160	17	17	17	17	15	16	16
162	17	17	17	17	16	14	15
163	10	17	9	9	14	14	11
164	17	17	17	17	17	15	16
166	17	17	17	17	16	16	14
167	13	17	13	13	17	14	13
169	13	14	13	13	10	0	0

Abiotic stress QTL in lettuce hybrids in greenhouse and field

Appendix 3. Continued

RIL	Greenhouse				Field		
	Control	Nutrient limitation	Drought	Salt 100mM	Control	Drought	Salt 100mM
170	17	17	17	17	17	15	13
171	17	17	17	17	17	13	14
172	15	17	15	16	17	15	15
173	9	17	8	8	6	5	4
175	17	11	17	17	12	14	13
176	17	17	17	17	16	13	12
177	17	17	17	17	16	14	11
178	17	17	17	17	14	13	10
179	12	15	12	11	13	14	14
180	17	17	17	17	17	16	11
181	8	4	8	11	14	8	10
182	17	17	17	17	17	16	16
183	17	17	17	17	12	11	12
186	17	17	17	17	15	15	15
187	13	15	13	14	15	14	13
190	17	17	17	17	16	15	13
192	15	13	17	17	17	17	14
193	17	17	17	17	16	15	12
194	17	17	17	17	16	13	15
195	17	17	17	17	16	14	12
196	17	17	17	17	13	14	15
197	17	16	17	17	15	14	12
199	10	14	16	16	16	11	13
201	17	17	17	17	16	16	14
202	17	10	17	17	16	16	16
203	17	17	17	17	13	11	14
204	17	17	17	17	16	14	15
205	13	17	17	17	15	14	11
206	15	17	15	15	16	15	17
207	17	14	17	17	16	15	16
209	17	17	17	17	17	15	14
211	17	17	17	17	15	15	10
212	17	17	17	17	17	14	13
213	17	11	17	17	15	14	12
214	12	17	12	12	9	10	9
215	14	15	14	14	13	12	9
217	14	12	14	15	15	13	16
218	12	17	12	12	17	16	16
219	17	17	17	17	16	11	14
220	16	17	17	16	17	14	14
221	15	17	15	15	15	13	15
222	17	17	17	17	16	11	14
223	13	17	11	12	13	14	14
227	12	14	13	13	16	14	13
228	14	9	15	12	10	11	11
average n treatment	14.8	15.7	15.0	15.1	14.7	13.0	12.8
average n experiment	15.2			13.5			