Theor Appl Genet (2010) 120:607–619
DOI 10.1007/s00122-009-1179-y

ORIGINAL PAPER

Identification of genetic factors influencing salt stress tolerance in white clover (*Trifolium repens* L.) by QTL analysis

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Received: 12 May 2009 / Accepted: 4 October 2009 / Published online: 29 October 2009

Abstract Allotetraploid ($2n = 4x = 32$) white clover (*Trifolium repens* L.) is the most commonly cultivated legume component of temperate pastures, sown in swards with a companion grass species. Genetic control of growth performance of white clover on saline land is highly important for dairy industries, due to increasing soil salinity problems. The objective of this study was to identify quantitative trait loci (QTLs) for salinity tolerance in terms of vegetative growth under stress. Two parental genetic maps consisting of 213 and 159 marker loci and spanning 1,973.0 and 1,837.6 cM, respectively, were constructed using simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers from a two-way pseudo-test cross F_1 population derived from pair-crossing of the $Haifa_2$ and LCL_2 genotypes. A total of 8 unique genomic regions on 8 linkage groups (LGs) of the $Haifa_2$ parental map and 6 unique regions on 5 LGs in the LCL_2 parental map were associated with plant growth under salt stress and relative growth under stress, as compared to control conditions. The results of this study indicate that salt tolerance in white clover is controlled by multiple QTLs, some at common locations, but each of limited magnitude. Location of these QTLs provides the genetic basis and potential for pyramiding of salt tolerance genes in breeding improvement.

Communicated by R. Varshney.

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Introduction

The global extent of salt-affected land under cultivation is sufficient to pose a threat to agriculture and food security, as many plants will not grow under highly saline conditions (Flowers 2004). An electrical conductivity (EC) value of 4.0 dS/m (equivalent to 40 mM NaCl) is used as a threshold to define saline soils, but some sensitive crops will show symptoms and reduced yields at even lower ECs. For example, yield of white clover is reduced significantly at 20 mM NaCl (Rogers et al. 1993). The objective of producing salt-tolerant crops has long been pursued, and due to increasing soil salinity and the requirement to feed growing human populations, plants with enhanced tolerance are in increasing demand (Munns 2005).

The underlying genetic systems that enable halophytic species to grow under extreme saline conditions, and the presence of significant genetic variation within a species, indicate that gains in plant performance and production through molecular-based breeding for salt tolerance are feasible (Flowers 2004). White clover is the most

commonly cultivated legume component of temperate pastures and its productivity is essential for the sheep meat, beef cattle and dairy production industries. White clover is often considered to be a salt-sensitive species, but salt-tolerant genotypes, cultivars and natural populations have been identified (Ab-Shukor et al. 1988; Rogers et al. 1993, 1997). Improving salt tolerance of white clover through a molecular-based breeding strategy is consequently possible. However, as for most other crop species, breeding for salt tolerance has so far obtained limited success. Salt tolerance is a complex trait to manipulate both physiologically and genetically, as there are many different evolved mechanisms in place to achieve tolerance, including ion homeostasis, osmotic balance and restoration, and metabolic and structural adaptations (Phang et al. 2008).

The use of molecular markers has revolutionised plant breeding through the ability to perform genetic analysis of complex traits and to select for target regions through marker-assisted selection (MAS). QTLs for salt tolerance or related physiological components have been reported in rice (Flowers et al. 2000; Koyama et al. 2001; Lee et al. 2006; Lin et al. 2004; Prasad et al. 2000; Zang et al. 2008), barley (Ellis et al. 1997, 2002; Mano and Takeda 1997), soybean (Lee et al. 2004), citrus (Tozlu et al. 1999a, 1999b), tomato (Foolad et al. 1998, 2001; Villalta et al. 2007, 2008), *Arabidopsis thaliana* (Quesada et al. 2002), and wheat (Ma et al. 2007; Quarrie et al. 2005). Detailed dissection of the genetic regulation of salt tolerance in white clover has not yet been performed. However, selections have been made for low shoot Cl^- (LCL) concentration from cultivar sources (Rogers et al. 1997).

Three white clover genetic maps with varying degree of coverage and marker types have been constructed, using either F_2 progeny derived from an intercross of inbred genotypes carrying a self-fertile mutation (Jones et al. 2003) or F_1 progeny from two-way pseudo-test crosses between two heterozygous genotypes (Barrett et al. 2004; Zhang et al. 2007). Subsequently, QTLs for seed production (Barrett et al. 2005) and for vegetative and reproductive morphogenesis have been identified (Cogan et al. 2006). Different nomenclature systems for the LGs generated from the current maps have been implemented, due to lack of a standardised system for white clover. Recently, alignment of expressed sequence tag (EST)-based genetic markers from white clover with the genome sequence of *Medicago truncatula,* demonstrating extensive macrosynteny, has provided a rational basis for alignment and ordering of LGs generated from genetic mapping studies (George et al. 2008).

The extant species most closely related to the diploid progenitors of white clover have been proposed to be *T. occidentale* D.E. Coombe and *T. pallescens* Schreber, based on a molecular phylogenetic study using nuclear ribosomal DNA internal transcribed spacer and chloroplast *trnL* intron sequences (Ellison et al. 2006). Comparison of white clover with *T. occidentale* and *T. pallescens* based on sequences from nine nuclear genes identified one group of haplotypes with high levels of sequence similarity to *T. occidentale*, leading to designation of the O sub-genome. The second sub-genome showed weaker affinity to both *T. pallescens* and *T. occidentale* and was consequently designated P' (Hand et al. 2008). The characterisation of sub-genomes has allowed refinements of genetic mapping and trait dissection studies and offers the possibility of attributing QTLs to homologous chromosomes within homoeologous groups (HGs), as well as determining allele and homoeoallele-specific gene expression effects.

Identification of QTL regions of genetic control of salt tolerance in white clover and molecular markers linked to the tolerance loci is an essential step towards greater understanding of plant performance in saline conditions, which will inform advanced breeding strategies for MAS. This study presents the results of genetic map construction from a trait-specific genetic mapping population and LG alignment in comparison to the genome of the model legume *M. truncatula*, and, where possible, sub-genome attribution to identify QTLs for vegetative growth of white clover under both control and salt stressed conditions. The number and location of regions detected and specificity to stress or control conditions are discussed. Identification of QTL-containing regions originating exclusively from sub-genome-specific locations is also discussed, along with the implications for applied breeding using molecular genetic markers.

Materials and methods

Plant materials

An F_1 mapping population comprising 232 progeny was derived from a two-way pseudo-testcross between a single genotype from the variety Haifa (hereafter referred to as $Haifa_2$) and a single genotype from the population LCL (hereafter referred to as LCL_2). Haifa is a cultivar that was widely grown in irrigation regions in south eastern Australia (Rogers et al. 1993) and was originally selected from material collected in Israel for persistence, adaptability, vigour, and tolerance to high temperature and moisture stress (Barnard 1972). LCL is the product of the second cycle selection for low shoot Cl^- concentration from cv. Haifa by Rogers et al. (1997). Both Haifa and LCL seeds were kindly supplied by Dr. Mary-Jane Rogers in the Department of Primary Industries (DPI) in Tatura, Victoria and crossing was performed in DPI Hamilton, Victoria.

Salt tolerance phenotypic analysis

The salt tolerance phenotypic trial of the mapping population was conducted in nutrient solution culture under glasshouse conditions. Each genotype was propagated asexually from a single plant, a total of twelve stolon tip cuttings for each genotype being cultured in half-strength Hoagland's solution (McFarlane et al. 2003) to allow root development for 2 weeks. Six uniform plantlets from each genotype were then selected and transferred into continuously-aerated half-strength Hoagland's solution. An incomplete block design was used with two NaCl concentration levels (0 and 75 mM) and three replicates per combination genotype-treatment resulting in a total of 1,404 plants. The parental genotypes ($Haifa_2$ and LCL_2) were also included in the experiments. The NaCl levels were randomly allocated to tubs, while genotypes were randomly allocated in blocks of 20, being the number of positions within a tub. A total of 72 tubs were randomly arranged in 8 benches in glasshouse. The 75 mM NaCl concentration was determined based on a pre-experiment test of response of parental genotypes to salt stress (data not shown). Salt treatments were established, with daily increments of 25 mM over 3 days. The pH levels of the culture solutions were monitored and regulated to 6.0 three times per week, and solutions were changed every 2 weeks. The experiment was conducted in glasshouse conditions with natural light and temperature 20 ± 3°C. Non-destructive measurements including leaf number (Leaf_No), maximum leaf width (MLW), maximum petiole length (MPL), and stolon number (Stolon_No), were obtained at the start of the treatment and at the time of harvest. Maximum stolon length (MSL) was measured at the time of harvest only. Plants were harvested 4 weeks after treatment. Leaf, petiole, stolon, root and flowers were separated for each individual plant and tissues were oven-dried for 48 h at 70°C, and leaf dry mass (LDM), petiole dry mass (PDM), stolon dry mass (StDM), root dry mass (RDM) and flower dry mass were measured. Shoot dry mass (ShDM) and whole plant dry mass (WPDM) were subsequently calculated.

Statistical analysis

Statistical analysis was carried out using the GenStat Edition 10.1 (Payne et al. 2007). Correlation analysis between pairs of traits was calculated using correlations procedure. Restricted maximum likelihood (REML) method and linear mixed model were used to test the effects of treatments. Logarithmic transformation was applied to the dry mass data and square root transformation was applied for non-destructive measures for best fit to the model. The initial leaf number was used as a covariate in the analysis, because significant correlations were detected between the final measurements and the initial leaf number. Predicted means were obtained for each genotype in both control (0 mM NaCl) and treatment (75 mM NaCl) conditions. To distinguish control and stress data sets, the suffixes –C and –S, respectively were added to the trait name. The –R suffix denotes relative measurements (percentage of trait value under stress as compared to trait value under control conditions).

Molecular genetic marker analysis and map construction

Genomic DNA was extracted using the DNeasy Plant 96-kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For SSR markers, PCR amplification and genotyping were performed as described previously (Wang et al. 2009). For SNP markers, reactions were performed using the SNuPe genotyping kit (GE Healthcare, Chalfont, St. Giles, UK) following manufacturer's instructions and as described in Hand et al. (2008).

Parental genetic linkage maps were established for $Haifa_2$ and LCL_2 using segregation data generated for 232 mapping population progeny, with polymorphic genomic DNA-derived SSR (Jones et al. 2003), EST–SSR (Barrett et al. 2004) and SNP (Cogan et al. 2007; Hand et al. 2008) markers. The F_1 ($Haifa_2$ x LCL_2) two-way pseudo-testcross population was analysed as an F_2 backcross population for the purpose of genetic map construction (Grattapaglia and Sederoff 1994). Marker segregation ratios were checked for deviation from Mendelian expectation (1:1) by χ^2 analysis. Map construction was conducted using MAPMAKER 3.0 (Lander et al. 1987). Data sets were inverted and merged with the normally-coded data in order to detect repulsion phase linkages. Using the group command, markers were grouped at a logarithm of odds (LOD) threshold of 5.0, and were subsequently ordered within groups at LOD $\geq$2.0. Final marker orders were confirmed using the ripple command. Map distances in centiMorgans were calculated using the Kosambi mapping function (Kosambi 1944). Linkage map orientation and nomenclature were aligned with the *M. truncatula* draft genome as described by George et al. (2008). LGs were attributed, if possible, to sub-genomes (O or P') on the basis of specific SNPs, as described by Hand et al. (2008). LGs that could not be unambiguously attributed to specific sub-genomes were arbitrarily denoted as .1 or .2, respectively.

QTL analysis

QTL detection was based on a pseudo-testcross model (Grattapaglia et al. 1995), with comparisons between (Q1Q3 + Q1Q4) versus (Q2Q3 + Q2Q4) for each parent.

Following genetic map construction, a sub-set of marker loci were used for simple interval mapping (SIM: Lander and Botstein 1989; Haley and Knott 1992) and composite interval mapping (CIM: Zeng 1994) as previously described (Cogan et al. 2006) using QTL Cartographer, version 2.5 (Basten et al. 1994). QTL location predictions for SIM were accepted for LOD values greater than 2.5. The LOD thresholds for each trait in CIM were determined by 1,000 permutation test at 0.05 confidence level. For each form of interval analysis, maximum LOD value, the location on the genetic map, additive marker value effects and the proportion of phenotypic variance attributable to the QTL were tabulated. The direction of the additive effect of the QTL is only in relation to the alternate alleles from each parental genotype, and is not comparable between the two parents. Unique QTLs were defined as the sub-set influencing correlated traits which mapped within 15 cM of one another (Sewell et al. 2000, 2002).

Results

Salt tolerance phenotyping

Eleven quantitative traits were measured in the 232 F_1 progeny under both control and salt-stressed conditions. The phenotypic correlations under control and salt stress are shown in Table 1. Under control conditions, the majority of the traits were significantly correlated ($P < 0.05$), with the exception of MPL with StDM, RDM, leaf number, and stolon number, and for leaf number with MLW. In contrast, under stress all measured traits were highly significantly correlated ($P < 0.001$). The correlations between LDM, PDM, ShDM, StDM, RDM and WPDM were positive and highly significant, most exhibiting correlation coefficients of $r > 0.90$. The correlation coefficients between leaf number and dry matters were slightly lower ($0.80 < r < 0.90$). Other non-destructive measurements generated low but significant correlations ($0.34 < r < 0.80$).

Salt stress had a highly significant effect ($P < 0.001$) on all the traits, reducing leaf number, MLW, MPL, MSL, stolon number, LDM, PDM, StDM, ShDM, RDM, and WPDM. Genotypic effects were highly significant ($P < 0.001$) for all the traits measured. Genotype-by-NaCl interaction was also significant ($P < 0.01$) for most traits except MLW ($P = 0.198$), suggesting that the leaf number, plant height, spread and yield decreased under salt stress, depending on the genotype.

Normal frequency distributions were observed for LDM-R, PDM-R, ShDM-R, StDM-R, RDM-R, and WPDM-R (Fig. 1). Largest effect of salt stress was observed for PDM, while the least effect was seen for RDM. For all trait distributions, the $Haifa_2$ parent was in the lower-value range of the distribution in terms of growth performance, while the tolerant parent (LCL_2) was consistently higher than the population mean, indicating that the distribution was not excessively transgressive (Fig. 1). Scatter plot analysis (Fig. 2) showed that $Haifa_2$ had a higher ShDM value in the control treatment, and a lower ShDM value in stress conditions, while LCL_2 exhibited low ShDM levels in both control and stress conditions, and so yield loss was relatively lower than for $Haifa_2$. Some progeny genotypes revealed higher ShDM levels in both control and stressed conditions, and will hence be ideal for breeding to improve production in saline soils.

Linkage map construction

The $Haifa_2$ genetic map contained 213 marker loci spanning a total length of 1,973.0 cM (Fig. 3). The LCL_2 genetic map contained 159 loci spanning a total length of 1,837.6 cM (Fig. 4). A total of 10 of the 17 LGs generated for the $Haifa_2$ genetic map could be attributed to a specific sub-genome (Fig. 3), but only 1 of the 19 LGs from the LCL_2 genetic map could be attributed. For both parental genetic maps, marker coverage was adequate for HGs 2, 3, and 4 to generate the expected number of groups of reasonable length, while increased marker coverage on HGs 1, 5, 6, 7, and 8 is required to resolve some anomalies in genetic map construction and in some instances increase total cM length.

Interval mapping of QTLs

A total of 51 putative QTLs for the 11 traits assessed under control and salt-stressed conditions have been identified using both SIM and CIM mapping approaches [30 on the $Haifa_2$ map (Table 2; Fig. 3) and 21 on the LCL_2 map (Table 3; Fig. 4)]. Many of these QTLs provide independent estimations of the same genomic region as many of these traits are highly correlated. In the $Haifa_2$ genetic map, 12 unique regions were identified, with 5 major clusters of 3 or more QTLs, accounting for 22 of the total. In the LCL_2 genetic map, 7 unique regions were identified, with 3 major clusters of 3 or more QTLs, accounting for 16 of the total. The range of phenotypic variation (V_p) accounted for was from 5 to 17%, with a mean of 8% across both genetic maps.

Vegetative growth under control conditions

On the $Haifa_2$ genetic map, a total of 5 unique QTLs for traits under control conditions were identified that account for 8 individual QTLs (Fig. 3; Table 2). These included two qMLW-C (on LG 1.1 and LG 1.2) of similar

Table 1 Phenotypic correlation between leaf dry mass (LDM), petiole dry mass (PDM), stolon dry mass (SDM), root dry mass (RDM), shoot dry mass (ShDM), whole plant dry mass (WPDM), leaf number (Leaf_No), maximum leaf width (MLW), maximum petiole length (MPL), stolon number (Stolon_No), and maximum stolon length (MSL) under control (lower matrix) and salt stress (upper matrix)

	LDM	PDM	StDM	ShDM	RDM	WPDM	Leaf_No	MLW	MPL	Stolon_No	MSL
LDM	–	0.95	0.89	0.98	0.90	0.98	0.89	0.53	0.53	0.75	0.72
PDM	0.96	–	0.85	0.95	0.85	0.95	0.85	0.53	0.64	0.70	0.73
StDM	0.92	0.86	–	0.94	0.82	0.93	0.82	0.42	0.42	0.71	0.80
ShDM	0.99	0.96	0.95	–	0.89	1.00	0.88	0.52	0.54	0.73	0.78
RDM	0.92	0.87	0.87	0.92	–	0.92	0.80	0.47	0.45	0.70	0.64
WPDM	0.99	0.96	0.95	1.00	0.94	–	0.88	0.52	0.53	0.74	0.76
Leaf_No	0.86	0.81	0.84	0.85	0.82	0.86	–	0.38	0.41	0.78	0.68
MLW	0.28	0.31	0.17	0.26	0.21	0.26	*0.07*	–	0.66	0.36	0.49
MPL	0.16	0.32	*0.06*	0.18	*0.07*	0.16	*0.01*	0.44	–	0.34	0.56
Stolon_No	0.77	0.72	0.75	0.76	0.75	0.77	0.82	0.10	*−0.01*	–	0.54
MSL	0.61	0.61	0.72	0.66	0.53	0.65	0.52	0.28	0.17	0.42	–

Values in italics denote not significant values at $P = 0.05$ level

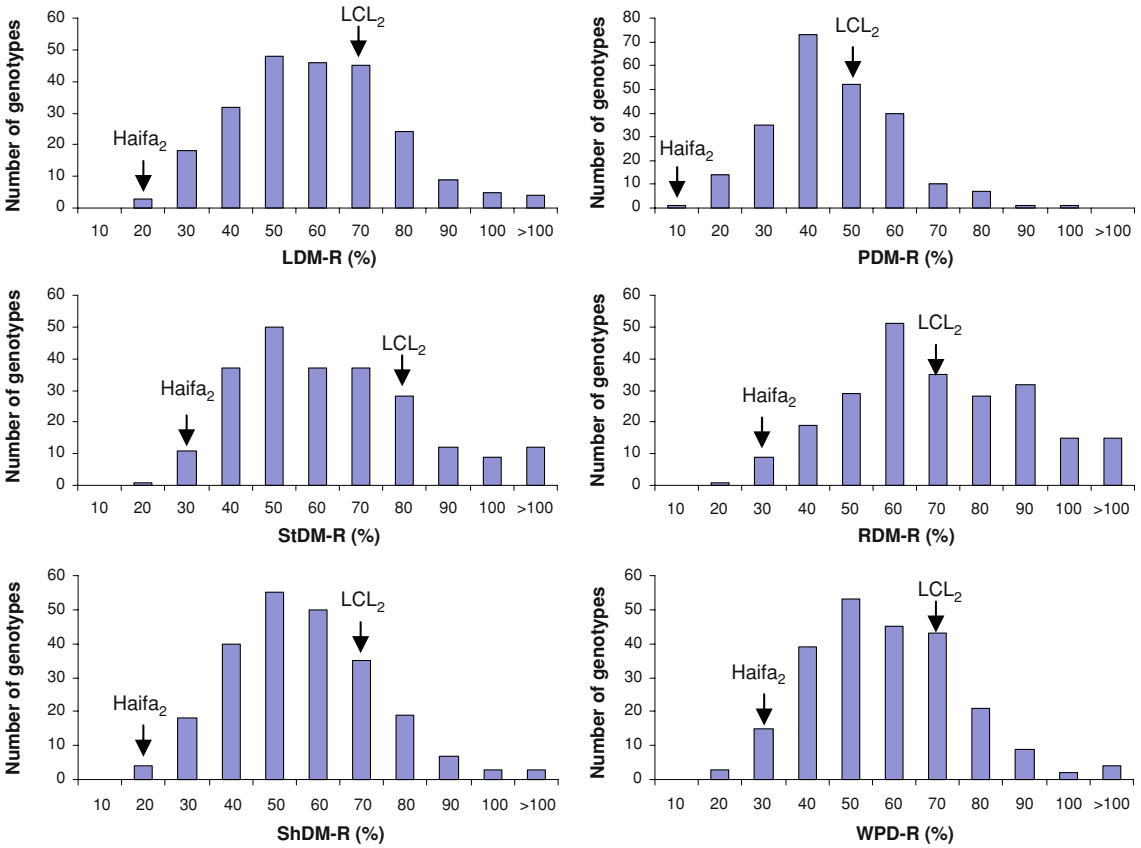

Fig. 1 Frequency distributions of relative dry mass of leaf, petiole, stolon, root, shoot and total plant in the F_1 progeny derived from the $Haifa_2$ and LCL_2 pair-cross. Mean parental values are indicated by *arrows*

magnitude of effect, qRDM-C on LG 6P', a cluster on LG 3O of qRDM-C, qPDM-C, qLeaf_No-C, qStolon_No-C, and qMSL-C on LG 3O. The V_p values explained by these QTLs varied from 6 to 12% depending on the trait.

On the LCL_2 genetic map, fewer QTLs were detected. A total of 3 unique QTLs were identified, accounting for 4 individual QTLs (Fig. 4; Table 3). The 3 unique QTL were detected on LG 4.1 (qStolon_No-C), on LG 7.1

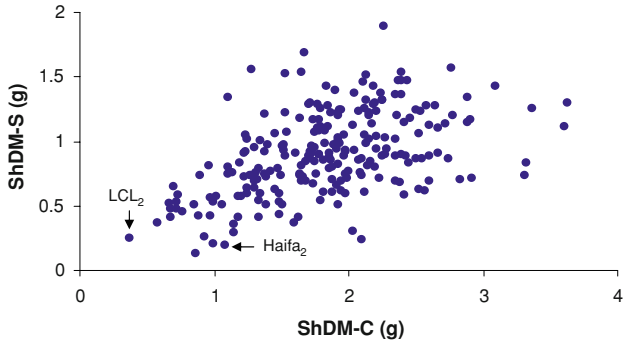

Fig. 2 Scatter plot of shoot dry mass under control (0 mM NaCl) and salt-stressed (75 mM NaCl) conditions

(qLeaf_No-C and qStolon_No-C) and LG 8.1 (qMSL-C). The V_p values explained by these QTLs varied from 5 to 15%.

Vegetative growth under salt stress

On the $Haifa_2$ genetic map, a total of 6 unique QTLs for traits under salt stress were identified relating to 15 individual QTL. An unique QTL region on LG 1.1 was associated with 7 traits (qLDM-S, qPDM-S, qRDM-S, qShDM-S, qWPDM-S, qMPL-S and qMLW-S; Fig. 3) with all of the positive additive effects originating from the same allele. A single QTL region for qMLW-S was detected on LG 1.2. Other unique QTL regions were detected on LG 3O (qShDM-S and qMPL-S), 4O (qLeaf_No-S and qStolon_No-S), 5O (qRDM-S) and 7P' (qLeaf_No-S and qStolon_No-S). The V_p values explained by these QTLs varied from 5 to 17%.

On the LCL_2 genetic map, a total of 3 unique QTL regions for traits under salt stress conditions were identified. A large cluster of 4 individual QTLs were located on LG 1.1 (qRDM-S, qShDM-S, qWPDM-S and qMSL-S) with all of the positive additive effects originating from the same allele. Other unique QTL regions were detected on LG 4.1 (qStolon_No-S) and on LG 8.1 (qMSL-S). The V_p values explained by these QTLs varied from 7 to 9%.

Relative vegetative growth of stress versus control

On the $Haifa_2$ genetic map, a total of 4 unique QTLs for relative growth traits were identified accounting for 7 individual QTLs. Regions of effect were identified on LG 3O (qStDM-R and qMPL-R), LG 4O (qStolon_No-R), LG 8.1 (qMLW-R), and a cluster on LG 8.2 with all of the positive additive effects originating from the same allele (qLDM-R, qPDM-R and qWPDM-R). The V_p values explained by these QTLs varied from 5 to 14%.

On the LCL_2 genetic map, a total of 3 unique QTL for relative growth traits were identified accounting for 11 individual QTLs. A cluster of 6 individual QTL on LG 2.1 was detected for qLDM-R, qStDM-R, qShDM-R, qRDM-R, qWPDM-R, and qLeaf_No-R, with all of the positive additive effects originating from the same allele and of approximately similar magnitude of effect. A single QTL was detected on LG 7.2 for qMSL-R, and a second cluster was detected on LG 8.1 for qPDM-R, qRDM-R, qShDM-R, and qWPDM-R. The V_p values explained by these QTLs varied from 5 to 9%.

Discussion

Effects of salinity and salt tolerance

Salt stress reduces the yield of most crops, including white clover. In the present study, approximately 50% shoot dry mass reduction was observed for plants growing in 75 mM NaCl, as compared to non-stressed conditions. All yield components, including leaf number, MLW, MPL, RDM, ShDM, stolon number and MSL, decreased to different extents during the course of the experiment. Although root growth has previously been commonly used as an indicator for salt stress tolerance, RDM was affected to a lesser extent than ShDM in this study. Symptoms such as leaf burn were not observed under the experimental conditions, but thinner and more curled roots were observed as a result of the salt stress treatment (data not shown).

Application of salt stress influenced the observed correlations between traits. Correlation between leaf number and MLW was not significant under control conditions, but became significant under stress, leading to both fewer leaves and smaller leaflet size. Similarly, MPL did not show correlation with StDM, RDM, leaf number and stolon number under control conditions, but the correlations became highly significant under stress treatment. The implication of this change of correlations is that sensitive genotypes with reduced MPL also exhibited lower StDM, RDM, leaf number and stolon number values. It could therefore be considered that MPL measurement may hence function as an effective indicator for evaluation of salt-stress tolerance in a non-destructive manner.

Genotype-by-NaCl concentration interactions were also significant, and those genotypes with the lowest reduction in trait values under saline conditions can be considered as viable sources for breeding improvement. Salt tolerance variation can be defined as genotypic difference in biomass production in saline as compared to non-saline conditions over prolonged periods of time (e.g., 3–4 weeks) (Munns and James 2003). However, some genotypes with limited tolerance could be prolific producers of vegetative biomass under control conditions and ultimately so exceed the performance of the most salt-tolerant genotypes. Therefore,

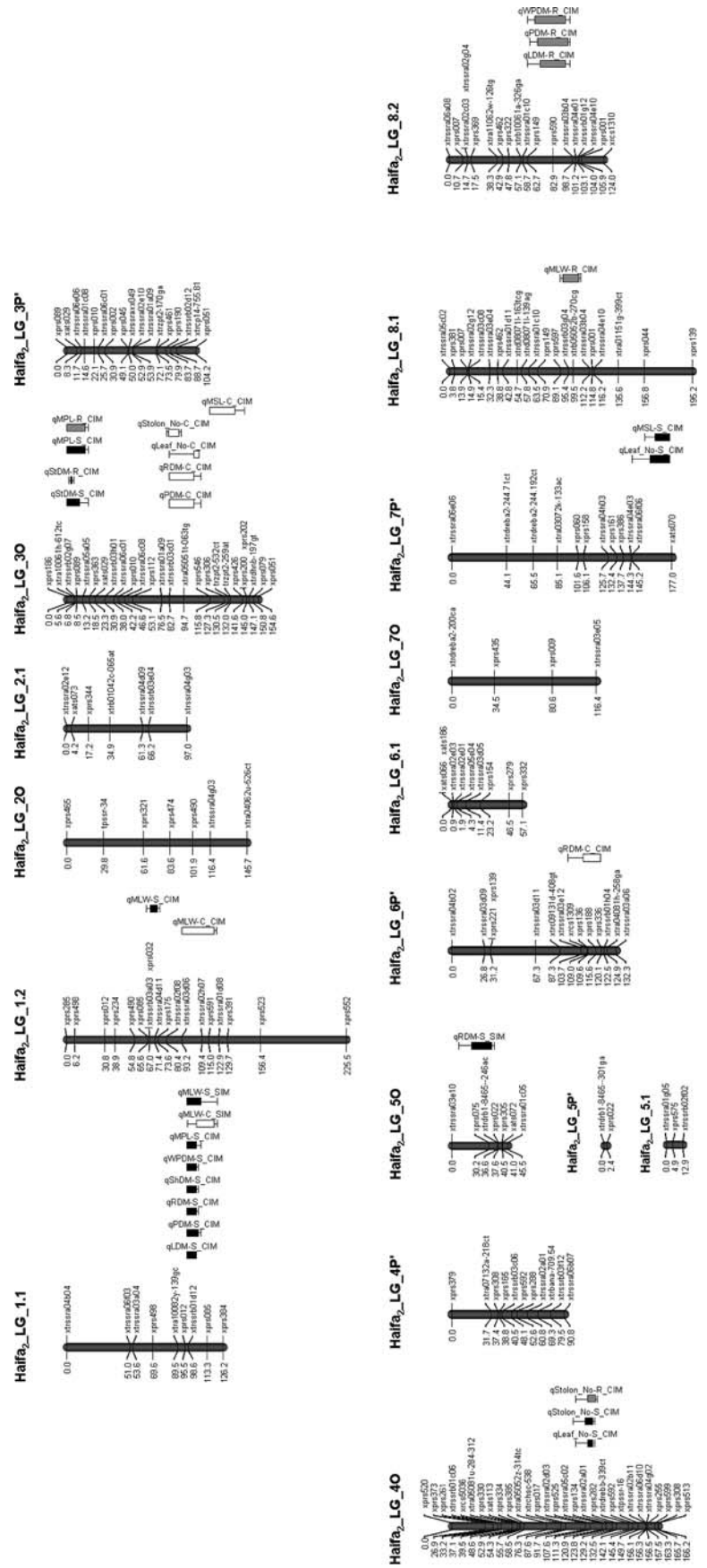

Fig. 3 QTLs identified on the Haifa$_2$ parental map. Linkage group (*LG*) orientation and nomenclature was determined through alignment with the chromosomally-assigned pseudomolecules of the *Medicago truncatula* genome draft. LGs were further attributed to the O or P' sub-genome, or denoted as .1 or .2 if the sub-genome origin was not determined. All marker loci (prefixed with an '*x*') indicate co-dominant marker types. Genomic DNA-derived SSR markers are indicated with xtrssr (Kölliker et al. 2001; Jones et al. 2003) and xats (Barrett et al. 2004) prefixes. EST-derived SSR markers (Barrett et al. 2004) are indicated with xprs prefixes. EST-derived SNP loci based on in silico prediction (Cogan et al. 2007) are named according to the format xtr-EST unique identifier (UI)-coordinate of variant base-variant base identity. SNPs derived from functionally-annotated genes based on in vitro discovery (Hand et al. 2008) are named according to the format xtr-gene name abbreviation—coordinate—variant base identity, with intronic locations denoted in form last coordinate of preceding exon.coordinate of variant base (e.g., xtrdreb-244.71ct). Genomic and EST-derived SSRs from red clover are indicated with xtpssr and xrcs prefixes, respectively (Kölliker et al. 2006; Sato et al. 2006). QTL nomenclature is adapted from McCouch et al. (1997) in the form q-TRAIT-method of analysis (*SIM* simple interval mapping; *CIM* composite interval mapping). *Bars* and *lines* represent 1 and 2 LOD unit drops from the maximum likelihood value. *White*, *black*, and *grey bars* indicate the traits under control (-C), salt stress (-S), and relative measurements of trait values under stress versus control (-R), respectively

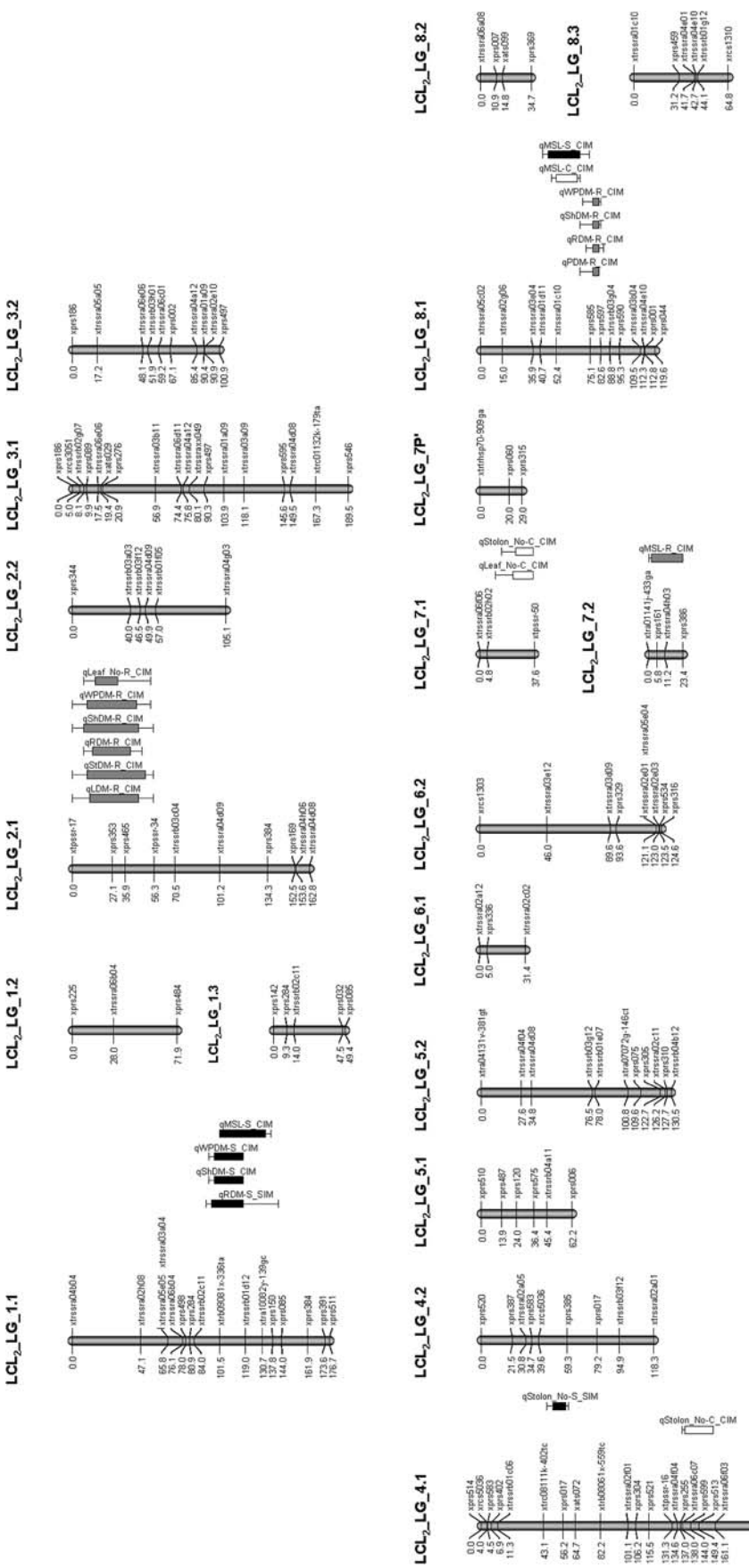

Fig. 4 QTLs identified on the LCL_2 parental map. Details are as described in the legend for Fig. 3

Table 2 Summary of QTL analysis for morphological traits on the Haifa$_2$ parental map

Description	Linkage group	SIM				CIM				
		Maximum LOD score	Position of maximum LOD	Additive effect	V_p	Threshold	Maximum LOD score	Position of maximum LOD	Additive effect	V_p
LDM-S	*LG_1.1*	*4.71*	*97.51*	*0.12*	*0.16*	*2.73*	*5.41*	*102.61*	*0.13*	*0.17*
LDM-R	LG_8.2					2.91	3.65	82.91	−15.14	0.09
PDM-C	LG_3O					2.93	3.41	88.71	1.27	0.08
PDM-S	*LG_1.1*	*3.28*	*97.51*	*0.05*	*0.12*	*2.93*	*3.91*	*97.51*	*5.41*	*0.12*
PDM-R	LG_8.2					2.93	3.79	82.71	−11.94	0.10
StDM-S	*LG_3O*	*2.50*	*6.01*	*0.04*	*0.05*	*3.01*	*3.20*	*2.01*	*4.81*	*0.06*
StDM-R	LG_3O					3.08	3.85	6.01	38.56	0.09
RDM-C	LG_3O					3.00	3.03	90.71	6.74	0.06
RDM-C	*LG_6P'*	*3.14*	*113.61*	*−0.05*	*0.07*	*3.00*	*3.11*	*113.61*	*−4.92*	*0.06*
RDM-S	*LG_1.1*	*3.22*	*97.51*	*0.04*	*0.11*	*2.92*	*3.80*	*97.51*	*4.51*	*0.11*
RDM-S	LG_5O	3.15	30.21	−0.03	0.06					
ShDM-S	*LG_1.1*	*3.24*	*97.51*	*0.22*	*0.12*	*2.98*	*3.85*	*97.51*	*2.23*	*0.12*
WPDM-S	*LG_1.1*	*3.38*	*97.51*	*0.27*	*0.12*	*2.93*	*3.47*	*97.51*	*2.53*	*0.11*
WPDM-R	LG_8.2					2.99	3.30	82.71	−14.26	0.09
Leaf No-C	LG_3O					2.94	5.36	88.71	13.11	0.12
Leaf No-S	LG_4O	2.99	111.31	−5.49	0.07					
Leaf No-S	*LG_7P'*	*3.31*	*175.21*	*5.81*	*0.08*	*2.91*	*3.03*	*175.21*	*5.17*	*0.06*
Stolon No-C	LG_3O					2.97	4.95	84.71	1.95	0.10
Stolon No-S	*LG_4O*	*3.04*	*111.31*	*−1.10*	*0.07*	*2.92*	*3.33*	*111.31*	*−1.08*	*0.06*
Stolon No-R	LG_4O					2.84	3.36	113.31	−28.88	0.14
MSL-C	LG_3O					2.92	3.23	121.81	−47.93	0.07
MSL-S	*LG_7P'*	*3.25*	*175.21*	*22.72*	*0.07*	*2.93*	*3.67*	*175.21*	*22.87*	*0.07*
MPL-S	*LG_1.1*	*2.82*	*97.51*	*13.22*	*0.09*	*2.73*	*2.73*	*98.61*	*9.54*	*0.05*
MPL-S	*LG_3O*	*2.94*	*6.81*	*10.45*	*0.06*	*2.73*	*3.65*	*6.81*	*10.98*	*0.06*
MPL-R	LG_3O					2.90	2.91	6.81	5.49	0.05
MLW-C	LG_1.1	3.56	108.61	1.76	0.12					
MLW-C	*LG_1.2*	*3.43*	*103.21*	*1.41*	*0.08*	*2.91*	*3.48*	*99.21*	*1.38*	*0.07*
MLW-S	LG_1.1	3.82	104.61	2.01	0.14					
MLW-S	*LG_1.2*	*2.55*	*69.61*	*1.32*	*0.06*	*2.91*	*4.59*	*71.41*	*2.63*	*0.09*
MLW-R	LG_8.1					2.87	3.81	99.51	−4.84	0.08

QTL identification based on SIM and CIM analysis was performed using the QTL Cartographer software. QTLs that were significantly identified by both analytical methods are italicised

V_p proportion of phenotypic variance explained by QTL; *additive effect* additive genetic effect of substituting alternative alleles at marker locus calculated with additive component

genotypes which either have greater salt tolerance or greater yield potential under non-saline conditions can be selected to maximise the yield (Rogers et al. 1993). In the present study, we found that some genotypes performed well in terms of both salt tolerance and yield potential and would be ideal source for selection.

QTL analysis

In the present study, an improved nomenclature system for both HG and LG attribution has been implemented (George et al. 2008; Hand et al. 2008), allowing sub-genome-specific origin to be identified for a large proportion of LGs. The nomenclature system will allow consistency and comparability between genetic maps in the future, and enhanced understanding of homoeologous gene function and regulation. To increase coverage and characterisation of the sub-genome origins of HGs that were not effectively populated in this study, additional genetic markers would need to be generated, as current public domain resources have largely been exhausted.

QTLs have for the first time been assigned to such a rationalised white clover genetic map. A total of 8 unique genomic regions on 8 Haifa$_2$ LGs and 6 unique regions on

Table 3 Summary of QTL analysis for morphological traits on the LCL_2 parental map

Description	Linkage group	SIM				CIM				
		Maximum LOD score	Position of maximum LOD	Additive effect	V_p	Threshold	Maximum LOD score	Position of maximum LOD	Additive effect	V_p
LDM-R	*LG_2.1*					2.83	3.31	29.11	9.53	0.06
PDM-R	LG_8.1					2.91	3.96	81.11	12.79	0.09
StDM-R	LG_2.1					2.82	3.87	31.11	14.42	0.08
RDM-S	LG_1.1	2.80	103.51	−0.03	0.07					
RDM-R	*LG_2.1*	*2.64*	*27.11*	*10.59*	*0.05*	*2.86*	*3.93*	*29.11*	*12.58*	*0.07*
RDM-R	LG_8.1					2.86	3.88	81.11	20.56	0.08
ShDM-S	*LG_1.1*	*2.60*	*107.51*	*−0.17*	*0.07*	*2.97*	*3.31*	*109.51*	*−0.18*	*0.08*
ShDM-R	*LG_2.1*	*2.50*	*29.11*	*8.27*	*0.05*	*2.85*	*3.36*	*24.01*	*9.28*	*0.07*
ShDM-R	LG_8.1					2.85	3.69	81.11	15.27	0.08
WPDM-S	*LG_1.1*	*2.71*	*107.51*	*−0.20*	*0.07*	*2.87*	*3.36*	*109.51*	*−0.22*	*0.08*
WPDM-R	*LG_2.1*	*2.63*	*29.11*	*8.67*	*0.06*	*2.83*	*3.58*	*29.11*	*9.64*	*0.07*
WPDM-R	LG_8.1					2.83	3.90	81.11	16.15	0.08
Leaf No–C	*LG_7*	*2.66*	*36.81*	*−7.28*	*0.07*	*2.83*	*3.09*	*36.81*	*−7.45*	*0.07*
Leaf No-R	*LG_2.1*	*2.59*	*27.11*	*9.92*	*0.05*	*2.91*	*4.37*	*27.11*	*12.53*	*0.08*
Stolon No–C	LG_4.1					2.80	2.96	148.01	−1.09	0.05
Stolon No-C	*LG_7*	*2.63*	*36.81*	*−1.21*	*0.07*	*2.80*	*2.99*	*36.81*	*−1.21*	*0.07*
Stolon No–S	LG_4.1	2.54	53.11	−1.17	0.08					
MSL-C	*LG_8.1*	*3.85*	*60.41*	*−48.93*	*0.14*	*2.91*	*4.66*	*60.41*	*−51.64*	*0.15*
MSL-S	*LG_1.1*	*2.86*	*111.51*	*−22.79*	*0.07*	*2.89*	*2.89*	*111.51*	*−21.13*	*0.06*
MSL-S	*LG_8.1*	*2.54*	*58.41*	*−23.72*	*0.08*	*2.89*	*2.95*	*60.41*	*−25.03*	*0.09*
MSL-R	LG_7.2					2.77	2.91	11.21	−8.47	0.05

QTL identification based on SIM and CIM analysis was performed using the QTL Cartographer software. QTLs that were significantly identified by both analytical methods are italicised

V_p proportion of phenotypic variance explained by QTL; *additive effect* additive genetic effect of substituting alternative alleles at marker locus calculated with additive component

5 LCL_2 LGs have been implicated in the genetic control of vegetative growth under salt stress or relative growth between treatments. Through comparison of the $Haifa_2$ and LCL_2 parental genetic maps, a common QTL for dry matter production of leaf, root, shoot and whole plant under salt stress was identified on LG 1.1 close to the xtrssrb01d12 locus. Other possible common QTLs between the two maps were identified. $Haifa_2$ LG 7P' and LCL_2 LG 7.2 both contain MSL QTLs, based on the shared marker xtrssra04h03. The markers xprs590 and xprs597 define a common region between HG 8 LGs ($Haifa_2$ LGs 8.1 and 8.2 with LCL_2 LG 8.1) containing relative growth QTLs. The presence of such a single region across different parental maps and both HGs of a single map suggests the potential role of one or more conserved homoeologous genes.

Considering the distribution of genomic regions containing relative growth QTLs, two major stress-specific unique regions were identified on LCL_2 LGs 2.1 and LG 8.1 for ShDM, accounting for 7 and 8% of V_p, respectively, in addition to the HG 8 regions previously described. Other relative rate QTLs observed in this study were qMPL-R and qStDM-R on LG 3O, and qStolon_No-R on LG 4O. In contrast, the major region identified containing QTLs for absolute vegetative growth values under salt stress, in terms of ShDM, was located on LG 1.1 of the $Haifa_2$ parental genetic map. In terms of RDM under stress, two unique regions were involved, on $Haifa_2$ LG 1.1 and LG 5O.

Chloride exclusion has been proposed as a major mechanism for salt tolerance in white clover. The tolerant parental base population (LCL) was selected from the Haifa variety through the criterion of low shoot Cl^- concentration (Rogers et al. 1993, 1997). By selection of the lowest shoot Cl^- concentration over two generations, levels were reduced by c. 20% Cl^-, and a corresponding increase of 10% in shoot dry matter was also observed (Rogers et al. 1997). Cl^- exclusion has been proposed to be controlled by a single dominant gene in the grain legume soybean *Glycine max* (Abel 1969) and a major salt tolerance QTL has been identified through visual score assessment (Lee et al. 2004). If Cl^- exclusion was also under simple genetic control in white clover and was the

major mechanism for salt tolerance, a major QTL would be expected in the present study. However, multiple QTLs for both absolute and relative measures were identified. It is possible that Cl^- exclusion may involve multiple gene effects in white clover, or that during the selection process for low Cl^- content, other salt tolerance genes may have been simultaneously selected. The presence of QTLs on both parental maps is also indicative of QTL heterozygosity within each parental genome, despite the relatively divergent phenotypes. The presence of a larger number of QTLs on the $Haifa_2$ map may represent a higher level of such heterozygosity prior to selection.

The ability to identify individual sub-genomes allows attribution of QTLs to specific homoeologous regions. QTLs have been identified which are specific to one, but not both HGs, in a given parental map (e.g., $Haifa_2$ LG 3O). Conversely, $Haifa_2$ HG 8 contains QTLs in coincident locations for both sub-genomes. The majority of QTLs were assigned to the O sub-genome, only 3 QTLs being attributed to the P' subgenome, while the others remain undetermined. As contemporary, *T. occidentale* has a strictly coastal distribution along the Gulf Stream coasts of Western Europe, in close proximity to the ocean (Williams et al. 2009), it is possible that the ancestral diploid may have contributed salt tolerance-associated allelic diversity to the nascent allotetraploid. In the QTL analysis of seed production, Barrett et al. (2005) also noted that the majority of the QTLs were specific to one homoeologue of white clover. However, the current lack of additional homoeolocus-specific markers to confirm sub-genome status has restricted the extent of confident attribution. Current activities aimed at generating large numbers of sub-genome-specific SNPs (Melanie Hand and Noel Cogan, unpublished) will permit continuous enhancement of the F_1 ($Haifa_2 \times LCL_2$) genetic maps and further resolution of this problem. This exercise will also be assisted through enhanced knowledge of comparative genetic relationships between Galegoid legumes, especially *M. truncatula*, which is located taxonomically in the same Fabaceae family tribe (Trifolieae) as white clover, and for which the majority of chromosomes reveals extensive macrosynteny with white clover HGs (George et al. 2008). Region-specific marker enrichment will be informed by white clover template gene prediction based on comparative analysis, although limited microsyteny (Febrer et al. 2007; Hand et al. 2009) may complicate this approach and require broader comparisons with other species such as *L. japonicus*.

The identification of QTL is recognised as the first step towards plant improvement through MAS, and provides useful target areas for gene discovery through physical mapping and positional cloning. However, in both situations the application of such data for the improvement of cultivar performance should not be expected to be straightforward. QTL introgression in breeding programs for self-incompatible plants presents major challenges, inability to self or backcross and the linkage drag effects of undesirable traits providing two examples (Asins 2002). Identification and implementation of diagnostic genetic markers which are functionally associated within the genes directly contributing to QTL effects will provide the most broadly applicable and efficient methods for molecular breeding of pasture species (Forster et al. 2008). Considering the scale of effort required to identify the underlying gene(s), regulating the phenotypic effect and the limited magnitudes of the QTLs identified, positional cloning in species such as white clover remains currently unfeasible. Exploitation of this first exercise in salt tolerance QTL identification for white clover will consequently require rigorous validation in different genetic backgrounds and environments prior to any implementation.

Acknowledgments This research was funded by the Department of Primary Industries, Victoria, Dairy Australia, the Geoffrey Gardiner Dairy Foundation, and Meat and Livestock Australia through the Australian Molecular Plant Breeding Cooperative Research Centre (MPB CRC).

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