Theor Appl Genet (2011) 122:609–622
DOI 10.1007/s00122-010-1473-8

ORIGINAL PAPER

Identification of QTLs for morphological traits influencing waterlogging tolerance in perennial ryegrass (*Lolium perenne* L.)

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Received: 15 June 2010 / Accepted: 11 October 2010 / Published online: 28 October 2010

Abstract Perennial ryegrass is a globally cultivated obligate outbreeding diploid species ($2n = 2x = 14$) which is subjected to periods of waterlogging stress due to flood irrigation during winter and the lead-up to summer. Reduction of oxygen supply to root systems due to waterlogging produces consequent deleterious effects on plant performance. Framework genetic maps for a large-scale genetic mapping family [$F_1(NA_x \times AU_6)$] were constructed containing 91 simple sequence repeat and 24 single nucleotide polymorphism genetic markers. Genetic trait dissection using both control and waterlogging treatments was performed in the glasshouse, a total of 143 maximally recombinant genotypes being selected from the overall sibship and replicated threefold in the trial. Analysis was performed for nine quantitative morphological traits measured 8 weeks after stress treatments were applied. A total of 37 quantitative trait loci (QTLs) were identified; 19 on the NA_x parental genetic map, and 18 on the AU_6 parental genetic map. Regions of particular interest were identified on linkage groups (LGs) 4 and 3 of the respective maps, which have been targeted for further analysis by selection of critical recombinants. This first study of genetic control of waterlogging tolerance in ryegrasses has important implications for breeding improvement of abiotic stress adaptation.

Communicated by P. Langridge.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1473-8) contains supplementary material, which is available to authorized users.

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is an important temperate pasture grass for livestock production industries, as the species is specifically adapted to withstand frequent close grazing from ruminant animals, and is rarely encountered in ungrazed areas (Breese 1983). Perennial ryegrass is a native species of Europe, temperate Asia and North Africa (Cunningham et al. 1994) but is now cultivated in the USA, UK, Japan, Australia, New Zealand, South Africa and South America (Wilkins and Humphreys 2003; Forster et al. 2008).

Perennial ryegrass is affected by waterlogging and drought, both of which are major abiotic stresses. Perennial ryegrass is subjected to periods of waterlogging during winter months and the lead-up to summer, during which plants are flood-irrigated (McFarlane et al. 2003). Subsequent adaptation to high heat frequently contributes to plant death, due to enhanced vulnerability to high-temperature effects (McFarlane et al. 2003). Field studies

of perennial ryegrass with simulated flood irrigation revealed that waterlogging can reduce dry matter yield (DMY) levels by up to 25%. When flood-irrigation is performed, lengthier periods of ponding (collection of water on the soil surface) lead to decreased oxygen diffusion rates, restricting root respiration for extended periods of time (Grieve et al. 1986; Dunbabin et al. 1997). Little research has to date been performed on waterlogging stress in perennial ryegrass, and although both waterlogging and drought are major problems, the current study has focused in the first instance on waterlogging.

When waterlogging occurs, air spaces in the soil are filled, resulting in anoxic stress to the plants due to reduction (up to 90%) of available oxygen levels (Cannell et al. 1984; Grieve et al. 1986; Adams and Akhtar 1994; Barnes et al. 2007). The rate of oxygen supply to the roots depends on soil porosity and the volume of air-filled space, as oxygen diffusion through air is approximately 10^4 times more rapid than through water (Adams and Akhtar 1994). It is believed that plant root activity increases the size and number of pores at the root-soil interface, assisting improvement of soil water-holding capacity during waterlogging (Macleod et al. 2007; Abberton et al. 2008). Measurements of oxygen diffusion rates in soil surrounding the root system of perennial ryegrass have revealed that waterlogging during field irrigation trials can affect plants as rapidly as 4 h after flooding occurs (Dunbabin et al. 1997).

Reduced oxygen levels during waterlogging may also lead to soil denitrification, carbohydrate fermentation, ethylene production (and hence to nitrogen deficiency later in plant development) and reduction of sulphate to sulphide, which is potentially phytotoxic to plants (Russell 1973; Tisdale and Nelson 1975; Tiedje et al. 1984; Grieve et al. 1986; Pearson and Havill 1988; Adams and Akhtar 1994).

During periods of waterlogging, pugging (penetration of the hooves of grazing animals into the pasture and soil surface during wet conditions) is exacerbated, leading to extensive damage (Nie et al. 2001). Medium-to-heavy pugging during winter months has been observed to cause a decrease of up to 42% in pasture regrowth during the following spring, as well as decreases in tiller density in ryegrass by up to 54% (Nie et al. 2001). Such events may cause plants to be buried, or foliage fouling to occur with the mud (Nie et al. 2001). Pugging during waterlogging also increases soil compaction, which reduces the water-holding capacity of the soil, thus reducing the content of oxygen-containing soil pores (Michael et al. 2006). Pulling, in which shoots and 2–5 cm of root system from whole shallow-rooted plants are removed by grazing animals, may also be problematic during waterlogging, as the soil is less compact (Thom et al. 2003; Faville et al. 2006).

The North African$_6$ (NA_6) and Aurora$_6$ (AU_6) parents of the $F_1(NA_6 \times AU_6)$ perennial ryegrass genetic mapping family (Faville et al. 2004), along with two F_1 progeny (2,178 and 2,182) from the cross were used to assess physiological and morphological responses under conditions of waterlogging (McFarlane et al. 2003). Root growth, shoot growth and photosynthesis were analysed, demonstrating that waterlogging reduced root growth and mass for all genotypes except AU_6 (Cannell et al. 1984; Grieve et al. 1986; McFarlane et al. 2003; Barnes et al. 2007). Waterlogging conditions reduced oxygen availability, which was observed to be associated with a reduced root length from 2 weeks following inception (McFarlane et al. 2003). Root mass continued to increase, indicating that adventitious root growth may be important for tolerance through assisting the plant to adapt to long-term stress. In addition, although shoot growth varied between genotypes, pseudostem weight was relatively similar (Donohue et al. 1985; Grieve et al. 1986; McFarlane et al. 2003). Photosynthetic rate was also observed to be reduced by 30–40% under waterlogging conditions (McFarlane et al. 2003). Apart from this initial description, the effects of waterlogging stress on perennial ryegrass have not yet been extensively analysed. Detailed trait dissection studies based on the use of comprehensive genetic linkage maps would permit the detection of QTLs for phenotypic traits related to tolerance to abiotic stresses such as waterlogging, allowing elucidation of the relevant mechanisms.

Identification of QTL related to morphological traits such as root length has been reported for rice (Price and Tomos 1997; Zheng et al. 2000, 2003; Nguyen et al. 2004), wheat (Börner et al. 2002; Hai et al. 2005), barley (Thomas et al. 1995; Buck-Sorlin 2002; Li et al. 2008) and maize (Schön et al. 2004; Mano et al. 2005), but equivalent studies in perennial ryegrass have yet to be performed through phenotypic measurements in a controlled environment (Faville et al. 2006). A broad range of perennial ryegrass genetic maps have been constructed (Bert et al. 1999; Jones et al. 2002a, b; Cogan et al. 2006), including the parental genetic maps derived from the $F_1(NA_6 \times AU_6)$ two-way pseudo-testcross population (Faville et al. 2004). The NA_6 parent was derived from a Moroccan ecotype which exhibits a long rooting system and early flowering time, leading to avoidance of summer water stress and increased drought tolerance (Anderson et al. 1999), while the AU_6 parent originated from the Aurora cultivar, bred in the United Kingdom from an ecotype from the Swiss Alps (Humphreys 1989; Smith et al. 1998, 2001, 2002; Guthridge 2004). The North African and Aurora populations are genetically distinct (Guthridge 2004), allowing maximisation of genotypic variation within the F_1 progeny set, and allowing for new and novel populations to be developed from these genotypes. Substantial trait variation

has been observed for a range of phenotypic characters, such as morphophysiological traits (Guthridge 2004), mineral content (Cogan et al. 2005) and fungal disease resistance (Dracatos et al. 2008, 2009).

Phenotypic studies for QTL detection typically require a large mapping population to achieve adequate power and to prevent detection of false-positives. However, such studies may be highly logistically demanding due to requirement for detailed measurements of multiple genotypes (Charmet 2000; Birolleau-Touchard et al. 2007; Cogan et al. 2007). Accuracy of QTL identification is dependent on the number of selected individuals, and so reduction of population size for phenotypic assessment without prior knowledge of genetic constitution can cause a failure to detect low-magnitude factors (Jin et al. 2004; Birolleau-Touchard et al. 2007). Selective phenotyping may be used to identify sub-sets of individuals with maximised recombination, permitting sample size reduction with full retention of genetic information from the larger population (Jin et al. 2004; Birolleau-Touchard et al. 2007; Cogan et al. 2007). This approach, supported by the mathematical software MapPop (Brown and Vision 1999), has previously been applied to construct a core collection for tomato (*Solanum esculentum* L.) (Doganlar et al. 2002), and for selection of sub-populations in pepper (*Capsicum annum* L.) both of which were successfully used for QTL detection (Barchi et al. 2007, 2009). Once QTLs have been identified, marker loci surrounding regions on specific LGs may be used to refine the selection of individuals with maximal recombination at a particular site, providing an improved set with increased QTL detection power (Jin et al. 2004). Assignment of additional markers to the genetic map will allow further refinement of QTL location (Jin et al. 2004)

The aim of this study was to rapidly develop a framework genetic map of a novel trait-specific family, through choice of ordered markers from previously published reference maps, and to identify a maximally recombinant collection of individuals for targeted phenotypic analysis of logistically challenging characters. Phenotypic analysis in control and waterlogged conditions has for the first time identified QTLs contributing to variation for morphological traits under both conditions in perennial ryegrass.

Materials and methods

Plant materials

An F_1 progeny population derived from the North African and Aurora base populations was generated using the AU_6 parent which was common with the $F_1(AU_6 \times NA_6)$ population (Faville et al. 2004) and an alternate North African parental genotype (NA_x). The $F_1(NA_x \times AU_6)$ sib-ship of 569 individuals was maintained at the premises of DPI-Hamilton.

Molecular genetic marker analysis and framework map construction

Genomic DNA was extracted using the DNeasy Plant 96-kit (Qiagen, Hilden, Germany) following manufacturer's instructions, following freeze drying of the plant material, with storage of genomic DNA at −20°C prior to usage. Single nucleotide polymorphism (SNP) marker analysis was performed using the SNuPe (single nucleotide primer extension) genotyping kit following the manufacturer's instructions and also as described by Hand et al. (2008). PCR amplifications for simple sequence repeat (SSR) genotyping was performed in 12 µl reactions containing 1.2 µl 10× Immolase buffer (Bioline, London, UK), 1.2 µl (2 mM) dNTPs, 0.05 µl (100 µM) forward (labelled with fluorochromes FAM™ [6-carboxyfluorescein], HEX™ [hexachloro-6-carboxyfluorescin] NED™ [7′,8′-benzo-5′-fluoro-2,4,7-trichloro-5-carboxyfluorescein] or TAMRA™ [6-carboxyrhodamine]), 0.05 µl (100 µM) reverse primer, 0.05 µl Immolase DNA polymerase (Bioline) and 10 ng genomic template DNA (5 ng/µl).

PCR products were diluted in 20 µl of sterile distilled water and then pooled so that each product contained one of each of the three dye-labelled PCR products. Aliquots of 2 µl of FAM-labelled, and 3 µl of HEX-labelled and NED-/TAMRA-labelled PCR products were added to each pool. PCR products were precipitated through the addition of 16 µl of 80% (v/v) isopropanol, and the pellet was then washed in 40 µl of 70% (v/v) ethanol and allowed to air dry. The dried product was resuspended in 5.25 µl of MegaBACE loading solution and 0.06 µl ET400-R size standard. SSR analysis was performed through product separation on the MegaBACE 4500 capillary sequencer and data were analysed using MegaBACE Fragment Profiler version 1.2 (GE Healthcare, Chalfont St. Giles, UK).

The $F_1(NA_x \times AU_6)$ population was analysed as a two-way pseudo-testcross (Grattapaglia and Sederoff 1994). Marker segregation ratios were checked for deviation from Mendelian expectation (1:1) by χ^2 analysis and map construction was performed using MAPMAKER 3.0 (Lander et al. 1987). For linkage groups that were difficult to construct due to the presence of five markers or less, JOINMAP 3.0 (Kyazma, Wageningen, The Netherlands) was employed. Data sets were inverted and merged with the normally coded data to detect repulsion phase linkages. Graphical displays of linkage groups were produced using MapChart 2.1 (Kyazma). With both software packages data were analysed using the group command and a minimum LOD threshold of 4.0 was accepted; markers not showing sufficient linkage were excluded.

Selection of recombinant individuals

MapPop version 0.9alpha (Brown and Vision 1999) was used to select for maximally recombinant individuals from the $F_1(NA_x \times AU_6)$ expanded sib-ship for the establishment of glasshouse trials. MapPop was programmed to run for 36,000 s (10 h) to select the 143 individuals with maximal recombination using the samplemax algorithm (Fig. 1).

Experimental design

A total of 143 F_1 progeny individuals with maximal genetic recombination were selected for the glasshouse-based experiment, in addition to the AU_6 parent, the NA_x parent being no longer available at the time of measurement. A total of 144 genotypes were arranged in a factorial incomplete block design with three replicates of each genotype for each treatment (control and waterlogging) giving a total of 864 plants for the experiment.

Plant culture

Clonal ramets consisting of nine tillers from the original individual plants of the $F_1(NA_x \times AU_6)$ sib-ship were sampled and placed in hydroponic culture, containing nutrients and water as required, for approximately 6 weeks to promote optimal growing conditions. After this period, two tillers were taken from each ramet, and were trimmed to 5-cm length for both roots and shoots. The sampled tillers were then transplanted into polyvinyl chloride (PVC) tubes which were filled with river sand. The plants were grown in optimal conditions in a glasshouse, with twice-daily watering and regular supplementation with a complete nutrient solution (2 mM $MgSO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 5 mM $Ca(NO_3)_4 \cdot 4H_2O$, 10 mM KNO_3, 2.5 mM $(NH_4)_2SO_4$, 0.1 mM FeNa(EDTA), 50 μM H_3BO_3, 1 μM $CuSO_4 \cdot 5H_2O$, 10 μM $MnSO_4 \cdot 4H_2O$, 0.07 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 μM $ZnSO_4 \cdot 7H_2O$, 2 μM $CaCl_2 \cdot 6H_2O$).

Plants were allowed to grow under optimal conditions for 5 weeks prior to imposition of treatments at 6–25°C under natural lighting conditions in the glasshouse. The experiment was conducted at Hamilton, Victoria, during autumn/winter of 2008. On commencement of the stress treatment, the PVC tubes containing half of the plant set were filled with water to a depth of approximately 10 mm above the soil surface to initiate the waterlogging treatment, which was maintained throughout the 8-week duration of the experiment. The control plant set continued to be watered with nutrient administration on a regular basis.

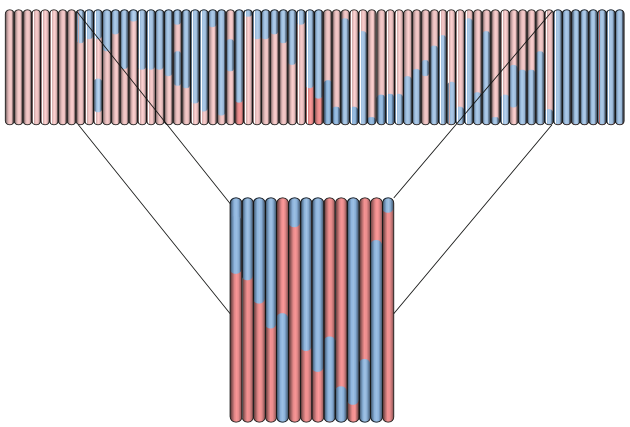

Fig. 1 Schematic representation of maximal recombinant individual selection through use of the MapPop algorithm, leading to relative reduction of non-recombinant chromosome prevalence and regular distribution of linkage bins. The alternate shading of chromosome segments represents contributions from either the first parent or the second parent of a genetic mapping population

Waterlogging morphological analysis

Eight weeks subsequent to treatment imposition, all plants were harvested. Following extraction from the PVC tubing sleeves and removal of sand from the root system by gentle washing in water, the plants were cut at the base to excise aerial tissue. Morphological measurements included maximum length in cm (RL) and dry weight in g (RG) of roots, number (TN) and dry weight in g (PG) of pseudostems, dry weight in g (LDG) and average area in cm^2 (ALA) of leaves, and dry weight of the 3 YFELs in g (3 YFEL DW), fresh weight of the 3 YFELs in g (3 YFEL FW) and area of 3 YFELs in cm^2 (3 YFEL A). Root length was measured manually using a metre rule, and YFEL leaf area was measured using a LI-3100 area meter machine (LICOR). All plant material was separated into roots, leaves, pseudostems and YFEL and was placed into individual paper bags for drying. Dry weight was measured by placing all collected plant material into a fan-forced oven at 100°C for 24 h.

Statistical analysis

Statistical analysis for both control and waterlogging data was performed using GenStat Edition 10.1 (Payne et al. 2007). Correspondence of data between the two treatments was calculated using a correlations procedure. Broad sense heritability was calculated according to the formula of Nyquist (1991). The restricted maximum likelihood (REML) method was used to test the effects of the treatments. Treatment factors were considered as fixed effects and experimental design factors as random effects (rows and columns). Predicted means were obtained for each genotype for both control and waterlogging treatments.

Using the predicted means from control and waterlogging measurements, least significant difference values (the difference between the control and waterlogging values for each genotype) were also obtained. Data from all three results sets were subsequently used for QTL analysis.

QTL analysis

For each of the parental genetic maps, single marker regression (SMR) analysis was initially undertaken to identify regions which showed significant variation associated with selected genetic markers, and simple interval mapping (SIM) (Lander and Botstein 1989; Haley and Knott 1992) and composite interval mapping (CIM; Zeng 1994) were performed for all phenotypic traits, to identify and confirm the presence of QTLs on the framework genetic map (Cogan et al. 2006). All analyses were performed using QTL cartographer 2.5 (Wang et al. 2007). For both SIM and CIM maximum LOD value, location on the framework genetic map, additive marker value effects and the proportion of phenotypic variance attributable to the QTL were recorded. Identification of a unique QTL was based on that sub-set for which QTLs for correlated traits were located within 15 cM of each other (Sewell et al. 2000, 2002; Wang et al. 2010).

Results

Framework genetic map construction and selection of maximally recombinant individuals

Genetic mapping was performed on the population, using assays for a total of 202 selected molecular genetic markers (105 SSRs, 97 SNPs). Due to locus reduplication, a total of 314 polymorphic loci were scored, of which 146 exhibited a $\chi^2 < 10$ for deviation from the expected (1:1) segregation ratio, and were hence suitable for map construction. The AU_6 framework parental genetic map contained a total of 73 marker loci spanning a total length of 579.3 cM, across all seven LGs, of which ten were coincident with other loci (Fig. 2). The NA_x framework genetic map contained a total of 42 marker loci spanning a total length of 389.7 cM which were mapped to five LGs, seven being coincident with other loci (Fig. 3). The two unresolved LGs were 5 and 6, each containing three loci.

Following map construction, a subset of 143 individuals with maximal recombination was selected from within the larger population using all relevant marker loci. A maximum bin length of 4.17 cM was obtained, and a clear shift was observed in the average number of recombination events per genotype, such that the majority contained eight or more recombinant chromosomes as compared with an average of seven in the full sib-ship, and the minimum number was four compared with one in the full sib-ship (S1).

Phenotypic analysis of control and waterlogging plants

Measurements of 13 quantitative traits were taken on the 143 maximally recombinant F_1 individuals under both control and waterlogging-stressed treatments. Morphological measurements under both treatments were found to be significantly correlated at $P < 0.05$ (S2).

A total of seven correlations between morphological measurements were observed with coefficients within the range $0.70 < r < 0.90$. For the control measurements, 12 correlations were within the range $0.50 < r < 0.69$, the highest values being observed between TN, ALA, LDG, PG and RG. Other measured traits obtained low but significant correlations, ranging from $0.11 < r < 0.50$. For the waterlogging treatment, six correlations showed coefficients varying between $0.70 < r < 0.90$. A total of seven correlations were observed within the range $0.50 < r < 0.69$, all of them relating to RG and TN. The remaining measurements also generated low, but significant, correlations within the range $0.11 < r < 0.50$ (S2).

Both waterlogging stress and genotype had a highly significant effect ($P < 0.001$) for all of the morphological traits. Genotype-by-waterlogging interaction was found to be significant ($P < 0.05$) for a number of the morphological traits, apart from TN ($P = 0.390$), PG ($P = 0.378$) and 3 YFEL DW ($P = 0.104$), which decreased under waterlogging stress depending on genotypic identity. Broad-sense heritability (H) estimates for each trait ranged from 0.182 to 0.327, the lowest value being for TN, and the highest for 3 YFEL DW (Table 1).

A decrease in mean value between the control and waterlogging stress treatments was observed for each measurement (Table 1; S3). The smallest decrement (28%) was observed for PG, and the largest decrement was observed for ALA (67%). For all other measurements, the mean value decrement was between 30 and 55% of the control value (Table 1).

Detection of QTLs

Of a total of 91 SSR marker loci, 25 were coincident with other mapped loci, and 24 SNP marker loci were used for framework genetic map construction. Loci separated by 5–10 cM on each LG were selected for QTL analysis. A total of 37 individual QTLs for the 13 traits assessed under control and waterlogging stress conditions were identified using SMR, SIM and CIM mapping approaches, giving a total of six unique QTLs. For both the AU_6 (Table 2; Fig. 2) and NA_x (Table 3; Fig. 3) parental genetic maps, three unique QTLs were identified. Many of

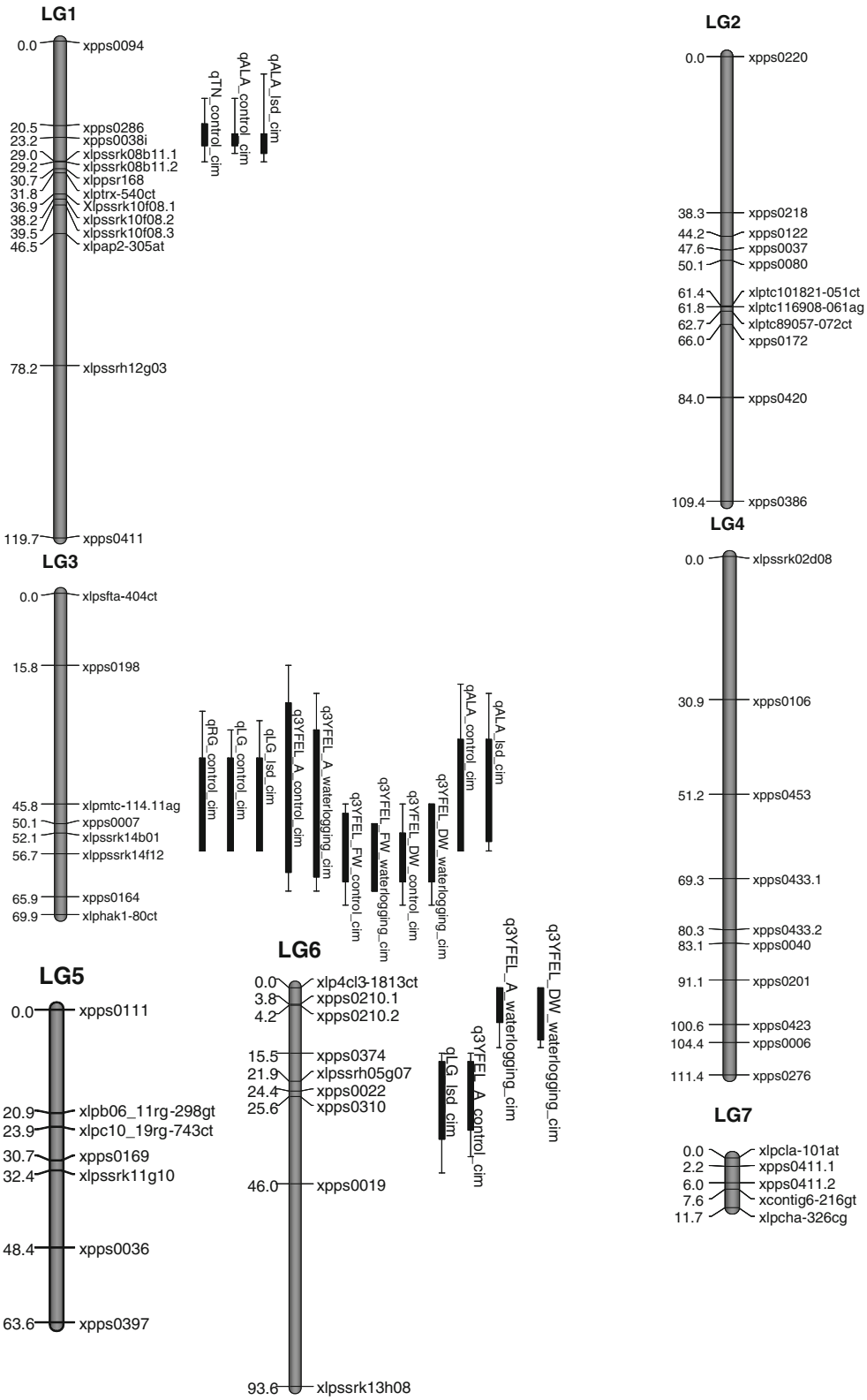

Fig. 2 QTLs identified on the AU_6 parental map. SNP marker loci are labelled according to the nomenclature xlp-gene name abbreviation- nucleotide coordinate. The nomenclature for DNA-derived and cDNA-derived simple sequence repeat (SSR) markers is as described in Faville et al. (2004) and Cogan et al. (2006). Only one of any given pair of coincident loci is depicted, for the purposes of clarity. QTL nomenclature is adapted from McCouch et al. (1997) in the form of q-trait-treatment-method of analysis. *Bars* on QTL represent 1 and 2 LOD drops form the maximum likelihood value

the individual QTL regions obtained independent evaluation of the same genomic region, due to high levels of correlation between the respective traits defining unique QTLs. The range of phenotypic variation (V_p) accounted by individual QTLs on each map ranged from 2.8 to 17.8%, with an average of 9.86 (AU_6) and 7.35% (NA_x).

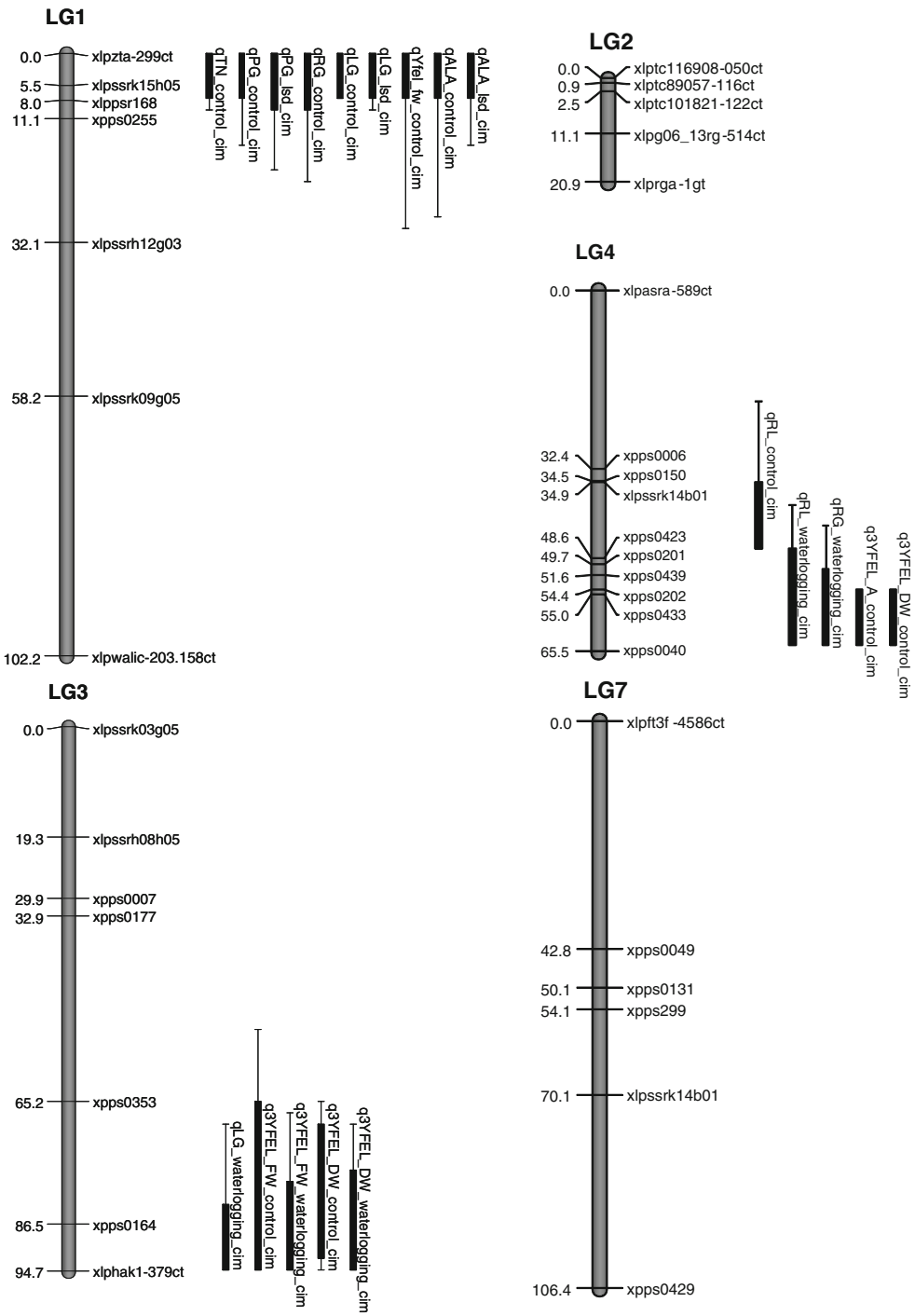

Fig. 3 QTLs identified on the NA_x parental map showing LGs 1, 2, 3, 4 and 7. Details are as indicated in the legend for Fig. 2

Vegetative growth under control conditions

The AU_6 framework parental genetic map contained a total of three unique QTLs that were assigned to LGs 1, 3 and 6 (Table 2; Fig. 2), representing eight of the nine measured traits. QTLs were observed on different LGs for the TN (LG1), ALA (LG1 and LG3), 3 YFEL A (LG3 and LG6) traits, while QTLs for the remaining traits (RG, LDG, 3 YFEL FW, YFEL DW) all mapped to LG3 and represent a single unique QTL. V_p values for individual QTLs varied from 6.3 to 17.8%.

The NA_x framework parental genetic map also contained unique three QTLs representing seven of the nine measured traits, located on LGs 1, 3 and 4 (Table 3; Fig. 3). The TN, PG, RG, LDG and ALA trait QTLs were located on LG1, RL and 3 YFEL A QTLs on LG4, 3 YFEL

Table 1 Heritability estimates and observed means for morphological phenotypic traits in both control and waterlogging stress treatments

Trait	Heritability (H)	Means	
		Control	Waterlogging
RL	0.265	42.19	21.43
RG*	0.197	1.15	0.685
LG*	0.243	1.34	0.603
PG*	0.193	1.10	0.790
TN	0.182	31.21	19.50
3 YFEL FW	0.292	0.110	0.062
3 YFEL DW	0.327	0.024	0.017
3 YFEL A	0.229	5.98	3.12
ALA	0.265	332.04	108.46

Bar-charts depicting distributions for each trait are provided in S3

* All measurements were taken on dried plant material

FW QTLs were observed on LG1 and LG3, while 3 YFEL DW QTLs were located on LG3 and LG4. V_p values for individual QTLs varied from 6.4 to 13.3%.

Vegetative growth under waterlogging conditions

A total of three unique QTLs were assigned to the AU_6 parental map, representing three of the measured traits: 3 YFEL A [LGs 3 and 6], 3 YFEL FW [LG3], and 3 YFEL DW [LGs 3 and 6] (Table 2; Fig. 2). V_p values for these QTLs varied from 6.1 to 11.3%.

The NA_x parental map contained a total of five unique QTLs, representing nine of the nine phenotypic traits observed. The QTLs were detected on LGs 1, 3, 4 and 7. The corresponding traits for each of these QTL regions were LDG [LG3], 3 YFEL FW [LG3], 3 YFEL DW [LG3], RL [LG4] and RG [LG4] (Table 3; Fig. 3). V_p values for these QTLs varied from 7.5 to 9.9%.

Least significant difference QTLs

QTLs for least significant difference (LSD) were observed on LGs 1, 3 and 6 of the AU_6 parental map (Table 2; Fig. 2). A total of three unique QTLs were observed for two phenotypic traits, these being observed for the LDG trait on LG 3 and 6 and for ALA on LGs 1 and 6. V_p values varied from 6.5 to 10.9%.

A total of two unique QTLs for lsd were observed on LGs 1 and 2 of the NA_x parental map (Table 3; Fig. 3), corresponding to three different phenotypic traits (PG, LDG and ALA). V_p values varied from 6.6 to 9.7%.

Discussion

Framework genetic map construction

Reasonable coverage was achieved for all AU_6 LGs apart from LG7, which hence requires further marker addition to increase total map length. The NA_x genetic map exhibited sufficient coverage for LGs 1, 4 and 7, but LG 2 required increased marker coverage for further resolution. LGs 5 and 6 were not represented on the NA_x genetic map, due to insufficient marker coverage to permit coalescence. Selection of marker assays for genotyping of the $F_1(NA_x \times AU_6)$ family was based on prior experience with $F_1(NA_6 \times AU_6)$ (Faville et al. 2004; Cogan et al. 2006), such that non-commonalities of polymorphic marker identity between the two NA-derived genotypes accounted for the deficit of map-assigned loci.

Selection of maximally recombinant individuals

MapPop identifies breakpoints (recombinational crossover-sites) within individuals from the genotypic data provided, ensuring that a maximal number of map distance bins are retained within the selected individuals (Vision et al. 2000). MapPop not only selects individuals with maximal recombination, but also those which contain unique recombination sites that have not occurred elsewhere within the population, to give a full representation of the genetic diversity. The ability to choose individuals with a more desirable distribution of crossover sites, rather then selecting individuals randomly, with no prior knowledge of the genetic background, permits maximum informative value during phenotypic studies, without a requirement for evaluation of the entire population.

Selection of maximally informative sets of individuals for phenotypic analysis is of particular importance as perennial ryegrass is an obligate outbreeding species. The pseudo-testcross structures necessary for genetic mapping in such a system (Jones et al. 2002a, b; Faville et al. 2004) offer inherently lower power for QTL detection, as fewer rounds of meiosis occur in an F_1 population as compared with cross structures which generate fully inbred genotypes, such as recombinant inbred lines (RILs; Barchi et al. 2007, 2009). For outbred species, levels of experimental replication and number of evaluated genotypes must hence be higher in order to obtain robust heritability estimates and to support accurate QTL detection. This outcome may be difficult to achieve, as many protocols are time-bound and may be costly. The logistics of sampling a large number of individuals may be difficult, as in some instances limited storage capacity and potential perishability of plant

Table 2 Summary of QTL analysis for morphological traits in the perennial ryegrass $F_1(NA_x \times AU_6)$ population relating to the AU_6 map

Trait	LG	SMR $P < 0.01$	Simple interval mapping				Composite interval mapping				
			Max LOD	Position	*a*	*R*	Threshold	Max LOD	Position	*a*	V_p
RL											
Control	3	na	1.025	45.81	3.395	0.032	2.235	1.312	45.810	3.737	0.038
Waterlogged	4	na	0.973	68.51	1.908	0.031	2.257	2.154	68.51	4.327	0.065
Least significant difference	3	45.8	1.490	43.81	4.001	0.051	2.365	1.580	45.810	3.823	0.045
TN											
Control	**1**	**20.5–23.2**	2.250	20.51	−3.549	0.070	**2.387**	**3.480**	**20.510**	**−5.011**	**0.099**
Waterlogged	7	0; 7.6	1.840	7.61	2.167	0.057	2.344	1.786	4.210	2.112	0.053
Least significant difference	1	na	1.010	22.51	−2.317	0.034	2.387	0.929	22.510	−2.175	0.029
PG											
Control	3	45.8–50.1	1.819	45.81	0.135	0.057	2.344	2.128	45.810	0.139	0.059
Waterlogged	6	15.5–25.6	2.070	25.51	−0.119	0.064	2.322	2.283	25.610	−0.118	0.063
	2	0	1.560	4.01	0.121	0.066	2.322	1.498	2.010	0.105	0.048
	5	na	0.103	0.01	−0.027	0.003	2.322	2.298	32.410	0.243	0.062
Least significant difference	6	4.2; 25.6	2.040	4.21	0.145	0.063	2.322	2.277	4.210	0.144	0.062
RG											
Control	6	4.2; 25.6	2.070	4.21	0.175	0.064	2.365	1.780	6.210	0.154	0.049
	3	**45.8–50.1**	1.650	47.81	0.158	0.525	**2.365**	**2.630**	**45.810**	**0.182**	**0.068**
Waterlogged	4	na	1.210	68.51	0.080	0.038	2.365	2.190	68.51	0.159	0.061
Least significant difference	6	4.2;25.6	1.770	4.21	0.162	0.553	2.235	1.627	8.210	0.155	0.050
LDG											
Control	**3**	**45.8–69.9**	**2.770**	**45.81**	**0.197**	**0.085**	**2.365**	**3.634**	**45.810**	**0.214**	**0.098**
Waterlogged	4	na	1.230	68.51	0.068	0.039	2.257	1.345	71.51	0.076	0.046
Least significant difference	**3**	**45.8–56.7**	2.080	45.81	0.162	0.064	**2.322**	**3.107**	**45.810**	**0.188**	**0.084**
	6	**4.2**; **25.6**	1.750	4.21	0.150	0.055	**2.322**	**2.431**	**25.610**	**0.165**	**0.065**
3 YFEL A											
Control	**3**	**15.8–69.9**	**3.041**	**52.11**	**0.748**	**0.101**	**2.192**	**4.013**	**52.110**	**0.812**	**0.116**
	6	**25.6**	1.470	25.61	0.507	0.046	**2.192**	**2.401**	**25.610**	**0.599**	**0.063**
Waterlogged	**3**	**45.8–69.9**	**3.077**	**47.81**	**0.344**	**0.096**	**2.279**	**3.476**	**41.810**	**0.373**	**0.113**
	6	**0–4.2**	2.420	0.01	0.31	0.078	**2.279**	**3.261**	**0.010**	**0.358**	**0.091**
Least significant difference	3	na	1.210	67.91	0.454	0.041	2.344	1.649	67.910	0.515	0.052
3 YFEL FW											
Control	**3**	**15.8–69.9**	**4.310**	**54.11**	**0.019**	**0.138**	**2.279**	**5.668**	**56.110**	**0.020**	**0.151**
Waterlogged	**3**	**45.8–69.9**	**4.270**	**60.71**	**0.010**	**0.141**	**2.387**	**4.110**	**54.110**	**0.009**	**0.110**
	6	0	1.570	0.01	0.006	0.050	2.387	1.449	25.510	0.010	0.036
Least significant difference	7	0–2.2	1.840	0.01	−0.010	0.057	2.322	2.044	2.010	−0.010	0.055
3 YFEL DW											
Control	**3**	**45.8–69.9**	**4.700**	**58.71**	**0.004**	**0.148**	**2.365**	**6.415**	**58.710**	**0.005**	**0.178**
	6	50.1	1.620	25.61	0.002	0.051	2.365	2.333	25.610	0.003	0.057
Waterlogged	**3**	**45.8–69.96**	**4.200**	**60.71**	**0.003**	**0.140**	**2.300**	**3.945**	**54.110**	**0.003**	**0.110**
	6	**0–4.2**	1.790	0.01	0.002	0.057	**2.300**	**2.314**	**0.010**	**0.002**	**0.061**
Least significant difference	5	na	1.380	20.91	0.002	0.044	2.257	1.953	20.910	0.003	0.059
ALA											
Control	**1**	**20.5–23.2**	**3.030**	**23.21**	**−53.700**	**0.092**	**2.235**	**4.445**	**23.210**	**−63.198**	**0.121**
	3	**45.8**	1.540	47.81	39.29	0.050	**2.235**	**2.564**	**45.810**	**45.934**	**0.065**
Waterlogged	2	na	1.330	4.01	14.560	0.054	2.279	0.994	0.010	10.725	0.028

Table 2 continued

Trait	LG	SMR $P < 0.01$	Simple interval mapping				Composite interval mapping				
			Max LOD	Position	a	R	Threshold	Max LOD	Position	a	V_p
Least significant difference	**1**	**20.5–23.2**	2.210	23.21	−44.390	0.068	**2.343**	**4.171**	**23.210**	**−71.616**	**0.109**
	3	**45.8**	1.470	45.81	36.41	0.046	**2.343**	**2.853**	**45.810**	**46.332**	**0.073**
	6	25.6	1.570	25.61	37.62	0.049	2.343	1.798	23.510	38.059	0.049

Putative QTLs that exceed the requisite LOD threshold values using QTL cartographer for both SIM and CIM are displayed in bold type

material may be factors (Birolleau-Touchard et al. 2007). It has been observed that storage of perennial ryegrass root tissue in 70% (v/v) ethanol causes a decrease in dry weight content by approximately 22.4%, compared with measuring dry weight after oven drying (Crush et al. 2010). If storage was required, loss of root weight could cause significant comparative errors, and a correction factor would be required (Crush et al. 2010). For these reasons, implementation of genetic algorithms for informative individual enrichment is an important development for trait-dissection in perennial ryegrass.

Effects of waterlogging on plant growth

Crop plants require free exchange of atmospheric gases for photosynthesis and respiration. Soils that are well-drained contain air pores around the roots which are interconnected with the atmosphere above, allowing oxygen to be transported rapidly around the rooting system of plants (Gambrell et al. 1991). When waterlogging occurs, these air spaces are filled and efficient transport of gaseous oxygen to the root system is no longer achieved and growth is highly inhibited. In this study, decreased values (between 30 and 67%) were observed for plants under waterlogging stress for all measured traits. Decreases in perennial ryegrass leaf and root morphological trait values were also observed in a smaller study performed on under the same conditions as used for the current trial (McFarlane et al. 2003).

Correlation coefficients for morphological traits were all found to be significant at $P < 0.05$. Application of the waterlogging treatment had a measurable (but in most cases small) effect on the extent of correlations, which were generally decreased under stress conditions. A larger decrement in correlation coefficients was observed between RG and RL (0.519 under control conditions changing to 0.261 under waterlogging conditions, $\Delta = -0.258$).

H values calculated for all measured traits were low to moderate in magnitude, possibly due to increased environmental variation caused by diurnal fluctuations in light, water and nutrient availability (Arntz and Delph 2001). In addition, due to the large scale of the experiment, there was limited ability to obtain all measurements on the same day, increasing environmental variance and hence potentially contributing to the observed H values.

QTL analysis

AU_6 LG1 contains a unique QTL relating to shoot characteristics (TN control, ALA control, ALA lsd), of which the QTL for TN was also located on LG1 of the NA_x map, based on a common location of the xlpssr168 marker locus between the two maps. LG3 contains the most individual QTLs on the AU_6 genetic map, mainly corresponding to the leaf-related traits, and suggesting that many of the leaf traits are controlled by a single region of the genome. In addition to the QTL located on AU_6 LG1, QTLs were also identified on NA_x LG1 for highly to moderately correlated traits (0.352–0.879). The occurrence of coincident QTLs on a single LG for both shoot and root-related morphogenetic characters may arise from allelic variation at a single pleiotropic locus, or there may be a number of alleles with similar types of effect at different loci on the same LG (Yamada et al. 2004). The root trait located in the AU_6 LG3 QTL was for RG under control conditions, the corresponding QTL being on NA_x LG1. The traits 3 YFEL A control and waterlogging and 3 YFEL DW waterlogging were also assigned to the QTL region located on AU_6 LG6, indicating that multiple areas of the AU_6 genome may control these traits.

Several traits in this study reveal QTL locations on alternate LGs between the respective parental maps, such as between NA_x LG1 and AU_6 LG3 for RG. Previous studies have revealed similar effects, such as QTLs for root weight-related traits on NA_6 LG7 as compared with AU_6 LGs 1, 6 and 7 of the $F_1(NA_6 \times AU_6)$ core sib-ship (Guthridge et al. 2003; Guthridge 2004). In addition, clusters of QTLs for common morphological traits were identified on a single genetic map, of p150/112 (Yamada et al. 2004). These studies provide evidence for multiple genomic locations controlling complex traits, with allelic variation in complementary genes arising both within and between specific genotypes.

Identification of QTLs associated with plants grown under both control and waterlogging stress conditions has previously been performed in cereals, such as wheat (Malik

Table 3 Summary of QTL analysis for morphological traits in the perennial ryegrass $F_1(NA_x \times AU_6)$ population relating to the NA_x map

Trait	LG	SMR $P < 0.01$	Simple interval mapping				Composite interval mapping				
			Max LOD	Position	*a*	*R*	Threshold	Max LOD	Position	*a*	V_p
RL											
Control	**4**	**34.9–54.4**	1.550	56.410	4.307	0.051	**2.148**	**2.180**	**34.910**	**4.775**	**0.062**
Waterlogged	**4**	**48.6–65.5**	**2.920**	**54.410**	**3.230**	**0.089**	**2.105**	**3.434**	**52.610**	**4.182**	**0.099**
Least significant difference	4	na	1.300	24.010	−5.720	0.100	2.062	1.165	34.910	3.257	0.034
TN											
Control	**1**	0.0	1.966	0.010	−3.450	0.066	**2.148**	**3.254**	**2.010**	**−5.418**	**0.109**
Waterlogged	7	na	0.510	106.110	−1.160	0.016	2.192	0.802	6.010	−2.468	0.055
Least significant difference	1	na	1.380	0.010	−2.680	0.045	2.105	2.024	2.010	−3.827	0.065
PG											
Control	**1**	**0.0–5.5**	**3.280**	**0.010**	**−0.191**	**0.111**	**2.105**	**3.335**	**4.010**	**−0.222**	**0.096**
Waterlogged	3	na	1.400	94.510	−0.099	0.045	2.127	1.299	94.510	−0.094	0.040
Least significant difference	**1**	na	1.160	4.010	−0.112	0.036	**2.148**	**2.238**	**4.010**	**−0.188**	**0.066**
RG											
Control	**1**	**0.0–8.0**	**2.600**	**2.010**	**−0.205**	**0.086**	**2.130**	**2.560**	**4.010**	**−0.234**	**0.072**
Waterlogged	3	94.7	1.910	94.510	−0.101	0.062	2.235	2.081	94.510	−0.098	0.057
	4	**54.4**	1.600	60.410	0.095	0.055	**2.235**	**2.902**	**54.410**	**0.139**	**0.077**
Least significant difference	7	na	1.500	34.010	0.181	0.071	2.127	1.360	36.010	0.161	0.054
LDG											
Control	**1**	**0.0–5.5**	**3.520**	**2.010**	**−0.236**	**0.120**	**2.170**	**3.936**	**2.010**	**−0.288**	**0.129**
Waterlogged	**3**	**86.5–94.7**	2.200	94.510	−0.090	0.069	**2.192**	**2.595**	**94.51**	**−0.095**	**0.075**
Least significant difference	**1**	**0.0–5.5**	2.160	2.010	−0.170	0.070	**2.170**	**3.022**	**2.010**	**−0.239**	**0.097**
3 YFEL A											
Control	**4**	65.5	1.600	60.410	0.561	0.056	**2.040**	**2.297**	**62.410**	**0.621**	**0.068**
Waterlogged	3	na	1.120	90.510	−0.220	0.039	2.192	1.492	94.510	−0.237	0.044
	4	na	0.921	48.610	0.190	0.029	2.192	1.569	48.610	0.240	0.045
Least significant difference	4	na	0.889	62.410	0.406	0.032	2.213	1.056	60.410	0.433	0.037
3 YFEL FW											
Control	**1**	**0.0–5.5**	2.270	2.010	−0.141	0.077	**2.105**	**2.524**	**2.010**	**−0.014**	**0.073**
	3	**94.7**	1.680	94.510	−0.012	0.054	**2.105**	**2.345**	**94.510**	**−0.013**	**0.064**
Waterlogged	**3**	**94.7**	1.920	94.510	−0.006	0.061	**2.148**	**2.733**	**94.510**	**−0.008**	**0.077**
Least significant difference	1	na	1.040	4.010	−0.008	0.033	2.127	1.043	4.010	−0.008	0.030
3 YFEL DW											
Control	**3**	**65.2–94.7**	2.450	79.210	−0.003	0.099	**2.127**	**3.66**	**77.210**	**−0.004**	**0.133**
	4	na	1.145	64.410	0.002	0.037	**2.127**	**2.803**	**64.410**	**0.004**	**0.081**
Waterlogged	**3**	**86.5–94.7**	**2.540**	**94.510**	**−0.002**	**0.080**	**2.148**	**3.333**	**92.510**	**−0.003**	**0.095**
Least significant difference	2	na	0.519	0.010	−0.001	0.017	2.061	0.526	0.010	−0.001	0.017
ALA											
Control	**1**	**0.0–5.5**	**2.710**	**2.010**	**−55.420**	**0.097**	**2.040**	**2.300**	**4.010**	**−45.969**	**0.064**
Waterlogged	4	na	0.667	14.010	−13.020	0.041	1.953	0.851	0.010	−13.377	0.033
Least significant difference	**1**	**5.5**	2.180	4.010	−44.840	0.067	**2.192**	**2.832**	**4.010**	**−60.450**	**0.080**

Details are as described in the legend for Table 2

et al. 2001), barley (Li et al. 2008; Xue et al. 2010) and maize (Qiu et al. 2007). A QTL for shoot biomass reduction under waterlogging conditions in barley was identified on chromosome 4H (Li et al. 2008). Based on comparative genetics (Jones et al. 2002a), barley 4H is syntenic with rice chromosome 3 and perennial ryegrass LG4. In the present study, QTL root dry mass and length under waterlogging stress were identified in LG4. However, it is still not certain whether these are orthologous QTLs, due to a lack of common markers and the difficulty of comparing

phenotyping protocols with different criteria between grain-filling crops and pasture species.

Identification of QTLs associated with stress conditions may assist the characterisation of key genomic regions for which plant improvement may be performed through marker-assisted selection (MAS). Molecular markers and quantitative genetic analysis have permitted accurate QTL mapping in plants and the application of the QTL data for use in MAS and in pre-breeding and breeding programmes (Asíns 2002). The current study of QTLs involved in response to waterlogging may also help to identify QTLs for improved drought tolerance in perennial ryegrass, as it has been observed previously that drought resistance is associated with a deeper rooting system. Some studies have shown that plants selected for heavier and deeper root systems generate progeny with the same characteristics, allowing for cumulative selection of plants with increased drought tolerance (Bonos et al. 2004).

The process of trait-dissection as performed in this study is constrained to QTL identification in a biparental population, and may consequently exclude other regions of effect present in more complex populations. However, the current study provides a basis to search for more extensive prevalence based on linked genetic markers in broader customised germplasm groups through linkage disequilibrium-based mapping. Finally, QTLs associated with traits such as root function, typically found to be associated with improved waterlogging and drought tolerance, can be used for the identification of underlying causal gene-specific variation. Fine-mapping of QTLs associated with specific traits may be further refined by more targeted phenotypic studies, with the aim of reducing the target genomic region and potential characterisation of candidate genes. In this context, the QTL cluster-containing regions on NA_x LG4 and AU_6 LG3 have been selected for identification of critical recombinants within the $F_1(NA_x \times AU_6)$ sib-ship. Diagnostic markers located in candidate genes can then be used for determination of perennial ryegrass genotypes with superior abiotic stress tolerance, for molecular breeding applications (Forster et al. 2008).

Acknowledgments This work was supported by funding from the Victorian Department of Primary Industries, Dairy Australia Ltd., the Geoffrey Gardiner Dairy Foundation, Meat and Livestock Australia Ltd. and the Molecular Plant Breeding Cooperative Research Centre (MPB CRC). The authors thank Prof. German Spangenberg for careful critical reading of the manuscript.

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