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Mapping of quantitative trait loci influencing frost tolerance in *Eucalyptus nitens*

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Abstract Regions of the genome influencing frost tolerance in an outbred family of *Eucalyptus nitens* have been identified. Two QTLs present on the same linkage group, but located 40 cM apart, were identified using single-factor analysis of variance. The QTLs explained between 7.7 and 10.8% of the phenotypic variation for frost tolerance in this family. Analysis of marker loci linked to the QTLs showed one of them to have a simple mode of action with the effect segregating from the male parent in the family. For the other QTL multiple alleles were identified. This QTL showed segregation from the female parent which gave a positive effect on frost tolerance; however, an allele segregating from the male parent was identified which showed a negative interaction with the allele for increased frost tolerance.

Key words Eucalypts · QTL · Frost tolerance · Genetic mapping

Introduction

The sensitivity of many eucalypt species to very low temperatures has been a major constraint to their use in cold-temperate areas throughout the world (Turnbull and Eldridge 1984). A number of eucalypt species are frost-tolerant; however their growth characteristics make most of them unsuitable for commercial plantations. Of the fast-growing species *Eucalyptus nitens* is considered suitable for planting on high-altitude sites where severe frosts and snow occur (Turnbull and Eldridge 1984). Frost tolerance is also a limiting factor in the more extensive use of *E. grandis* on cooler sites (Eldridge et al. 1994). There has been interest in the selection of more-frost-tolerant families of *E. nitens* for planting at higher-altitude sites in Tasmania (Volker et al. 1994), and in the introgression of frost tolerance into the more-fast-growing commercial species, such as *E. globulus*/*E. nitens* and *E. globulus*/*E. gunnii* hybrids (Tibbits et al. 1991).

Whilst early assessment of frost tolerance relied on field data or freezing-chamber experiments (Tibbits and Reid 1987), the development of a laboratory method of measuring frost tolerance under controlled conditions has enabled a more reliable assessment of the trait (Raymond et al. 1986, 1992 a). This method measures leaf damage in terms of the electrical conductivity of leaf discs exposed to freezing temperatures. A comparison of individuals using the electroconductivity method and frost damage due to simulated frosts in a radiation frost room showed that the results from the laboratory method were highly correlated with the leaf damage obtained from the frost-room study (Raymond et al. 1992 a).

E. nitens occurs naturally in areas of high altitude in eastern Australia (Pederick 1979) and is planted on higher-altitude sites in Tasmania where conditions are not suitable for the more commercially favoured *E. globulus* (Tibbits 1986). There is variation within *E. nitens* for the degree of frost tolerance, with the northern New South Wales and central Victorian provenances exhibiting higher frost tolerance than the southern New South Wales and Errinundra provenanes (Tibbits and Reid 1987). Raymond et al. (1992 b) also

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found the northern New South Wales provenance to have the highest levels of frost tolerance, but identified frost-tolerant families in all provenances of the species.

Identification of regions of the genome influencing frost tolerance will lead to a greater understanding of the trait and enable further selection and manipulation of the trait in breeding programs. In the present study regions of the genome influencing frost tolerance were identified in a controlled-cross family of *E. nitens*. An analysis of variance with mapped RFLP markers was used to identify QTLs affecting the trait in this family.

Materials and methods

Plant materials

The plants relevant to this study were part of a larger experiment involving four controlled crosses and open-pollinated seed from the same parents of *E. nitens*. Progeny of one of the controlled-cross families of *E. nitens* was used in this study. The structure of this family is shown in Fig. 1 and details of the natural distribution of the species is given in Byrne and Moran (1994). No information about the level of frost tolerance of the first-generation parents of the family was available. Seed was cold/moist stratified for 3 weeks and germinated in sand under mist. The seedlings were planted in 5-inch pots in a medium of sieved sand/vermiculite/perlite/peatmoss (1 : 1 : 1 : 1) that had been steam-sterilised at 60°C for 1 h. In all, there were 480 plants arranged in a row-column design with four replicates, in order to assess possible variation both across and down the glasshouse. Plants were initially grown in the glasshouse at a 24°C day temperature and 16°C night temperature with individual pot irrigation using a 1/5 Hoagland solution (Went 1957) with full-strength micro-nutrients and iron. After 3 months the plants were weaned off the nutrient solution and the day- and night-time temperatures were gradually reduced until the plants were at ambient temperatures.

Frost-tolerance measurement

Since frost damage to eucalypts in southern Australia occurs primarily due to radiation frosts in winter, frost measurements were made

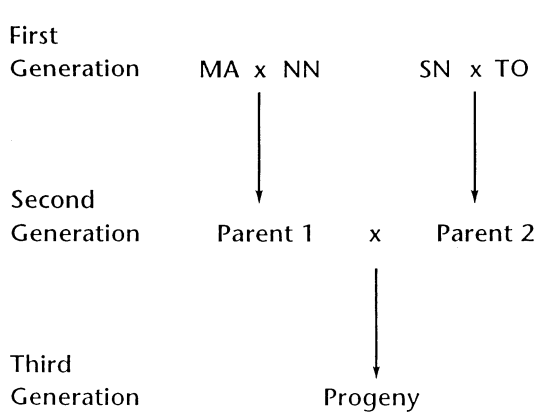

Fig. 1 Structure of family used to investigate QTL effects in *E. nitens*. The first-generation trees come from four different populations: *MA*, Macalister; *NN*, northern New South Wales; *SN*, southern New South Wales; *TO*, Toorongo

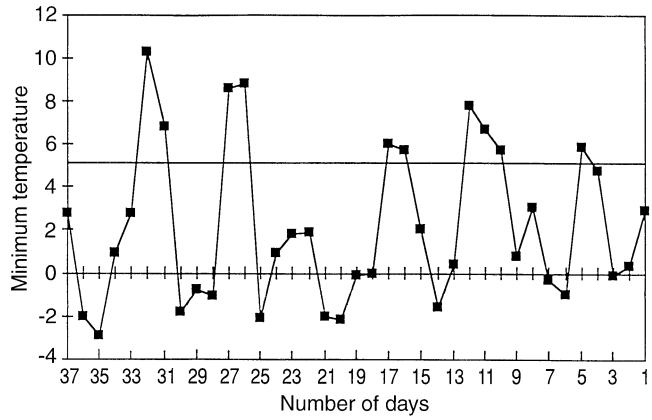

Fig. 2 Minimum temperatures for the 30 days prior to the commencement of frost screening and the 7days of screening. The minimum temperature required for hardening of plants (5°C) is indicated as a *line across the graph*

on hardened plants. At the beginning of winter the plants were moved to an open shade-house for natural winter hardening. Details of the minimum temperatures in the 30 days prior to screening and the 7 days of screening are presented in Fig. 2. All the 480 plants were screened for frost tolerance in August 1993 at 6 months of age. A total of 24 leaf discs were taken from each plant, 12 from each of two leaves with the first leaf being from the youngest fully expanded leaf pair and the second leaf from the next leaf pair below. The leaf discs were assigned at random to three temperatures, -5.5°C, -7.0°C and -8.5°C, i.e. there were eight samples for each plant/temperature combination. Following Raymond et al. (1992a), two measures of electrical conductivity were obtained: the conductivity at the test temperature (ct) and an absolute conductivity (ck) after heating the leaf disc to 80°C for 10 min. The modified relative conductivity $RC^* = [(ck - ct)/ck]^{1/2}$ was then used for analysis.

DNA genotyping and QTL analysis

DNA genotypes were determined for 118 plants from the controlled cross. Leaves of the seedlings were sampled 3 months after planting out. DNA extractions were carried out as described in Byrne et al. (1993). The grandparents, parents and progeny of the cross were genotyped for 210 RFLP loci, and linkage analysis and mapping of these loci was carried out as described in Byrne et al. (1995). The variation in the phenotypic data was assessed for all 480 plants to determine if there were any glasshouse effects. Single-factor analyses of variance (Edwards et al. 1987) for the DNA genotypes and phenotypic data were carried out for the controlled cross. The position of the significant markers on the genetic map (Byrne et al. 1995) was determined to locate regions of the genome influencing frost tolerance. The mode of action of the QTLs was determined by analysis of the allelic constitution of the marker loci in the three generations.

Results

Phenotypic data

The variate RC* was analysed using the four-row column design to determine the effect of position within the glasshouse. The data for each test temperature were

analysed separately. The middle temperature (− 7.0°C) was designed to provide the most discrimination between genotypes, with the lower (− 8.5°C) designed to identify the least-tolerant genotypes and the higher (− 5.5°C) temperature designed to identify the most-tolerant genotypes. An analysis of the − 7.0°C temperature showed good discrimination between individuals with significant replicate effects (due either to glasshouse variation or to the replicate-by-replicate order of conductivity testing of leaf discs). There were significant ($P < 0.001$) differences between the RC* values for the four crosses. The narrow-sense heritability was 0.141.

QTL analysis

Single-factor analysis of variance of frost tolerance at − 7.0°C identified seven loci significant at the 5% level and three loci significant at the 1% level (Table 1). The genomic position of these loci was determined using the *E. nitens* map (Byrne et al. 1995), and showed clustering of the loci in four regions of the map, on linkage groups 5, 6 and 10. The markers showing significance at the 1% level were located in two regions of linkage group 6, 40 cM apart (Fig. 3). This indicates the position of two putative QTLs affecting frost tolerance located on this linkage group. Both regions contained markers that were fully informative, i.e. segregation from both parents could be unambiguously determined, as well as markers segregating from either the male or the female parent only. For the first QTL on linkage group 6, markers segregating from the male parent show no association whereas markers segregating from the female parent show a strong association (e.g. *g063*). The allele originating from the Macalister grandparent was associated with the significant effect at this locus. The fully informative marker in this region (*c514*) was also significantly associated with frost tolerance, but the association is not as significant as for the female-segregating markers close by. For *c514* the two genotype classes with the allele from Macalister (allele 1) have higher mean values than the two classes with the alternate female allele (Fig. 4 a). However, the two classes with the allele from Macalister (genotypes 12 and 13)

Table 1 Loci that showed a significant association with frost-tolerance measurements. FI, fully informative; F, female segregation; M, male segregation; H, heterozygous for the same alleles. Linkage groups from the *E. nitens* map (Byrne et al. 1995)

Locus	Segregation type	Linkage group	*F* probability
c010L	M	10	0.044
c165C	F	6	0.006
c373	M	10	0.044
c456A	F	5	0.036
c514	FI	6	0.032
g063	F	6	0.006
g093	FI	6	0.005
g221	H	6	0.015
g339B	F	5	0.012
g373	FI	6	0.018

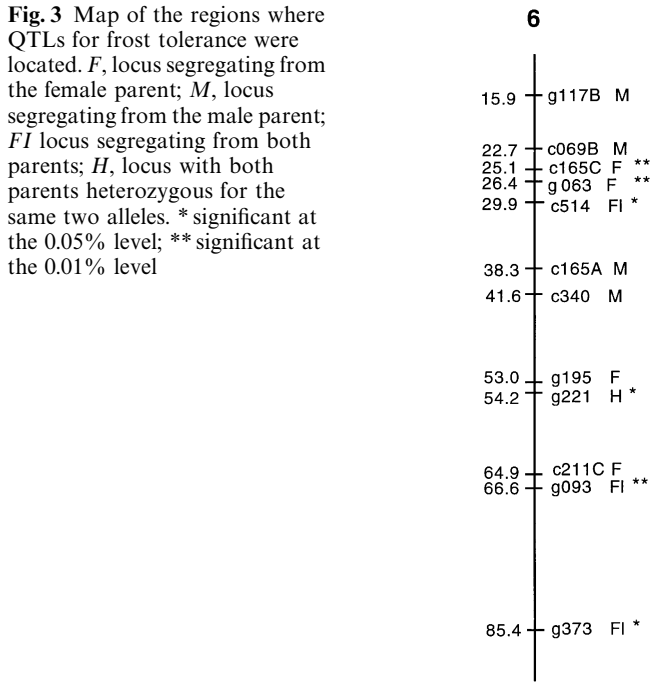

Fig. 3 Map of the regions where QTLs for frost tolerance were located. *F*, locus segregating from the female parent; *M*, locus segregating from the male parent; *FI* locus segregating from both parents; *H*, locus with both parents heterozygous for the same two alleles. * significant at the 0.05% level; ** significant at the 0.01% level

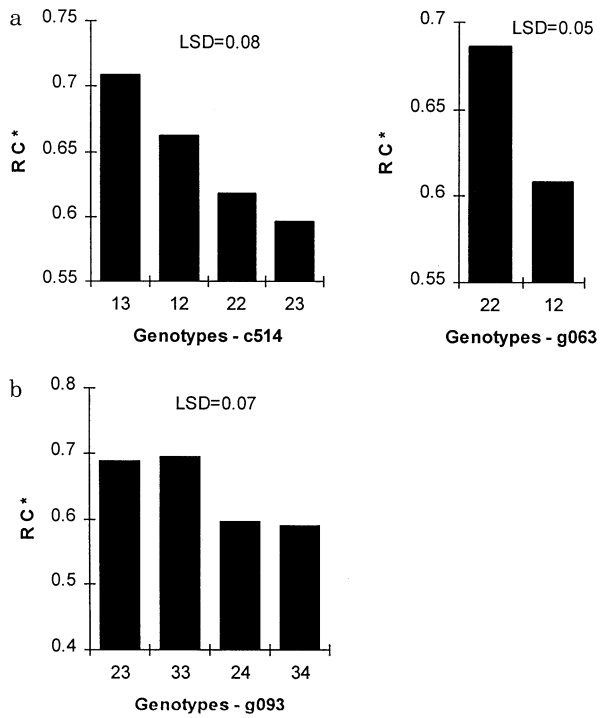

Fig. 4 Mean relative conductivity (*RC**) for genotype classes of 118 progeny at **(a)** marker loci *c514* and *g063* and **(b)** marker locus *g093*

Table 2 Location and effect of QTLs influencing frost tolerance. Origin of effect is from grandparent: SN, southern New South Wales; MA, Macalister

Linkage group	Flanking marker	Segregation type	% Variation explained	% Increase	Origin of effect
6a	*c514*	FI	7.70	7.11	MA
6b	*g093*	FI	10.77	8.56	SN

also show a large difference between them. This indicates the presence of more than two alleles at this locus and an interaction between the Macalister allele and the Toorongo allele segregating from the male parent, which reduces the effect of the Macalister allele. The two genotype classes for the female-segregating marker locus *g063* show a significant difference (Fig. 4a).

For the second QTL the markers segregating from the male parent were significantly associated with frost tolerance, whereas the markers segregating from the female parent showed no association. The fully informative marker in this region (*g093*) showed significant association for the allele segregating from the male parent that originated from the southern New South Wales grandparent (allele 3) (Fig. 4 b). Analysis of *g093* shows that the two classes with the southern New South Wales allele (classes 23 and 33) have similar values, which are significantly higher than the values for the two classes with the alternate allele from the male parent.

The amount of phenotypic variation explained by these two QTLs was calculated for a marker flanking each QTL and was 7.70% and 10.77% (Table 2). The mean increase in relative conductivity conferred on the genotypic class containing the QTL effect compared to the mean relative conductivity of all individuals was 7.1% and 8.56% (Table 2). To test for epistatic interactions between the QTLs a pairwise analysis of variance was carried out using the fully informative marker locus that showed the most significant association for each QTL. There was no significant interaction between the two QTLs.

Discussion

Two regions of the genome have been identified as having an effect on frost tolerance in a family of *E. nitens*. These two regions were on the same linkage group and the results could arise from the influence of a single QTL with a large effect spread across a large region of the linkage group. However, the two regions are well separated and the fully informative markers in each region show different modes of action for the effects of the QTLs in those regions. Therefore it is more likely that there are two QTLs for frost tolerance located on linkage group 6 in this *E. nitens* family. In an outbred pedigree, such as the one investigated here, QTLs may segregate for up to four alleles. The mode of action of one of the QTLs, as revealed by analysis of the marker loci, showed a simple effect of a two-allele locus with one allele having a positive effect on the trait. For the other QTL, analysis of the associated marker locus showed multiple alleles with an interaction between two of the alleles. The presence of multiple alleles at a QTL is not surprising for a long-lived outbreeding tree species. Other investigations using outbred pedigrees have demonstrated the presence of multiple alleles at a QTL, e.g. *E. nitens* (Byrne et al. 1997), loblolly pine (Groover et al. 1994) and potato (van Eck et al. 1994). Interactions between alleles at a QTL have also been identified in QTLs for growth traits in *E. nitens* (Byrne et al. 1996) and wood specific gravity in *Pinus taeda* (Groover et al. 1994). Non-additive versus additive genetic effects cannot be determined in the present experiment. However, additive genetic effects have been identified in the analysis of controlled- and open-pollinated families of *E. nitens* and *E. globulus* (Volker et al. 1994) and in eucalypt interspecific hybrids (Tibbits et al. 1991) using the electro-conductivity method of assessment, and in an analysis of field frost damage in *E. grandis* (van Wyk 1976). In contrast a non-additive genetic component was identified in a full-sib family of *E. regnans* using the electro-conductivity method of assessment (Raymond et al. 1992 a).

Frost tolerance is an important trait for some crops and QTLs affecting this trait have been located in several species. In wheat a gene of large effect on frost tolerance is known to be located on chromosome 5A and RFLP mapping has identified several loci closely linked to this gene (Galiba et al. 1995). QTLs influencing winter hardiness in barley have been located in the region of chromosome 7 that is homoeologous to the region of wheat chromosome 5A where the major frost-tolerance gene was mapped (Hayes et al. 1993). A QTL controlling freezing tolerance in *Brassica rapa* has also been identified (Teutonico et al. 1994). Genes with a major effect on vernalisation response have also been identified in the same regions as the frost-tolerance genes in wheat, brassica and barley (Hayes et al. 1993; Galiba et al. 1995; Teutonico and Osborn 1995).

The QTLs identified in the present study each explained between 7.70 and 10.77% of the phenotypic variation for frost tolerance in this family. No information on the differences in frost tolerance in the parents of this family was available prior to screening. The size of the effects of the QTLs detected here are similar to

the size of effects detected for QTLs in intra-specific families of other forest-tree species, e.g. height and leaf area in *E. nitens* (Byrne et al. 1997) and wood specific gravity in *P. taeda* (Groover et al. 1994). Larger QTL effects in forest trees have been observed in studies where interspecific hybrids have been used, e.g. growth and phenology in poplars (Bradshaw and Stettler 1995) and propagation traits in tropical eucalypts (Grattapaglia et al. 1995).

The origin of the alleles with a positive effect for the two QTLs are from two different populations of the species. In a study of the genetic variation in frost tolerance in a breeding population of *E. nitens* Raymond et al. (1992 b) found that the northern New South Wales provenance was more frost tolerant than other provenances. However, the study also identified frost-tolerant families in all provenances tested including northern New South Wales, southern New South Wales, and the central Victorian provenances of Macalister, Toorongo and Rubicon (Raymond et al. 1992 b). Therefore, it is not unlikely to find the origin of effects from several provenances, particularly in this family which results from a wide inter-population cross. Our study shows that quantitative trait loci afecting an adaptive trait such as frost tolerance can be identified in a family where no previous knowledge of the trait was available. Further investigations in other families are required to identify other QTLs effecting frost tolerance in *E. nitens*.

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