

Genetic mapping provides evidence for the role of additive and non-additive QTLs in the response of inter-specific hybrids of *Eucalyptus* to *Puccinia psidii* rust infection

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Abstract Eucalypts are susceptible to a wide range of diseases. One of the most important diseases that affect *Eucalyptus* plantations worldwide is caused by the rust fungus *Puccinia psidii*. Here, we provide evidence on the complex genetic control of rust resistance in *Eucalyptus* inter-specific hybrids, by

analyzing a number of full-sib families that display different patterns of segregation for rust resistance. These families are totally unrelated to those previously used in other inheritance studies of rust resistance. By using a full genome scan with 114 genetic markers (microsatellites and expressed sequence tag derived microsatellites) we also corroborated the existence and segregation of a resistance locus, explaining 11.5% of the phenotypic variation, on linkage group 3, corresponding to *Ppr1*. This find represents an additional validation of this locus in totally unrelated pedigree. We have also detected significant additive × additive digenic interactions with LOD >10.0 on several linkage groups. The additive and epistatic QTLs identified explain between 29.8 and 44.8% of the phenotypic variability for rust resistance. The recognition that both additive and non-additive genetic variation (epistasis) are important contributors to rust resistance in eucalypts reveals the complexity of this host-pathogen interaction and helps explain the success that breeding has achieved by selecting rust-resistant clones, where all the additive and non-additive effects are readily captured. The positioning of epistatic QTLs also provides starting points to look for the underlying genes or genomic regions controlling this phenotype on the upcoming *E. grandis* genome sequence.

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Introduction

Eucalyptus tree species and hybrids have over the past five decades become the most widely planted hardwood trees in the world, mainly in the tropics and subtropics. These fast-growing plantations are today supporting a multi-billion industry based on eucalypt pulp and paper, charcoal for the steel industry and solid products (Grattapaglia and Kirst 2008). Eucalypts are however, susceptible to a wide range of diseases including fungal and bacterial pathogens. One of the most important diseases that currently affect eucalypts plantations is caused by the rust fungus *Puccinia psidii* Winter (Coutinho et al. 1998; Glen et al. 2007). This rust affects members of the *Myrtaceae* family and rapidly adapted to eucalypts after their introduction into Brazil and other countries. *Eucalyptus grandis* (Hill) Maiden, one of the most planted eucalypt species, is also among the most-susceptible, making this rust the most-damaging disease to eucalypt plantations in tropical regions nowadays (Zauza et al. 2010). Rust is also a potential threat to other tropical and subtropical areas with natural or cultivated eucalypts, where the disease has not yet been reported, such as Australia and South Africa (Glen et al. 2007). Rust occurs mainly on seedlings in nurseries, on young trees in the field, on coppice and also on shoots in clonal hedges (Coutinho et al. 1998; Glen et al. 2007). The first symptoms shows up several days after the host contact with fungus urediniospores and the first sign of infection is the appearance of pale yellow specks on the leaf blades. Within 10–12 days, the specks deepen in color to a characteristic egg-yolk yellow. Thereafter, the pustules increase in size due to the radial growth of the fungus (Coutinho et al. 1998), biochemically limiting the carbon fixation at the chloroplast level and photo-inhibiting the photosynthesis (Alves et al. 2011). Although fungicide applications are able to reduce losses in nurseries and on coppice (Zauza et al. 2008) the use of resistant genotypes is the best alternative for rust control.

Considerable attention has been given to the understanding of the genetic control of disease resistance in crop plants. Much less is known about the inheritance and molecular aspects of disease resistance in forest trees, in spite of important advances especially in poplar and pines (Azaiez et al. 2009; Jorge et al. 2005; Kayihan et al. 2005; Liu

and Ekramoddoullah 2009). Identifying the genetic basis of disease resistance is, however, essential when breeding for resistance. In long-living forest trees most of the existing knowledge on the genetic architecture of disease resistance comes from quantitative trait loci (QTL) and gene mapping studies in pines (Devey et al. 1995; Liu et al. 2006; Li et al. 2006), poplar (Cervera et al. 1996; Newcombe et al. 1996; Dowkiw and Bastien 2004; Jorge et al. 2005; Lefèvre et al. 1998) and eucalypt (Freeman et al. 2008; Mamani et al. 2010; Junghans et al. 2003a). Resistance to rust occurs naturally in many eucalypt species, and since *P. psidii* is native from South America and eucalypts are from Australia, it is thought to be a type of exapted resistance, i.e. it was not favored by selection for its current role, but rather evolved for other functions (Newcombe 1998). Those other functions might involve resistance to pathogens and, or pests native to the ranges of *Eucalyptus* in Australia. Rust resistance is conferred by a hypersensitive reaction (HR) (Xavier et al. 2001), which is a common type of response triggered when plant resistance genes (R genes) are challenged by pathogen avirulence genes (Avr genes) (Hammond-Kosack and Parker 2003; Jones and Dangl 2006). A major locus for rust resistance, *Ppr1*, was indeed mapped in *E. grandis* using RAPD markers (Junghans et al. 2003a). It has since been positioned on a microsatellite reference map, allowing it to be screened and validated in independent crosses (Mamani et al. 2010). Despite these important advances there are still many relevant aspects of rust resistance to be elucidated. As previously pointed out by Junghans et al. (2003a) there is evidence of a more complex pattern of inheritance, as the expression of the major resistance gene seems to be dependent upon the genetic background. At least in one case, the segregation of resistance did not fit to a simple Mendelian model (an excess of susceptible plants was observed), even though the family was derived from the tree that carried *Ppr1* (G21) (Junghans et al. 2003a). Similar effects have been reported in families of *Pinus monticola* inoculated with *Cronartium ribicola* (Kinloch et al. 1999), and also in resistance to three races of *Melampsora larici-populina* in hybrid families of *Populus deltoides* and *P. trichocarpa* (Lefèvre et al. 1998). The effect of genetic background has been explained by the existence of suppressor and modifier genes affecting the resistance

gene (Kolmer 1996). Moreover, recent studies have suggested that epistatic effects contribute to disease resistance in many pathosystems (Wilfert and Schmid-Hempel 2008). Examples are soybean resistance to the cyst nematode (*Heterodera glycines*) (Wu et al. 2009); wheat resistance to the stripe-rust (*Puccinia striiformis* f. sp. *tritici*) (Lu et al. 2009) and *Arabidopsis thaliana* resistance to clubroot (*Plasmodiophora brassicae*) (Jubault et al. 2008). In that context, a number of methods have been proposed to map interacting loci (Li et al. 2008). Perhaps then, the putative suppressor and modifier genomic regions affecting the eucalypts resistance to rust could be identified using such analysis.

In this paper, we provide evidence of a complex genetic control of rust resistance in *Eucalyptus* inter-specific hybrids by analyzing a number of full-sib families that display different patterns of segregation for rust resistance and also of a complex genetic architecture of the *Eucalyptus* response to rust by genetic mapping of additive and epistatic QTLs in an inter-specific bi-parental cross.

Materials and methods

Resistance evaluation

We first phenotyped 10 inter-specific *Eucalyptus* families to gain insights on the genetic control of rust resistance, and also to select a suitable segregating population in which we could carry out a full QTL mapping analysis. Resistance or susceptibility responses of the inter-specific families were assessed by artificial inoculation. It is noteworthy that these 10 families are totally unrelated to those studied by Junghans et al. (2003a) and Mamani et al. (2010). The 10 crosses involved two *E. grandis* genotypes (G and G1), one *E. dunnii* genotype (D), three *E. globulus* genotypes (GL, GL1 and GL2), three *E. urophylla* genotypes (U, U1 and U2), one *E. camaldulensis* genotype (C) and one *E. tereticornis* genotype (E) developed by the Genolyptus consortium (Table 1). Initially 20 plants per family were evaluated and later some families were expanded in size and re-inoculated to confirm the segregation patterns obtained in the screening phase. No rust-resistance information was available for the parent trees, and these trees weren't available for inoculations. Greenhouse-grown

eucalypt seedlings (4–5 months old) were spray inoculated with an inoculum suspension at 2×10^4 urediniospores ml^{-1} of a single pustule-isolate of *P. psidii* (UFV-2) (Junghans et al. 2003a). Inoculated plants were incubated for 24 h in a moist chamber at 25°C in the dark and then transferred to a growth chamber at $22 \pm 2^\circ\text{C}$ under a 12 h photoperiod at 40 $\mu\text{mol photons s}^{-1} \text{m}^{-2}$ of light intensity (Ruiz et al. 1989). Disease severity was assessed 20 days after inoculation (DAI), using a visual disease severity scale (DSS) based on the one developed for rust-severity evaluation by Junghans et al. (2003b). The DSS encompasses five phenotypic classes: S0 = immune plants, S0HR = those that display a hypersensitive reaction; S1 = plants with small pustules; S2 = plants with medium-size pustules and S3 = plants with large pustules (Fig. 1). In the inheritance studies, plants S0, S0HR and S1 were considered resistant, while plants S2 and S3 susceptible following Junghans et al. (2003b). A two-tail Fisher exact test ($P \leq 0.05$) for small progenies and chi-square test ($P \leq 0.05$) for large progenies were used to test the hypothesis of Mendelian inheritance of the disease phenotypes (S0, S0HR and S1 versus S2 and S3). Fisher exact tests were performed with the aid of an online tool at <http://www.langsrud.com/fisher.htm>. Chi-square tests were performed with the software GENES (Cruz 1998).

Based on the phenotypic value of each plant derived from the cross 8 [(D × G) × (U × GL)] (Table 1) assessed by DSS in two different inoculations (20 DAI each) the heritability of rust resistance in that specific cross was estimated by means of its repeatability coefficient (20 DAI repeatability coefficient). Both inoculations were performed on the same plants. For that, after the first assay, the plants were pruned and re-conducted. The second inoculation was then performed when new shoots were expanding. In this process 17 plants were lost, and the repeatability coefficient was calculated based on data of 188 instead of the initial 205. The repeatability coefficient was obtained from an analysis of variance (ANOVA). The following statistical model was adopted:

$$Y_{ij} = m + g_i + a_j + e_{ij},$$

where Y_{ij} is the observation that refers to the individual i and assessment j (rust severity—S0, S0HR, S1, S2, S3 converted to 1, 2, 3, 4 and 5); m is

Table 1 Segregation of rust resistance in full-sib families of inter-specific crosses of *Eucalyptus* inoculated with a single pustule isolate of *Puccinia psidii* and evaluated 20 days after inoculation

	Family	No. of plants/severity class (S0:S1:S2:S3)	Ratio ^a	Expected ratio R:S ^b	P value ^d
1	C1 × (U × GL) ^c	18:1:1:0	19:1	1:0	0.99 ^{ns}
2	G1 × GL2	2:2:5:11	4:16	1:3	1 ^{ns}
3	U2 × E135	0:6:3:11	6:14	1:3	1 ^{ns}
4	U2 × GL1	0:5:3:12	5:15	1:3	1 ^{ns}
5	(D × G) × GL2	0:1:2:17	1:19	0:1	0.99 ^{ns}
6	G1 × (U × GL)	13:2:5:0	15:5	3:1	1 ^{ns}
7	U1 × (U × GL)	18:2:0:0	20:0	1:0	1 ^{ns}
8	(D × G) × (U × GL)	15:2:3:0	17:3	3:1	0.6947 ^{ns}
9	(D × G) × C1	0:2:8:10	2:18	0:1	0.4872 ^{ns}
10	(D × G) × U2	0:2:10:8	2:18	0:1	0.4872 ^{ns}

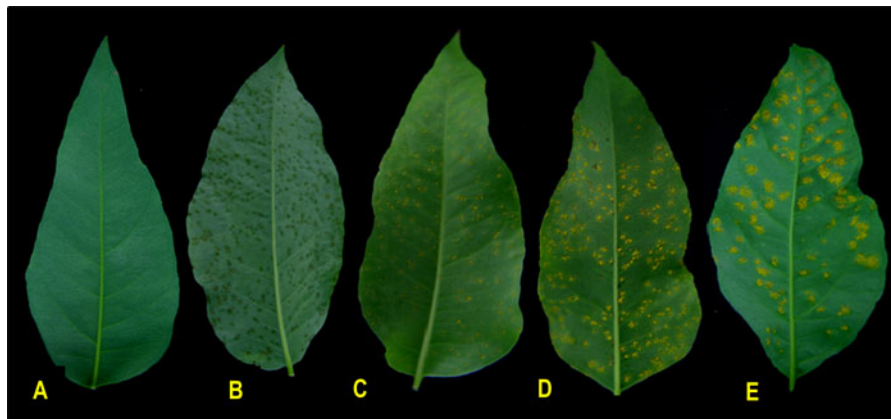
^a The Resistant:Susceptible (R:S) ratio is based on R = S0 + S1 and S = S2 + S3

^b The resistance was hypothesized to be controlled by a major dominant gene

^c *E. grandis* (G), *E. dunni* (D), *E. globulus* (GL), *E. urophylla* (U), *E. camaldulensis* (C) and *E. tereticornis* (E)

^d P value of Fisher exact test (small progenies)

ns Not significant at 5% of probability

**Fig. 1** Disease severity scale (DSS) used for rust-severity evaluation. (a) S0 = immune, (b) S0HR = hypersensitive reaction, (c) S1 = small pustules, (d) S2 = medium-size pustules and (e) S3 = large pustules

the general mean; g_i is the random effect of the individual i under influence of permanent environmental conditions ($i = 1, 2, \dots, 188$ individuals); a_j is the effect of the assessment j ($j = 1, 2$) and, e_{ij} is the experimental error associated with observation Y_{ij} .

The repeatability coefficient is given by $r = \frac{\widehat{Cov}(Y_{ij}, Y_{ij'})}{\sqrt{\widehat{V}(Y_{ij}) \cdot \widehat{V}(Y_{ij'})}} = \frac{\widehat{\sigma}_g^2}{\widehat{\sigma}_y^2} = \frac{\widehat{\sigma}_g^2}{\widehat{\sigma}_e^2 + \widehat{\sigma}_g^2}$, and resembles trait heritability when permanent environmental conditions do not considerably affect the phenotype, as it is

expected for rust resistance. σ_g^2 was estimated by $(MSG - MSR)/n$, where n is number of assessments, MSG is the mean square of genotypes and MSR is the mean square of the residual. These analyses were performed with the software GENES (Cruz 1998).

For QTL mapping, the 188 individuals of the inter-specific cross $(D \times G) \times (U \times GL)$ that segregated for rust resistance were inoculated and scored following the procedure previously described.

Linkage analyses and parental maps construction

DNA extractions of expanded leaves from the 188 plants of the cross (D × G) × (U × GL) and both parents were carried out as described previously (Grattapaglia and Sederoff 1994). A total of 106 microsatellite markers, chosen to provide even genome coverage, were selected based on the reference linkage map (Brondani et al. 2006) and on the microsatellites developed by CSIRO (Thamarus et al. 2002). An additional set of eight EST derived markers (Embra915, 954, 979, 1468, 1851, 1928, 1977 and 2014) recently developed (Faria et al. 2010, 2011) were also used, totalizing 114 loci. Genotyping was carried out in multiplexed systems with four to six co-amplified loci, each one labeled with a specific fluorochrome (6-FAM, HEX or NED). PCRs contained 2.5 µl of the Multiplex PCR Kit (Qiagen®) Master Mix; 0.5 µl of Q-Solution; 0.1 µl of each primer at 10 µM; 0.4 µl of RNase-free water and 2.0 ng of genomic DNA (1µl). The total reaction volume was 5 µl. A hot start PCR program was used with a 5 min denaturation step followed by 35 cycles of 1 min at 94°C, annealing for 1 min at 65°C and extension for 1 min at 72°C and a final step of 15 min at 72°C in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). An 1 µl aliquot of PCR product was mixed with 1 µl of ROX size standard (Brondani and Grattapaglia 2001) and 8 µl of Hi-Di formamide. The mixture was electro-injected in an ABI 3100XL genetic analyzer (Applied Biosystems, Foster City, CA, USA), and data collected using DataCollection and analyzed with Genemapper software (Applied Biosystems, Foster City, CA, USA).

Genetic mapping was performed using the pseudo-testcross mapping approach (Grattapaglia and Sederoff 1994). Separate linkage maps for each parent tree were constructed based on microsatellites that were heterozygous and thus segregating in the expected 1:1 ratio. Linkage analyses were performed using Map-Maker (Lander et al. 1987). Linked markers were first placed onto linkage groups using the “group” command with a threshold LOD score of 3.0 and a maximum recombination fraction (θ) of 0.30. The “first-order” and “compare” commands were then used to identify the most probable marker order within a linkage group. The “ripple” command was

used to verify the log likelihood support for local order. Recombination fractions were transformed to estimated map distances by the Kosambi map function. Linkage group numbering followed the one established for the reference linkage map of *Eucalyptus* (Brondani et al. 2006).

QTL mapping strategies

Disease scores given to each plant according to the DSS, S0, S0HR, S1, S2 and S3 were converted into 1, 2, 3, 4 and 5, respectively, and used as phenotypic values of each plant in QTL mapping. Based on the pseudo-testcross maps, QTLs analyses were performed using the modified algorithm ICIM (Inclusive Composite Interval Mapping) (Li et al. 2007). The parameters used for one dimensional ICIM were a threshold LOD = 3 to declare significant QTL, a 1 cM genome scanning step, a P -value ≤ 0.01 for entering variables in the forward–backward stepwise regression of residual phenotype on marker variables and a P -value ≤ 0.02 for removing variables in the forward–backward stepwise regression of residual phenotype on marker variables. We then used two dimensional ICIM (Li et al. 2008) for mapping digenic interacting QTLs (epistatic QTLs). Extensive simulations have shown that ICIM is an efficient method for epistasis mapping, and that epistatic QTLs can be identified no matter whether the two QTLs have any additive effects (Li et al. 2008). The parameters for ICIM of digenic QTLs (two dimensional ICIM) were the same as for the one dimensional ICIM but we used a 5 cM genome scanning step and a threshold LOD of 3.5. Only epistatic QTLs resulting from the interaction of regions located in different linkage groups were considered.

The following parameters were analyzed when a significant QTL was detected: (i) parental genome; (ii) linkage group on which the QTL is localized; (iii) marker interval; (iv) most likely QTL position corresponding to LOD peak, in cM distance from leftmost marker and distances to the flanking markers; (v) one likelihood of odds (LOD) confidence interval of the QTL position; (vi) genetic additive effect (a) of the QTL; (vii) LOD score for the genetic additive effect; (ix) percentage of the phenotypic variation explained (PVE) and (x) percentage of the genetic variation explained given by $g = \frac{m^2}{h^2} = \frac{\sigma_a^2}{\sigma_a^2}$.

Table 2 Segregation of expanded families 8, 9 and 10 of interspecific crosses of *Eucalyptus* spp. inoculated with a single pustule isolate of *Puccinia psidii* to rust resistance and evaluated 20 days after inoculation

	Family	No. of plants/severity class (S0:S1:S2:S3)	Ratio ^a	Expected ratio R:S ^b	χ^2	P value
8	(D × G) × (U × GL) ^c	144:28:19:14	172:33	3:1	8.665	0.3263 ^{ns}
9	(D × G) × C1	1:4:43:189	5:232	0:1	– [§]	–
10	(D × G) × U2	1:0:19:138	1:157	0:1	–	–

^a The Resistant:Susceptible (R:S) ratio is based on R = S0 + S1 and S = S2 + S3

^b The resistance was hypothesized to be controlled by a major dominant gene

^c *E. grandis* (G), *E. dunni* (D), *E. globulus* (GL), *E. urophylla* (U) and *E. camaldulensis* (C)

^{ns} Not significant at 5% of probability by a chi square test

[§] Chi-squared tests could not be calculated because one of the expected frequencies is zero

Results

Inheritance of rust resistance in inter-specific crosses

Among the 10 inter-specific families initially challenged with *P. psidii*, three (families 5, 9 and 10) were evaluated as susceptible and two as fully resistant (families 1 and 7). The other families segregated for rust resistance either in a 3R:1S or a 1R:3S ratio (Table 1). Although these segregation patterns may be individually explained based on the hypothesis of major loci, the results from families 5, 8, 9 and 10 taken together suggests a more complex pattern of inheritance. Assuming a simple monogenic control for rust resistance, while the results from family 8, indicates that the D × G parent is a heterozygote, the results from the other families involving this hybrid, indicates that this tree is a recessive homozygote. To confirm the phenotypes we expanded some of these families and re-inoculated them (Table 2). The results confirmed that families 9 and 10 are completely susceptible to rust, not only confirming the accuracy of the screening inoculation, but also supporting a more complex inheritance pattern. Although the resistance seems to be controlled by major loci, the phenotypic variation in all 10 families also points to the existence of minor effect loci since there is variation within each phenotypic class, i.e. resistant plants can be classified in S0, S0HR or S1, while susceptible plants in S2 or S3. Based on the phenotypic score of each plant derived from family (D × G) × (U × GL) in two distinct inoculations (20 DAI) the heritability of rust

resistance in that family was estimated by means of its repeatability coefficient as equal to 84.5% (20 DAI repeatability coefficient). Phenotypic variance, genotypic variance and phenotypic mean in that specific family were estimated as 1.28, 1.03 and 0.74, respectively.

Linkage mapping

Out of 114 segregating loci a total of 89 (78%) were fully informative. No markers were observed segregating 1:2:1, i.e. equally heterozygous in both parents. A higher heterozygosity was observed in the U × GL parent tree compared to D × G. In total, 95 markers were heterozygous in D × G and 108 in U × GL, i.e. 11% more than D × G. At the statistical stringency adopted for linkage analysis, the maternal D × G map had a total of 93 markers organized into 11 linkage groups while the paternal U × GL map had a larger number of markers, 100, in 11 linkage groups (n = 11). The female map with 93 markers covered an observed length of 1112.1 cM with a mean distance between adjacent markers of 11.9 cM, calculated as the arithmetic mean of the map distances between adjacent markers in each linkage group and not just simply by dividing the total map length by the number of markers. For the U × GL male map the total recombination map distance covered was of 1580 cM with an average distance between adjacent markers of 15.9 cM. A paired t-test revealed significant difference in the mean recombination fraction between adjacent markers (11.9 for D × G and 15.9 for U × GL) when comparing the parental maps. The lengths of our

Table 3 QTL for rust resistance in the U × GL parental map of family (D × G) × (U × GL) (*Eucalyptus dunnii* × *E. grandis*) × (*E. urophylla* × *E. globulus*) as determined by inclusive composite interval mapping analysis

LG ^a	Parent	Marker interval (distance in cM)	Pos ^b	Conf. Int. ^c	LOD Peak	a ^d	PVE ^e	g ^f
3	U × GL	Embra1656 (16.4) Embra1071 (1.4)	37.0	28.0-38.6	5.63	-0.3141	11.48	13.6

^a Linkage group

^b Most likely QTL position, corresponding to the LOD peak, in cM, from the leftmost marker of the linkage group

^c One likelihood of odds confidence interval

^d Genetic additive effect

^e Percentage of the phenotypic variation explained

^f Percentage of the genetic variation explained calculated by $g = \frac{m^2}{h^2} = \frac{\sigma_m^2}{\sigma_a^2}$

maps are in agreement with the lengths of *E. grandis* and *E. urophylla* maps (1814.5 and 1133.4 cM, respectively) and with the reference map of *Eucalyptus* (1567.7 cM) (Brondani et al. 2006). As the reference eucalypt map coverage was estimated as 93%, our maps provided enough genomic coverage to perform a whole genome QTL scan for rust resistance.

QTL mapping

To perform QTL analysis, disease severity was assessed based on a DSS (Fig. 1). This visual DSS is based on pustules size and is derived from the one originally published by Junghans et al. (2003b). Based on the pseudo-testcross maps and on the DSS evaluation, one QTL with significant effect on rust resistance was located by ICIM analysis, on linkage group 3 of the hybrid U × GL (Table 3) between the markers Embra1656 and Embra1071. This QTL explained 11.5% of the phenotypic variance in rust resistance. When we used two dimensional ICIM (Li et al. 2008) for mapping digenic interacting QTLs (epistatic QTLs), we detected three pairs of interacting QTLs based on the U × GL parental map and nine pairs epistatic QTLs on the D × G parental map (Table 4). We found that the proportion of the phenotypic and genetic, variation explained by the epistatic QTLs pairs is sometimes as large as the proportion explained by the additive QTL (Table 4). No additive effect was evident for any of the interacting QTLs, and therefore, these QTLs can be viewed as QTLs with significant epistatic effects but no significant additive effects.

Discussion

The phenotypic analyses carried out in this study involved a number of families displaying different patterns of segregation for rust resistance. In four families (5, 8, 9 and 10), all involving the hybrid D × G, the segregation ratios were not compatible with a single-gene inheritance. If that were the case the 3R:1S pattern of cross 8, would indicate that the hybrid D × G is heterozygous for the resistance locus while the 0:1 pattern in crosses 5, 9 and 10, would suggest that this same tree is a recessive homozygote for a resistance locus. Similar incongruence was earlier observed when *Ppr1* was first detected and mapped (Junghans et al. 2003a). These additional observations of a more complex pattern of inheritance led us to investigate the occurrence of epistasis in the determination of rust resistance in *Eucalyptus*. Assuming that both the hybrid D × G and the hybrid U × GL are heterozygous for a resistance locus, in agreement with results from the expanded family 8, the absence of segregation in the other families could occur if the resistance locus interacts epistatically with other loci. Furthermore, an excess of susceptible plants in families 2, 3 and 4, in a relatively limited sample of screened plants, also suggests a more complex pattern of inheritance. In intra-specific crosses, however, the occurrence of such epistatic phenomenon does not seem to be determinant of the resistant phenotype (Junghans et al. 2003a), possibly because of the close relationship between the trees genetic background, that may guarantee that the same alleles in the epistatic loci are passed to the progeny.

Table 4 Summary of the digenic interacting QTLs for rust resistance in the pseudo-testcross maps of *Eucalyptus dunnii* × *E. grandis* (D × G) and *E. urophylla* × *E. globulus* (U × GL) hybrids as determined by two-dimensional inclusive composite interval mapping

P ^a	LG1 ^b	QTL1 Pos ^c	Marker Interval (Distance)	LG2 ^d	QTL2 Pos ^e	Marker Interval (Distance)	Add-QTL1 ^f	Add-QTL2 ^g	Add-Add ^h	LodHaa ⁱ	PVE ^j	g ^k
U × GL	1	25.0	En06 (15.0) Embra676 (1.2)	2	5.0	Embra390 (5.0) Embra172 (13.2)	-0.0921	-0.0785	0.2512	5.23	6.9	8.2
U × GL	7	5.0	Embra42 (5.0) Embra623 (42.7)	9	165.0	Embra357 (19.2) Embra2014 (1.1)	-0.0431	-0.0882	0.2214	3.95	5.4	6.4
U × GL	9	50.0	Embra3 10 (5.4) Embra954 (10.2)	11	5.0	Embra6 69 (5.0) Embra165 (0.8)	0.0816	-0.0049	-0.2265	4.16	6.0	7.1
D × G	1	70.0	Embra1639 (3.2) Embra219 (4.4)	2	60.0	Eg86 (2.0) Embra6 48 (9.3)	-0.0092	-0.0061	0.2183	10.66	5.5	6.5
D × G	2	15.0	Embra172 (8.8) Embra390 (1.5)	8	45.0	Embra53 (1.1) Embra696 (26.3)	-0.0518	0.0068	-0.4001	18.85	10.7	12.6
D × G	2	50.0	Embra333 (22.8) Embra27 (0.2)	3	25.0	Embra1656 (9.3) Embra98 (0.7)	-0.0045	-0.015	-0.1711	7.13	3.2	3.8
D × G	2	55.0	Embra201 (0.2) Eg86 (3.0)	10	120.0	Embra127 (10.0) Embra155 (4.6)	0.0181	-0.0146	-0.2373	10.02	5.5	6.6
D × G	3	10.0	Embra122 (0.4) Embra1656 (5.7)	4	60.0	Embra662 (5.6) Embra179 (1.2)	-0.0129	0.0237	-0.126	4.40	1.8	2.2
D × G	5	90.0	Embra214 (0.6) Embra209 (2.6)	6	60.0	Embra950 (3.0) Embra324 (3.0)	-0.0153	-0.0195	0.2344	9.55	4.7	5.6
D × G	5	100.0	Embra208 (0.6) Embra37 (1.8)	7	90.0	Embra1761 (30.6) Embra347 (3.8)	-0.0088	0.008	0.2769	14.88	7.8	9.2
D × G	6	40.0	Embra196 (2.3) En16 (11.8)	7	30.0	Embra623 (4.0) Embra128 (16.2)	0.0506	-0.0159	-0.1652	5.86	3.1	3.6
D × G	9	20.0	Embra210 (4.8) Embra310 (0.3)	10	110.0	Embra127 (0.0) Embra155 (14.6)	0.0055	-0.0061	0.1464	5.88	2.5	2.9

^a Parental genome

^b Linkage Group on which the first interacting QTL is localized

^c Most likely first interacting QTL position, corresponding to the LOD peak, in cM, from the leftmost marker of the linkage group

^d Linkage Group on which the second interacting QTL is localized

^e Most likely second interacting QTL position, corresponding to the LOD peak, in cM, from the leftmost marker of the linkage group

^f Genetic additive effect of the QTL1

^g Genetic additive effect of the QTL2

^h Genetic additive by additive effect from two-dimensional scanning

ⁱ LOD score for genetic additive by additive effect from two-dimensional scanning

^j Percentage of the phenotypic variation explained

^k Percentage of the genetic variation explained; $g = \frac{\sigma_g^2}{\sigma^2} = \frac{\sigma_a^2}{\sigma_e^2}$

This hypothesis is supported by observations that progenies derived from intra-specific crosses often display the expected ratios of segregation (R:S - 1:1, 3:1 and 1:0 for example) when inoculated artificially, while progenies derived from inter-specific crosses often display ratios that are not normally expected (e.g. 1:3) (Alfenas AC—unpublished).

Because epistatic effects apparently contribute to disease resistance in many pathosystems (Wilfert and Schmid-Hempel 2008), we tested the hypothesis that an additive locus along with epistatic loci are involved in the rust-resistance response. We attempted to map additive and epistatic interacting QTLs using a full linkage map scan. It is interesting to note that to date, no full genome scan was performed aiming the identification of genes/QTLs affecting rust resistance. With this strategy, we first detected a QTL for rust resistance on linkage group 3 explaining a moderate proportion of the genetic variation (11.5%) located between markers Embra1656 and Embra1071 (16.4 and 1.4 cM away, respectively) (Table 3). This mapping result agrees with the recent positioning of *Ppr1* on linkage group 3 in the same map interval (Mamani et al. 2010), as *Ppr1* was positioned on the reference map between these same markers. Our mapping result indicates then that the mapped QTL might correspond to the *Ppr1* locus, and thus provide additional validation of the existence and location of such locus in a totally unrelated pedigree. As the QTL detected on LG3 is inherited from U × GL parent, which belongs to a different species than the one where *Ppr1* was originally identified (*E. grandis*), the gene underlying the QTL likely corresponds to the same locus but to a different allele or to a closely linked locus. The possibility that the regions with QTL of large effect represent clusters of closely linked genes cannot be dismissed since resistance genes are commonly clustered in plants (Mondragon-Palomino and Gaut 2005).

Besides corroborating the *Ppr1* position, the full genome scan adopted in our study allowed the detection of various digenic interacting QTLs, with no additive effect, explaining moderate proportions of the phenotypic variation (Table 4). The additive QTL and epistatic QTLs identified explain together between 29.8 and 44.8% of the phenotypic variability for rust resistance in the U × GL and D × G parents, respectively. The remaining genetic variability is likely due to minor effect QTLs, higher-order epistatic effects or also due to epistatic effects between pairs of QTLs

inherited from different parents that are not detectable in our experimental design. It has been recently suggested that disease resistance, both in plants and animals, can be strongly affected by epistatically interacting loci that do not show additive gene action (Wilfert and Schmid-Hempel 2008). In our study, the additive by additive genetic effect at many of the epistatic QTLs was as large as the additive effect of *Ppr1* detected by one-dimensional ICIM (Tables 3 and 4), suggesting that the effects of interacting secondary loci have a similar contribution to the genetic architecture of rust resistance in eucalypts as the effect of *Ppr1*. It is noteworthy that in our study in the same way that we have verified some epistatic QTLs with an additive by additive genetic effect favorable to rust infection contention, the additive by additive genetic effect of many other epistatic QTLs seem to be unfavorable to rust infection contention. Perhaps then, some of this unfavorable interacting QTLs may act as suppressors and modifiers genomic regions early speculated by Junghans et al. (2003a). Then, it is tempting to speculate that the outcome of the *P. psidii*-*Eucalyptus* interaction, at least in inter-specific crosses, is determined by the presence of the favorable allele on the R locus (*Ppr1*), but also by the prevalence of favorable or unfavorable interacting QTLs.

The detection of epistasis has been usually attempted via QTL mapping procedures and a number of methods have been proposed with this purpose. However, the power of most methods to detect epistasis between QTL in mapping populations is typically low. Several factors contribute to weak detection of epistasis including those pointed out by Mackay (2001): (a) Even large mapping populations contain few individuals in the rarer two-locus genotype classes; (b) segregation for other QTLs can interfere with detecting epistasis between the pair of loci under consideration; (c) after adjusting the significance threshold for the multiple statistical tests involved in searching for epistatic interactions, only extremely strong interactions remain significant; and (d) even strong epistatic interactions contribute little to the epistatic variance. However, the observations of epistasis between QTLs from experimental designs that are not optimal for detecting interactions suggest that genotype-specific QTL effects can be rather common and important (Mackay 2001). Recently, many theoretical papers have proposed improved methods for mapping interacting QTLs. In that

respect, two-dimensional ICIM for digenic interacting QTLs (Li et al. 2008) has been shown to be efficient through extensive simulations. Still, not all epistatic interactions detected are potentially biologically relevant (Carlborg and Haley 2004). In our study, we used the two-dimensional ICIM methodology and could find significant digenic interactions with LOD scores in excess of $\text{LOD} > 10.0$ in several linkage groups (Table 4), thus providing strong evidence for the relevance of epistasis in the control of rust resistance in *Eucalyptus*. It is however difficult to validate the putative biological role of the epistatic loci. With the upcoming release of *E. grandis* reference genome, coupled to an e-QTL mapping approach (Kirst et al. 2004) for rust resistance, gene-level investigations of the genetic architecture of rust resistance in *Eucalyptus* are warranted.

In most eucalypt breeding programs, rust resistance has not been a trait specifically targeted by breeding until recently. Rust resistance has been usually assessed in the final stages of clonal trials, when susceptible clones are recommended for areas with no history of rust epidemics or simply discarded. With the expansion of clonal plantations rust epidemics have become more frequent. Several breeding programs in the tropics now specifically include rust resistance as a selection criterion, although no heritability estimates for rust resistance have been reported to date. In this study, we have provided an initial broad sense heritability estimate of 84% for rust resistance in an inter-specific cross showing a strong genetic control. We have also shown, however, that non-additive effects are at least as important as additive ones in the outcome of the disease phenotype. It is well known that breeding for epistatic interactions is difficult both in selfing and outcrossing species, even if heritability is high (Holland 2001, 2007; Mackay 2001). Modern *Eucalyptus* breeding programs, however, are based on a combination of hybrid breeding and clonal propagation of elite individuals, therefore capturing all the additive and non-additive effects (Grattapaglia and Kirst 2008). For this reason, epistasis does not represent a practical obstacle in breeding resistant clones, and this fact might also explain the success achieved so far in selecting rust-resistant clones for operational deployment.

Finally, the recognition of epistasis as an important contributor to rust-resistance variation in eucalypts

reveals the increased complexity of this host-pathogen interaction and provides starting points to look for the underlying genes or genomic regions controlling this phenotype on the upcoming *E. grandis* genome sequence.

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