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Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers

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Abstract Rust is one of the most-damaging eucalypt diseases in Brazil and is considered a potential threat to eucalypt plantations worldwide. To determine the mode of inheritance of resistance in the *Eucalyptus grandis*—*Puccinia psidii* pathosystem, ten full-sib families, generated from crosses between susceptible and resistant trees, were inoculated with a single-pustule isolate of the pathogen and rust severity was scored. The observed segregation ratios in segregating families suggested major gene control of rust resistance, although clearly incomplete penetrance, variable expressivity and minor genes are also involved in the global rust-resistance response. To identify markers linked to the resistance locus, screening of RAPD polymorphisms was conducted using bulked segregant analysis in a large full-sib family. A linkage group was built around the *Ppr*1 gene (*P. psidii* resistance gene 1) encompassing six RAPD markers, with a genetic window spanning 5 cM with the two most-closely linked flanking markers. Besides these two flanking markers, RAPD marker AT9/917 co-segregated with *Ppr*1 without a single recombinant in 994 meioses. This tightly linked marker should prove useful for marker-assisted introgression and will provide an initial lead for a positional cloning effort of this resistance allele. This is the first report of a disease resistance gene identified in *Eucalyptus*, and one of the few examples of the involvement of a major gene in a non-coevolved pathosystem.

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Introduction

Eucalypts are the most-widely planted hardwood trees in the world. Estimates in 2000 (FAO 2000) indicated that the eucalypt plantation area was globally greater than 17.8 million hectares with India being the largest planter with 8.0 million hectares, mostly in extensive low-productivity plantations, followed by Brazil with 3.0 million hectares and intensive hybrid-clonal plantations reaching average productivities of 45–60 m^3/ha/year (Mora and Garcia 2000). Elite hybrid clones involving *Eucalyptus grandis* are extensively used by the cellulose and paper industry, because of its wood quality, rapid growth and high volumetric yield. *E. grandis*, however, is one of the most-susceptible species to rust disease caused by *Puccinia psidii* Winter such that this disease is currently one of the most-damaging to *Eucalyptus* plantations in Brazil. *P. psidii* rust is also a potential threat in other tropical and subtropical areas with a natural or cultivated eucalypt (Coutinho et al. 1998). The disease occurs on seedlings in nurseries, on young trees in the field, on coppice and also on shoots in clonal gardens (Alfenas et al. 1997). Different provenances of *E. grandis* show high variability in response to rust inoculation under natural and greenhouse conditions (Ferreira and Silva 1982).

Although selection of resistant clones has been successful by the forest industries in Brazil, nothing is known about the inheritance of resistance in the *P. psidii–Eucalyptus* pathosystem. Knowledge on the genetic control of resistance is essential for establishing breeding strategies involving conventional as well as marker-assisted introgression of resistance genes into specialized breeding populations, currently composed mainly by susceptible individuals. In this paper, we provide genetic evidence for the segregation of a major gene involved in rust resistance in *E. grandis* and identify RAPD markers tightly linked to this locus.

Materials and methods

Plant material

Ten genetically divergent full-sib families with 115 to 120 plants each, obtained from crosses between susceptible and resistant trees of *E. grandis* were used. Susceptibility or resistance was determined previously by controlled inoculations (Alfenas et al. 1997). One segregating family (G38×G21) best fitting a 1:1 Mendelian segregation ratio for resistance expression, was selected for identification of molecular markers linked to a potential resistance locus. In this family, the initial number of 118 individuals tested was increased to 1,000 plants, to confirm the inheritance model and to allow the discovery of recombinants for fine-scale genetic mapping.

Inoculation and rating scale

Greenhouse-grown eucalypt seedlings (4–5 months old) were inoculated by spraying to run-off the upper and lower surface of the third and fourth apical leaves with 2×10^4 uredinio-spores/ml of a single pustule-isolate of *P. psidii* (UFV-2). This isolate was obtained from *E. grandis* at Itapetininga, São Paulo State, and is available upon request. Inoculated plants were incubated for 24 h in a mist chamber at 25°C in the dark and then transferred to a growth chamber at 22°C and a 12-h photoperiod, at 40 μM photons $m^{-2}s^{-1}$ light intensity.

Disease severity was evaluated 12 days after inoculation (d.a.i.), using a rating scale of rust-severity evaluation: S0 = immune or hypersensitive reaction (HR) with fleck or necrosis; S1 = smalt pustules, <0.8 mm diameter, with one or two uredinia; S2 = medium-size pustules, 0.8 to 1.6-mm diameter, with about 12 uredinia; and S3 = large pustules, >1.6-mm diameter, with 20 or more uredinia, on leaves petioles and or shoots. The segregating families were re-evaluated twice, more at 14 to 16 and at 24 d.a.i. Plants of S0 or S1 classes were considered resistant, and S2 and S3 were susceptible.

A χ^2 test was used to test the hypothesis of Mendelian inheritance of the disease phenotypes (S0 and S1 versus S2 and S3). Since one family (G38×G21) best fit the simple 1:1 Mendelian inheritance model, clonal ramets of the male parent, which proved to be heterozygous for resistance against isolate UFV-2, were further tested against 21 single-pustule isolates of *P. psidii* obtained from different hosts and regions in Brazil.

DNA isolation and identification of RAPD markers linked to a rust resistance locus

DNA extractions and RAPD marker were carried out as described previously (Grattapaglia and Sederoff 1994). The strategy for identifying RAPD markers linked to a rust resistance locus essentially followed Harkins et al. (1998) using the Bulk Segregant Analysis (BSA) technique (Michelmore et al. 1991). Within the selected (G38×G21) segregating family of 118 plants, the DNA of ten resistant S0 plants and ten susceptible S3 plants were extracted individually, and mixed in equimolar quantities. Thus two DNA bulks one from resistant plants (called R or the resistant bulk) and one from susceptible plants (called S or the susceptible bulk) were obtained. The two DNA bulks were screened for polymorphic RAPD markers using 980 arbitrary primers (OPA-01 to OPAZ-20 and OPAA-01 to OPAX-20; Operon Technologies). For fine genetic mapping, DNA of an additional set of 876 individuals of the same family was amplified using only those primers that generated tightly linked RAPD markers in coupling to the resistance gene in the initial set of 118 plants. Markers linked in coupling are those where the RAPD band co-segregates with the resistance allele. The linkage analysis between the rust resistance gene and the RAPD marker was done using a LOD score threshold of 3.0 and a recombination fraction of θ=0.4, as a limit for grouping the markers. The Kosambi mapping function was used to convert the recombination fraction to approximate map distances between the RAPD markers and the resistance gene. Nomenclature of the markers followed the convention of primer designation followed by the fragment size in base pairs (Grattapaglia and Sederoff 1994).

Results

Inheritance of resistance

Among the ten families challenged with isolate UFV-2, four were evaluated as susceptible, three fully resistant, and three segregated for rust resistance (Table 1). All three fully resistant families have tree G26 as a common

Table 1 Segregation of resistance to rust in full-sib families of *E. grandis* inoculated with a single pustule isolate of *P. psidii* and evaluated 12 days after inoculation

Family		Number of plants/severity class (S0:S1:S2:S3)	R:S[a]	Expected ratio[b]	χ^2	*P* value
1	(G38×G15)	0:0:34:81	0:115	0:1		
2	(G38ss×G15)	0:0:34:85	0:119	0:1		
3	(G38×G33)	0:4:44:71	4:115	0:1		
4	(G38×G45)	0:6:38:73	6:111	0:1		
5	(G9×G26)	108:11:1:0	119:1	1:0		
6	(G38×G26)	96:20:3:0	116:3	1:0		
7	(G38ss×G26)	98:12:7:1	110:8	1:0		
8	(G9×G35)	50:22:35:9	72:44	1:1	6.76	0.009
9	(G38×G21)	46:13:25:34	59:59	1:1	0.0	1.000
10	(G38ss×G21)	25:21:27:46	46:73	1:1	6.13	0.013
11	(G21×G21)	11:1:2:2	12:4	3:1	0.0	1.000

[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

Table 2 Segregation of resistance to rust in three families of *E. grandis* seedlings inoculated with a single pustule isolate of *P. psidii* (UFV-2) under controlled conditions and evaluated at three times after inoculation

Family		Days after inoculation	Plants/Severity class (SO:Sl:S2:S3)	R:S	Expected ratio	χ^2	*P* value
8	(G9×G35)	12	50:22:35:9	72:44	1:1	6.76	0.009
		16	43:20:19:34	63:53	1:1	0.86	0.353
		24	50:12:11:43	62:54	1:1	0.55	0.458
9	(G38×G21)	12	46:13:25:34	59:59	1:1	0.0	1.000
		14	32:23:23:40	55:63	1:1	0.54	0.461
		24	37:24:19:38	61:57	1:1	0.13	0.713
10	(G38ss×G21)	12	25:21:27:46	46:73	1:1	6.13	0.013
		15	22:24:24:49	46:73	1:1	6.13	0.013
		24	23:23:21:52	46:73	1:1	6.13	0.013

Table 3 Segregation of rust resistance in famity G38×G21 of *E. grandis* seedlings inoculated with a single pustule isolate of *P. psidii* (UFV-2) and evaluated 12 days and confirmed 24 days after inoculation

Family		Plants/Severity class (SO:Sl:S2:S3)	R:S	Expected proportion	χ^2	*P* value
9	(G38×G21)	269:212:165:354	481:519	1:1	1.44	0.229

parent, while all four susceptible ones have tree G38 or G38ss as the common parent. A selfed progeny of G21 showed a 3:1 segregation ratio of resistant to susceptible, thus indicating that it is indeed heterozygous for resistance at a single major-effect gene.

In family 8 involving tree G35, the segregation ratios observed also fit a simple Mendelian inheritance model at later evaluation dates (Table 2), suggesting that also in this genetic background there is at least a major gene for resistance to rust with dominant expression. The data indicate that a rust-resistance allele is inherited from trees G35 and G21, since trees G9 and G38ss in other crosses did not contribute to observable resistance. In family 10, also derived from a cross involving resistant tree G21, segregation of resistance did not fit a simple Mendelian model. An excess of susceptible plants in all the three evaluation dates was observed (Table 2), suggesting a more complex pattern of inheritance.

The major effect gene in family 9 was confirmed by the co-segregation analysis of a significantly larger number of progeny individuals (Table 3). Tree G21 was found resistant to all other 20 isolates of *P. psidii* (Table 4). In most cases the resistance was manifested as a hypersensitive response (HR) with fleck formation 12 d.a.i. that developed to very small necrotic patches in 22 to 24 d.a.i. (data not shown).

Table 4 Response of *E.grandis* tree G21 to different single pustule isolates of *P. psidii* obtained from different hosts and geographic areas of Brazil

Isolate	Host[a]	Origin	Severity class
1	*Eucalyptus*	Guaiba, RS	S0 (fleck)
UFV-2	*Eucalyptus*	Itapetininga, SP	S0 (fleck)
16	*Eucalyptus*	Guaiba, RS	S0 (fleck)
21	*Eucalyptus*	Aracruz, ES	S0 (fleck)
36	*Eucalyptus*	lpatinga, MG	S0 (fleck)
38	*Eucalyptus*	lpatinga, MG	S0 (fleck)
39	*Eucalyptus*	lpatinga, MG	S0 (fleck)
7116	*Eucalyptus*	lpatinga, MG	S0 (fleck)
8	Guava	Luiz AntÃnio, SP	S0 (fleck)
32	Guava	Santa Maria de JetibÃ¡, RS	S0 (fleck)
40	Guava	GuanhÃ£es, MG	SO (fleck)
42	Guava	Belo Oriente, MG	S0 (fleck)
15	Guava	Passo Fundo, RS	S0 (fleck)
3	Jaboticaba	LenÃ§Ã³is Paulista, SP	S0 (fleck)
5	Rose Apple	ViÃ§osa, MG	S0 (fleck)
7	Rose Apple	Mogi-GuaÃ§Ãº, SP	S0 (immune)
17	Rose Apple	Porto Alegre, RS	S0 (immune)
31	Rose Apple	BrasÃlia, DF	S0 (fleck)
13	Java plum	GuaÃba, RS	S0 (fleck)
19	Brazil cherry	Passo Fundo, RS	S0 (fleck)
20	Brazilian cherry	Porto Alegre, RS	S0 (fleck)

[a] Guava = *Psidium guajava* L.
Jaboticaba = *Myrciaria jaboticaba cautiflora* (C. Martius) O. Berg.
Rose Apple = *Syzygium jambos* (L.) Alston
Java plum = *Syzygium cumini* (L.) Skeels
Brazil cherry = *Eugenia uniflora* L.
Brazilian cherry = *Eugenia dombeyi* Skeels

Identification of RAPD markers linked to a rust resistance locus

Among 980 primers screened for polymorphism between the resistant and susceptible bulks of family G38×G21, 45 (4.6%) did not generate any amplification product; 868 (88.6%) generated only monomorphic bands in both bulks and 67 (6.8%) generated at least one polymorphic band. In a second screening step, of the 67 primers used with DNA of the individual plants composing the bulks, only 20 generated clearly polymorphic bands that co-segregated with the resistance phenotype. However only 13 primers were ultimately selected for mapping work. These primers yielded markers that relative to phenotype (susceptible versus resistant) showed five or less recombinants in 20 test-individuals.

These primers generated 13 markers in a single linkage group that also contained the rust resistance gene

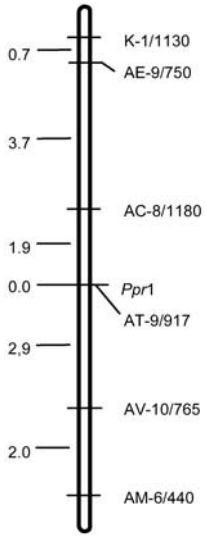

Fig. 1 Linkage group of RAPD markers in parent G21 around the *Ppr*1 gene, which confers *P. psidii* rust resistance in *E. grandis*. This map was constructed from co-segregation data between RAPD markers and rust resistance in 994 individuals of family G38×G21. Map distances are in centiMorgans (cM)

designated as *Ppr*1 (*P. psidii* resistance gene 1). Eight of the 13 markers were linked in coupling and five in repulsion with the *Ppr*1 gene. Out of the eight coupled markers, six were selected for a full-scale co-segregation analysis of the additional 882 individuals. As a result, a linkage group containing the *Ppr*1 gene with a genetic window of 11.2 cM was obtained (Fig. 1). Besides marker AT9/917 for which no recombinants were observed, markers AC8/1180 and marker AV10/765 closely flanked the resistance gene, bracketing it within a window of less than 5 cM.

Marker AT9/917 (Fig. 2) was the most informative as a potential marker to be used in marker-assisted introgression experiments, as it fully co-segregated with zero recombinants with the *Ppr*1 gene (Fig. 2). This amplified RAPD fragment was cloned and sequenced as a first step to develop a sequence-tagged-site marker. The sequence obtained did not show homology to any other sequence deposited in GenBank (Altschul et al. 1990).

Discussion

This is the first report of a disease resistance gene identified in *Eucalyptus* and one of the still relatively few examples among forest species (Benet et al. 1995; Devey et al. 1995; Cervera et al. 1996; Newcombe et al. 1996; Wilcox et al. 1996; Harkins et al. 1998; Tabor et al. 2000) as compared to crop plants. Moreover, the analysis carried out in this study, involved a number of families displaying different patterns of segregation for resistance. The observation of three fully resistant families, all of them derived from crosses involving tree G26 as a parent, suggests homozygosity of the resistance gene in this tree. The observation of the putative homozygous tree for resistance in a relatively limited sample of screened trees, further suggests that the frequency of this resistance allele in *E. grandis* populations is expected to be relatively high. The identification of homozygous resistant trees could have a great impact and immediate application in *Eucalyptus* breeding. Such trees would be used as females to generate resistant families even in open-pollinated seed orchards where seeds would be harvested only from these trees.

In spite of the observed variation in segregation for resistance, once plants were classified into susceptible and resistant data were consistent with the hypothesis of a major gene with Mendelian behavior. The phenotypic variation of response to rust in the various families studied suggests that such a major gene shows potentially incomplete penetrance and/or expressivity depending upon the genetic background, i.e. putative modifier genes of secondary effect. Plants with the major gene and a favorable combination of minor genes would show the resistance phenotype classified as S0, while plants with the major gene but with an unfavorable combination of minor genes would display a phenotype classified as S1 in the rating scale. For the same reason, plants without the major gene but with a favorable combination of minor genes would be expected to produce phenotype S2. Some of the plants containing genes that delay disease initiation or manifestation, could be erroneously classified as resistant, as observed in the first evaluation in family 8. The existence of such minor genes is also substantiated by the variation in the mean latent period of rust in other studies (Castro et al. 1985; Ruiz et al. 1989).

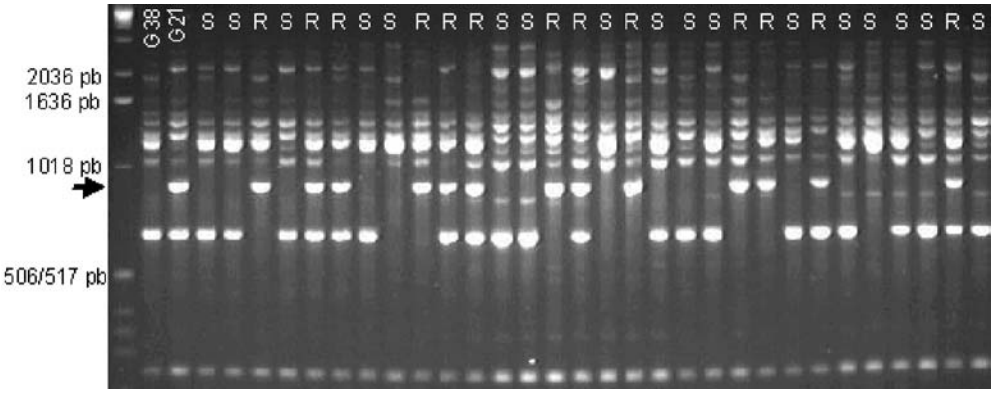

Fig. 2 DNA amplification with primer AT-9 in part of the family G38×G21. The first line is a 1-kb DNA Ladder size standard (Invitrogen). The second and third lanes are the susceptible and resistant parents. The *arrow* indicates marker AT9/917 that co-segregates with the *Eucalyptus* rust resistance locus. *R* = resistant and *S* = susceptible

Segregation for resistance in family 10, unlike family 9, did not support the hypothesis of one heterozygous major gene, despite the fact that both families shared the same male parent. Possibly, incomplete penetrance of the resistance allele in family 10 is due to the specific genetic background of tree G38ss. 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 *Populus trichocarpa* (Lefreve 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). Since the segregation data of a large number of plants in family 9 supports the hypothesis of a major-effect locus, designation of *Ppr*1 is proposed for this resistance gene derived from G21. Allelism tests should be carried out to verify if the same gene also controls resistance in G35.

Loci for rust resistance in forest species generally are either isolate or race-specific, such as the gene *Fr*1 in *Pinus taeda* (Wilcox et al. 1996), or resistance is of a wider spectrum, such as the gene *Mer* in *Populus* (Cervera et al. 1996). The response of G21 to different isolates of *P. psidii* suggests, however, that the resistance gene identified in *E. grandis* is not isolate-specific or that very little variation in pathogen virulence exists in the rust population. The wide-spectrum resistance of the parent tree G21 is of great relevance for breeding programs of *E. grandis* in Brazil and worldwide. However, because eucalypt forests are established predominantly in large clonal plantations, the use of a unique source of resistance to rust is certainly not an adequate long-term strategy for management of this disease considering the possibility of natural selection for virulent isolates (Pinon et al. 1987; Steenackers et al. 1994; McIntosh and Brown 1997) and also due to existence of physiological specialization in *P. psidii* (Castro et al. 1985; Xavier et al. 2001). New sources of resistance should be looked for and incorporated into *Eucalyptus* breeding programs.

Plant-pathogen interactions conferring resistance through single genes are usually interpreted as a gene-for-gene relationship (Flor 1971). Such a type of relationship usually results from co-evolution of the pathogen and the host, resulting in a high degree of specialization in the majority of pathogens. This premise, however, does not appear to be valid in the *P. psidii—Eucalyptus* pathosystem. *Eucalyptus* originated in Australia and neighboring islands. lt was introduced into Brazil for commercial purposes by the end of the 19th century. *P. psidii*, on the other hand, is native to South America and attacks various species of native or exotic *Myrtaceae*. Up to now, this pathogen has not been found in Australia where *Eucalyptus* grows naturally (Coutinho et al. 1998).

The major resistance gene identified in the *P. psidii—Eucalyptus* pathosystem is similar to monogenic resistance genes found in *Pinus* and *Populus* that did not co-evolve with the pathogen. The resistance in these cases can be conferred by genes of resistance to similar pathogens found in the centers of origin of the host. The resistance to *C. ribicola* identified in *P. lambertiana* (Devey et al. 1995) appears to be the first case of a non-co-evolved pathosystem expressing monogenic resistance. At the same time there is a history of co-evolution between the genus *Pinus* and the genus *Cronartium* that goes back to their ancestors in the northern part of Central Asia (Leppik 1970). This is not the case with the *Eucalyptus—P. psidii* pathosystem. A plausible explanation in this case is that gene *Ppr*1 confers resistance to other pathogens in the center of origin. Recent studies indicated that the same gene could confer resistance to different species of organisms, as the *Mi* gene in tomato conferring resistance to *Meloidogyne incognita* and aphids (Rossi et al. 1998).

The combination of RAPD markers and the BSA approach proved to be very useful for the identification of several markers linked to the *Ppr*1 gene of *E. grandis*, in tree G21. This methodology has also been used for mapping resistance genes in other forest species, such as the *Lrd*1 locus in *P. deltoides* (Tabor et al. 2000), the gene *R* in *P. lambertiana* (Devey et al. 1995), the gene *Fr*1 in *P. taeda* (Wilcox et al. 1996) and a resistance gene in Chinese elm (*Ulmus parvifolia*) (Benet et al. 1995). The BSA technique was also used in the identification of AFLP markers linked to the gene *Mer* in hybrids of *P. deltoides* × *Populus nigra* L. conferring resistance to diverse races of *M. larici-populina* (Cervera et al. 1996).

Marker AT9/917 showed complete genetic linkage to the *Ppr*1 gene. This RAPD marker could be converted into a SCAR (Sequence Characterized Amplified Region) (Paran and Michelmore 1993) to allow targeted screening for the presence of the resistance allele by PCR. This approach has been used in some vegetable crops (e.g. Martin et al. 1993), and in forest species when attempting to clone the locus *Mmd*1 of *P. trichocarpa* (Stirling et al. 1999; Tabor et al. 2000; Zhang et al. 2001). However, although a SCAR marker could prove useful to search for new allelic variation at the linked *Ppr*1 gene, the success of this approach will depend on the detection of polymorphism at the SCAR marker, which is usually low. A more promising approach is to map a number of fully transferable and hypervariable microsatellite markers (Brondani et al. 1998, 2002) close to AT9/917 in tree G21, and then use a pair of closely linked microsatellites as an efficient screening tool for new allelic variation at the *Ppr*1 gene in other trees.

Based on the size of the segregating family used in this study to map the linked RAPD marker, the maximum-estimated possible distance between AT9/917 and *Ppr*1 is of 0.462 cM (α=0.01). Although the relationship between map distance and physical distance is typically non-linear, if we consider that in *E. grandis* a genetic distance of 1 cM corresponds to a mean physical distance of 395 kb (Grattapaglia and Bradshaw 1994), the AT9/917 marker may be located within a physical distance of 200 kb from *Ppr*1. This distance is within the limits of insertions cloned in Bacterial Artificial Chromosome (BAC) vectors. Thus positional cloning of a genomic region that

contains *Ppr*1 should be successful using a relatively small series of superimposed BACs starting from a BAC that contains RAPD markerAT9/917 amplified specifically from tree G21.

The cloning and eventual generation of a transgenic eucalypt with this gene would confirm its biological function in rust resistance response, and contribute to a better understanding of the molecular basis of rust resistance in *Eucalyptus* and trees in general after a comparative analysis with other disease resistance genes cloned from annual plants.

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