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ORIGINAL PAPER

Genetic and physical localization of an anthracnose resistance gene in *Medicago truncatula*

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Abstract Anthracnose of alfalfa, caused by the fungal pathogen *Colletotrichum trifolii*, is one of the most destructive diseases of alfalfa worldwide. An improved understanding of the genetic and molecular mechanisms underlying host resistance will facilitate the development of resistant alfalfa cultivars, thus providing the most efficient and environmentally sound strategy to control alfalfa diseases. Unfortunately, cultivated alfalfa has an intractable genetic system because of its tetrasomic inheritance and out-crossing nature. Nevertheless, the model legume *Medicago truncatula*, a close relative of alfalfa, has the potential to serve as a surrogate to map and clone the counterparts of agronomically important genes in alfalfa—particularly, disease resistance genes against economically important pathogens. Here we describe the high-resolution genetic and physical mapping of *RCT1*, a host resistance gene against *C. trifolii* race 1 in *M. truncatula*. We have delimited the *RCT1* locus within a physical interval spanning ~200 kb located on the top of *M. truncatula* linkage group 4. *RCT1* is part of a complex locus containing numerous genes homologous to previously characterized TIR-NBS-LRR type resistance genes. The result presented in this paper will facilitate the positional cloning of *RCT1* in Medicago.

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Introduction

Alfalfa (*Medicago sativa* L.) is the most important and widely grown forage legume worldwide. In the United States, alfalfa ranks third in dollar value after corn and soybeans (USDA Crop Values, 2005 Summary). In addition to providing highly nutritious hay and pasture for animal and dairy production, alfalfa is also an integral component of crop rotations because of its capacity for symbiotic nitrogen fixation, underlying its importance as a source of nitrogen in natural and agricultural ecosystems.

Alfalfa is susceptible to numerous damaging pests and pathogens, causing significant losses in forage production (Nutter et al. 2002). An improved understanding of genetic and molecular mechanisms underlying host resistance will facilitate the development of resistant cultivars, thus providing the most efficient and environmentally sound strategy to control alfalfa diseases. Unfortunately, cultivated alfalfa has an intractable genetic system because of its autotetraploid ($2n = 4x = 32$) and out-crossing nature. The model legume *Medicago truncatula*, a close relative of alfalfa, has the potential to serve as a surrogate for genetic analysis of disease resistance in alfalfa and to provide new sources of host resistance (Zhu et al. 2002).

Medicago truncatula (barrel medic) is native to the Mediterranean basin and has long been cultivated as winter forage in Australia. It was chosen as a model legume because of its annual habit, diploid ($2n = 2x = 16$) and self-fertile nature, short lifecycle (2–3 months), ample seed production, relatively small genome (~500 Mb), abundant natural variation, and close phylogenetic relationships to the major crop legumes (Cook 1999). In the past decade, abundant genetic and genomic tools and resources have been developed for this model legume (Vandenbosch and Stacey 2003; Young et al. 2005). Since *M. truncatula* and alfalfa

share many common pathogens (Yaege and Stuteville 2000, 2002; O'Neill and Bauchan 2000; O'Neill et al. 2003; Vandemark and Grünwald 2004; Tivoli et al. 2006), it is potentially feasible to clone resistance genes in *M. truncatula* that are active against alfalfa pathogens. Due to the close phylogenetic relationship between the two species, it is likely that functional resistance genes can be moved across species boundaries by transgenic approaches (Zhu et al. 2002).

Anthracnose of alfalfa, caused by the fungus *Colletotrichum trifolii*, is one of the most destructive diseases of alfalfa worldwide. The disease causes lesions on stems and leaves, and in advanced stages results in crown and root rot which eventually kills the plant (Stuteville and Erwin 1990). Severe infection in susceptible alfalfa varieties can cause up to 25–30% losses in forage yield as well as losses in plant stand and vigor (Barnes et al. 1969). Two races of *C. trifolii*, races 1 and 2, were identified in North America (Ostazeski et al. 1979). Resistances to the two races in alfalfa were reported to be controlled by two dominant genes, *An1* and *An2* (Elgin and Ostazeski 1985). *An1* conditions resistance to race 1, whereas *An2* confers resistance to both race 1 and race 2. Race 3 of *C. trifolii* was reported in 1982 (Allen et al. 1982), but this fungus was subsequently reported to be likely *C. destructivum* (O'Neill 1996b). Most recently, a new *C. trifolii* race, named race 4, was identified in Australia and in the US (Mackie et al. 2003; Ariss and Rhodes 2006). *An1* may also confer resistance to race 4 (Mackie et al. 2003, 2007). Few follow-up studies were performed on mapping and characterization of *An1* and *An2* in alfalfa. Mackie et al. (2007) recently described mapping of quantitative trait loci (QTLs) that condition resistance to the *C. trifolii* races 1, 2, and 4 in autotetraploid alfalfa.

Defense responses in the alfalfa-*Colletotrichum* pathosystem include both hypersensitive reactions (incompatible interactions) and induced resistance mechanisms (compatible interactions) (Esquerré-Tugayé et al. 1992; O'Neill 1996a). Resistance was also associated with the production of pterocarpan and isoflavonoid phytoalexins following fungal infection in both compatible and incompatible interactions (O'Neill 1996a; Salles et al. 2002). Several genes required for fungal pathogenicity were isolated from *C. trifolii* (Dickman 2000; Dickman et al. 2003), but little is known about how host resistance genes recognize the pathogen and trigger resistance responses. Cloning and characterization of the host resistance genes will help to gain a better understanding of the process of host recognition and to develop novel mechanisms for disease control.

Detailed characterization of the *M. truncatula–C. trifolii* pathosystem revealed that the infection process and resistant reactions were similar to those observed in alfalfa and other annual *Medicago* species (Mould and Robb 1992; O'Neill and Bauchan 2000; Torregrosa et al. 2004). The resistance of Jemalong, a *M. truncatula* genotype, to *C. trifolii* race 1 was associated with a hypersensitive response (HR) and likely controlled by a single dominant gene (Torregrosa et al. 2004). In this report, we describe the high-resolution genetic and physical mapping of *RCT1*, a host resistance gene against *C. trifolii* race 1 in *M. truncatula*. We delimited the *RCT1* locus within an ~200 kb interval located on one end of the *M. truncatula* linkage group 4. *RCT1* is part of a complex locus containing numerous genes homologous to previously characterized TIR-NBS-LRR type resistance genes. The result presented here will facilitate the cloning of *RCT1* in Medicago.

Materials and methods

The mapping population

An F2 mapping population was derived from the cross between the two *M. truncatula* genotypes Jemalong A17 (resistant) and F83005.5 (susceptible). Seedlings of parents and the segregating population were grown in a growth chamber with a 16 h light, 23°C/8 h dark, 20°C regime for about 6 weeks before inoculation.

Disease reaction assay

Colletotrichum trifolii Bain and Essary race 1 (isolate 2sp2), as determined with alfalfa cultivars, was kindly provided by Dr. Nichole O'Neill (USDA-ARS, Beltsville, MD, USA). Mycelium was routinely grown on ANM plates (malt extract 2%, bactopeptone 0.1%, glucose 2%, and agar 2%) in the dark at 23°C in Petri dishes. Conidia were produced after a week at 23°C on YPSS medium. Spores were collected and washed three times in sterile water with the final concentration being adjusted to 2×10^6 spores per ml. The six-week-old plants were inoculated by injection of spores into the stems of living plants using a latex free syringe with a thin needle (0.4 mm × 13 mm) (1 ml 27G1/2, Becton Dickinson & Co.) (Ostazeski and Elgin 1982; Mackie et al. 2003, 2007). At least two stems of each plant were inoculated. Inoculated plants were then transferred to a growth chamber programmed for a 16 h light, 23°C/8 h dark, 20°C regime with >90% humidity. Symptoms were recorded 7 days post inoculation. The plants were scored as either resistant (no symptom) or susceptible (stem collapse).

Marker development and genetic mapping

Initially, we mapped SSR (simple sequence repeat) markers with known genetic position to localize the approximate position of *RCT1*, according to the procedures described by

Mun et al. (2006). Additional markers were then developed from ESTs (expressed sequence tags) and BAC (bacterial artificial chromosome) sequences that were mapped close to the *RCT1* locus (Zhu et al. 2002; Choi et al. 2004; Mun et al. 2006). Markers were based on SNPs (single nucleotide polymorphisms) identified between the two parents, which were converted to CAPS (cleaved amplified polymorphic sequences) markers, as described elsewhere (Zhu et al. 2002; Choi et al. 2004). Only susceptible plants (homozygous recessive for the susceptible alleles) were used for genetic mapping. The initial mapping population consisted of 93 susceptible F2 plants. The size of the mapping population was increased to include 390 susceptible individuals for fine mapping. Genetic map was constructed using the software MAPMAKER version 1.0 (Lander et al. 1987). All markers described in this paper are listed in Table 1.

Physical mapping and sequence analysis

For this experiment, we took advantage of the database for the integrated genetic and physical map of the *M. truncatula* genome (Mun et al. 2006; http://www.medicago.org). The genomic and BAC-end sequences allowed us to directly anchor the mapped markers onto the BAC contigs. Sequencing of BACs H2-144L3 (AC203223) and H2-152N14 (AC203224) were carried out at the Advanced Center for Genome Technology, Department of Chemistry and Biochemistry, University of Oklahoma. Gene prediction was performed using the FGENESH program (Solovyev and Salamov 1997). Domains were predicted using Pfam 21.0 (Bateman et al. 2004) with an initial *E* value cutoff of 0.1. Sequence alignments and phylogenetic analysis were performed using the ClustalX (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbor-joining method as implemented in ClustalX with 1,000 bootstrap sampling steps.

Results

Disease reaction assay and segregation analysis

The ability to unambiguously distinguish between resistant and susceptible phenotypes is crucial for accurate mapping and subsequent positional cloning of a disease resistance gene. To assay for disease resistance and susceptibility, we used an inoculation method based on the injection of inoculum into the stems of living plants (Ostazeski and Elgin 1982; Mackie et al. 2003, 2007). This inoculation technique allowed for unequivocal differentiation between resistant and susceptible phenotypes. Seven days post inoculation, the inoculated stems of the susceptible genotype

Table 1 Molecular markers described in this study

Marker name	Template sequence accessions no.	Marker type	Restriction enzyme	A17 restriction pattern of CAPS	F83005.5 restriction pattern of CAPS	Forward primer	Reverse primer
MtB331	AC144503	SSR	N/A	N/A	N/A	GGCTTCCTGATGCTGGTTAG	ACAAGCAGGTTGGACACACA
MtB99	AC127674	SSR	N/A	N/A	N/A	CTTGGCAAAATGTCAACTCT	GGAAAGGGGTTAGGTGAGTA
AW257289	AW257289	CAPS	*Bsm*AI	341 + 137	478	CTTCGGACCTTCAGCAAAACACAG	CGGGTGACAGATTATTTGGTGACATC
CAP20	AC124959	Dominant	N/A	N/A	N/A	GGTGCTATTTTTCTTTGAAGTGTGT	TTTCGAAGAAGCTGAACTTAGTTGT
CAP25	AC140914	CAPS	*Hin*fI	518 + 46	394 + 170 + 46	AAATTCACTCCTAAACAACCAGCTAAGT	CCGGTATAACAACATTAATTCACACTTC
CAP29	AC165943	Dominant	N/A	518	0	AGTGTTGGTTGGCAGGATCT	TGCTTTGAAACCTGCACACT
CAP30	AC138016	CAPS	*Dra*I	156 + 400	556	AAATATGTGAACCAAAATTGAAGGA	TACTTAGACGGCCAAAACAATTAAG
61P8L	CG952991	Dominant	N/A	463	0	AAGTATTGCAAGATTCTTTGGATTG	AGTCATTTTCCTGACTTCACCATAG
71O16R	CG959738	CAPS	*Stu*I	249 + 78	327	GGCCTATAAGTAGGCTTGCAG	ATGGCTCTGGCTGCTGTTAG
71O16L	CG959746	CAPS	*Bbv*I	312 + 140	452	ATTCTATGTCCCGTAAGTTTCTGC	CCTCTGATTGGCTTTCATTTACTT
61P8-R	CG928897	CAPS	*Nla*III	307 + 180 + 32 + 26	190 + 190 + 117 + 32 + 26	AGAGCCTCCTAGTTGTGATCTTTTT	AAAGTACAACTTCAATACCCATCCA
81B21R	CG929447	CAPS	*Dra*I	436 + 190	626	CAATACCAGTTCCATACCCATACAT	CTTTTCAACAAGCAAGAGTGATACA
h2_119h6a	AC149473	SSR	N/A	N/A	N/A	CGCACGAGTTGGATATGATG	CGTCGCACGAGTTTACTGAT
h2_13m22a	AC164520	SSR	N/A	N/A	N/A	TCAAACTCAAGCCACCACAA	GCTCGAGTCATGGAGGGTAA

(F83005.5) formed large lesions at the inoculation site and collapsed with severe anthracnose symptoms, while the inoculated stems of the resistant genotype (Jemalong A17) grew normally and were completely symptomless. The resistance was clearly associated with a hypersensitive response (HR) at the inoculation site in which infected host cells underwent rapid cell death and further fungal colonization was arrested. In contrast, the fungus can successfully colonize on susceptible stems with well-developed dark acervuli. Support of this observation was the fact that abundant spores could be collected from the inoculated sites of the susceptible plants, while sporulation never occurred from the inoculation sites of resistant plants (data not shown). Consistent with the Koch's rules, the spores collected from susceptible plants successfully re-infected and colonized susceptible parental lines and exhibited similar anthracnose phenotypes. The advantage of this inoculation technique was its consistency to cause the break off and subsequent death of the inoculated stems of susceptible plants, but not causing such symptoms for the inoculated stems of resistant plants. Through this assay, we were able to score each of the F2 individuals as either resistant or susceptible.

The resistant genotype Jemalong A17 and the susceptible genotype F83005.5 were crossed to produce an F2 mapping population. Initial analysis of 231 F2 plants identified 51 susceptible and 180 resistant individuals. The segregation of resistance and susceptibility fits 3:1 ratio ($\chi^2 = 1.12$, $df = 1$, $P = 0.29$), suggesting that a single dominant gene controls the anthracnose resistance in Jemalong A17. The resistance gene in Jemalong A17 was named as *RCT1* (for resistance to *Colletotrichum trifolii* race 1).

Genetic mapping of the *RCT1* locus

Of the F2 mapping population, we only selected individuals that were susceptible to pathogen infection for genetic mapping of the *RCT1* locus. One advantage of this strategy was that the susceptible plants were homozygous recessive for the susceptible alleles (*rct1*/*rct1*) and thus were more informative to detect recombination events, while the resistant plants can be either homozygous (*RCT1*/*RCT1*) or heterozygous (*RCT1*/*rct1*). Furthermore, selection of the susceptible plants for genetic mapping avoided the possible experimental errors that might occur during the phenotyping process, because a susceptible plant was surely susceptible, while it was possible, though unlikely, that a plant scored as resistant was indeed susceptible due to escape of infection.

As a first step to localize the *RCT1* locus, our strategy was to map selected SSR markers that were evenly distributed among the eight linkage groups of the *M. truncatula* genome (Mun et al. 2006). These SSRs were originally developed from the sequenced BAC clones and have been used to integrate genetic and physical map of Jemalong A17, the reference genotype of the *M. truncatula* genome project and the resistant parent of this study (Mun et al. 2006). During the mapping process, we gave preferences to the SSR markers that were linked to the clusters of resistance gene homologs (Zhu et al. 2002; Mun et al. 2006).

Initial mapping of a base population comprising 93 susceptible individuals in a 96-well PCR plate (also included are three DNA samples from the two parents and an F1 plant) identified four SSR markers, MtB99, H2-119H6a, H2-13M22a, and MtB331, on the top of linkage group 4 that were linked to the *RCT1* locus. Thus, our further effort has been focused on developing and mapping molecular makers that were mapped around the *RCT1* region in the F2 population from the cross of Jemalong A17 × A20 (Zhu et al. 2002; Choi et al. 2004; Mun et al. 2006). Through this process, we were able to identify three SNP-based markers, CAP25, CAP30, and AW257289 that were closely linked with the *RCT1* locus (Fig. 1a). In particular, AW257289, an EST-based marker, was co-segregated with the *RCT1* locus, while a single recombinant event was detected between CAP25 and *RCT1* and between CAP30 and *RCT1*. Based on this initial mapping experiment, we inferred that the *RCT1* is located between the markers CAP30 and CAP25.

Fine mapping and physical localization of the *RCT1* locus

For the purpose of accurate delimiting the *RCT1* locus relative to the closely linked flanking markers, we increased the mapping population to include 390 susceptible F2 plants. Despite the use of a larger mapping population, we were not able to detect any recombination events between AW257289 and *RCT1*, while nine and five recombinants were identified between *RCT1* and CAP25 and between *RCT1* and CAP30, respectively (Fig. 1a). This observation indicated that AW257289 is tightly linked to the *RCT1* locus. We therefore decided to use AW257289 as a query to electronically search for *M. truncatula* BAC clones that harbor AW257289 and to initiate physical mapping of the *RCT1* locus.

The availability of a high-throughput (~20×) physical map and abundant genomic, BAC-end and EST sequence information in *M. truncatula* offered an in silico approach to physically localize the *RCT1* locus. Multiple-step "sequence walking" through BLAST searching of the *M. truncatula* Gene-index (http://compbio.dfci.harvard.edu/tgi/) and the NCBI BAC-end sequence (Genome Survey Sequence or GSS) database allowed us to anchor the contiguous sequence of AW257289 onto one end of the *M. truncatula* BAC clone H2-152N14. Searching the *M. truncatula* physical map

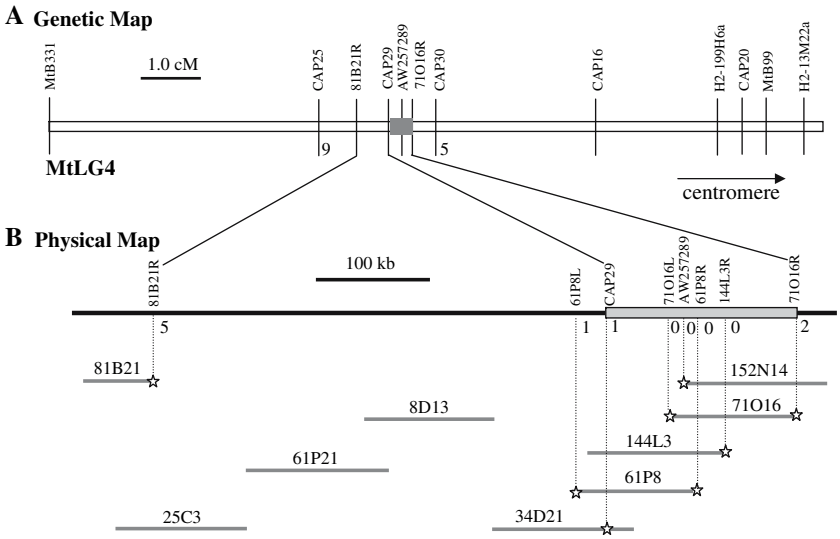

Fig. 1 Genetic and physical mapping of the *RCT1* locus. **a** Genetic map of the *RCT1* region. *RCT1* is located on one end of the *M. truncatula* molecular linkage group 4. The position of the *RCT1* gene was delimited to an ~0.4 cM region between markers CAP29 and 71O16R (as indicated by the *solid box*). **b** Physical map of the *RCT1* locus. The BAC contig covers the genetically defined interval containing *RCT1* (as indicated by the *open box*). *Numbers* indicate the number of recombination breakpoints separating the marker from *RCT1*. The maps are drawn to scale

database (http://www.medicago.org) using the BAC ID H2-152N14 as a query enabled us to assign H2-152N14 onto to a single BAC contig of ~700 kb (contig 1357; Fig. 1b).

The BAC-end sequences as well as high-throughput sequences from several BAC clones of this contig (e.g., H2-61P21, H2-8D13, and H2-34D21) served as templates to develop new SNP markers for fine mapping of this physically defined region. Genotyping the 390 susceptible plants did not reveal any recombination events between *RCT1* and markers developed from the BAC ends H2-71O16L, H2-152N14L (AW257289), H2-61P8R, and H2-144L3R. Nonetheless, we observed one common recombinant event between *RCT1* and the markers developed from the BAC end H2-61P8L and the BAC sequence of H2-34D21 (CAP29). Furthermore, two independent recombination events were also detected between H2-71O16R and *RCT1*. Therefore, we determined that the *RCT1* is located between 71O16R and CAP29 that span ~200 kb. We have now completely sequenced the BACs H2-144L3 (AC203223) and H2-152N14 (AC203224) that cover the 200-kb interval, from which 16 genes were identified (Table 2). Five of the predicted genes are members of NBS-LRR gene family as described below.

RCT1 region is rich in NBS-LRR genes

The majority of plant disease resistance (*R*) genes identified to date belong to the nucleotide binding site (NBS)-leucine rich repeat (LRR) family (Hulbert et al. 2001). NBS-LRR genes can be further divided into two subfamilies based on their N-terminal structural domains, namely, TIR-NBS-LRR and CC-NBS-LRR (Meyers et al. 1999). Annotation of the ~570-kb contig assembled from the BAC sequences of H2-61P21, H2-8D13, H2-34D21, H2-144L3, and H2-152N14 identified 16 NBS-LRR genes of the TIR-type. Ten of the 16 predicted genes contain complete open reading

Table 2 Predicted genes in the *RCT1*-region

Homology	Copy no.	*E* value
Translation elongation factor (*Arabidopsis*)	1	0.0
Aberrant lateral root formation 4 (*Arabidopsis*)	1	5e-90
AT hook motif-containing protein-related (*Arabidopsis*)	1	0.0
Nucleic acid-binding, OB-fold (*M. truncatula*)	1	0.0
Protein kinase family protein (*Arabidopsis*)	1	e-142
mRNA capping enzyme family protein (*Arabidopsis*)	2	2e-89
Defective in exine formation 1 (*Arabidopsis*)	1	0.0
NBS-LRR homologs		
(*M. truncatula*)	5	0.0
Hypothetical protein (*Arabidopsis*)	1	3e-54
Proton-dependent oligopeptide transport (POT) family protein (*Arabidopsis*)	1	0.0
Copper ion binding/oxidoreductase (*Arabidopsis*)	1	0.0

frames (ORFs), while the remaining are truncated genes lacking either a TIR or LRR domain. The distribution of the predicted NBS-LRR genes was indicated in Fig. 2a. This set of NBS-LRR genes formed a monophyletic clade in the phylogenetic tree consisting of ~200 *M. truncatula* TIR-NBS-LRR genes (S.B. Cannon, personal communication), indicating a recent common ancestor of this gene cluster. Phylogenetic analysis of the ten complete TIR-NBS-LRR (TNL) genes revealed two minor clades (Fig. 2b). Overall, the phylogenetic distances are correlated with physical proximity, suggesting that independent gene duplication has played a role in radiation of this gene cluster. In particular, the 200-kb interval, where the *RCT1* was predicted to be located, contains five tandem duplicated NBS-LRR genes (l to p, Fig. 2a), three of which (l, m, and p) contain complete ORFs and share ~80% identity with each other at amino acid level. These three genes serve as the strong candidates of *RCT1*.

Discussion

In the present study, we finely mapped the *RCT1* locus in *M. truncatula* that confers resistance to *C. trifolii* race 1. Our ability to accurately delimit the *RCT1* locus within a small physical interval was attributed to the use of the stem injection inoculation method (Ostazeski and Elgin 1982; Mackie et al. 2003, 2007). This inoculation technique resulted in qualitative disease reactions and thus allowed reliable discrimination between resistant and susceptible phenotypes in the F2 mapping population. However, the use of spray inoculation or detached leaf assay on the same genotypes caused quantitative disease responses (Torregrosa et al. 2004; Mackie et al. 2007). This difference could be due to differential resistance mechanisms performed by the plants when inoculated by different inoculation methods (Mackie et al. 2007). Stem injection inoculation by-passed the pre-penetration and penetration process, thus likely resulting in only gene-for-gene type responses (Dickman et al. 2003; Mackie et al. 2007). In contrast, the disease reactions from spray inoculation and detached leaf assay might also involve genes and/or environmental and physiological factors associated with penetration events and pathogenecity. It was reported that the resistance response to *C. trifolii* occurred at the time of penetration of the cuticle and epidermal cell by the penetration peg (Churchill et al. 1988). Dickman et al. (2003) isolated a lipid-induced protein kinase (LIPK) from *C. trifolii*, which was specially induced by plant cutin. The LIPK was required for appressorium formation, and the mutants of LIPK were unable to

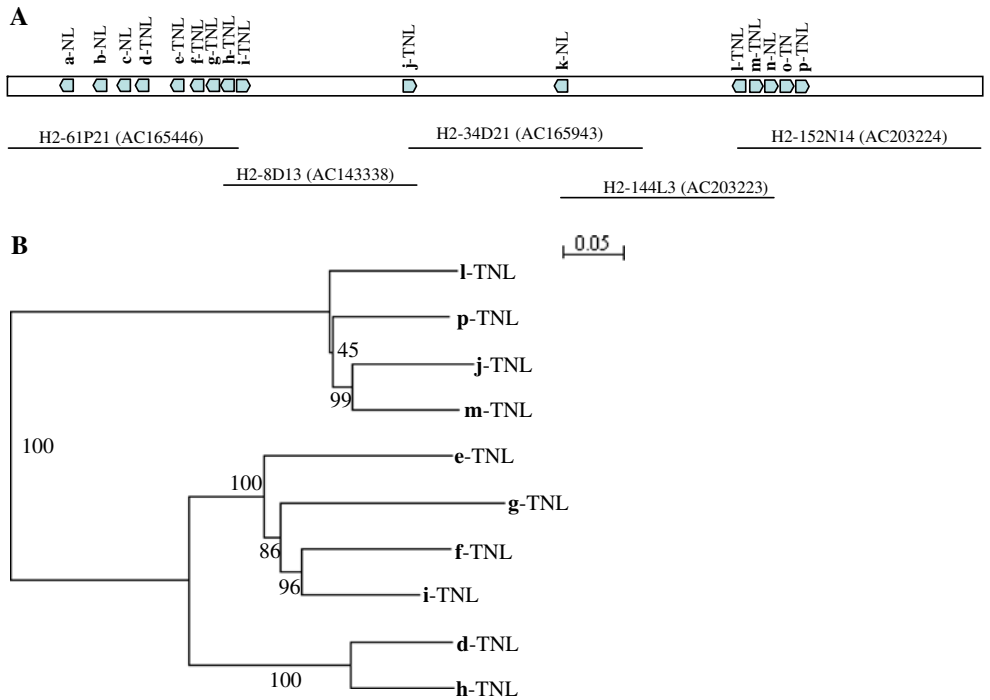

Fig. 2 Distribution and phylogeny of NBS-LRR genes in the *RCT1* region. **a** Organization of the NBS-LRR genes around the *RCT1* locus. Only 16 predicted NBS-LRR genes (represented by *letters* a through p) are shown. The orientations of the genes are indicated by *arrows*. *TNL* TIR-NBS-LRR; *NL* NBS-LRR lacking a TIR domain; *TN* TIR-NBS lacking a LRR domain. **b** Phylogeny of TIR-NBS-LRR genes in the *RCT1* region. Phylogenetic analyses of protein sequences were performed using the ClustalX program (Thompson et al. 1997), and trees were constructed using the neighbor-joining method. Numbers are the percentage of 1,000 bootstrap replications supporting the particular nodes

infect intact host tissue, but able to colonize host tissue following artificial wounding (Dickman et al. 2003). Despite the quantitative reactions observed by spray inoculation and detached leaf assay, it was evident that there exist major genes (or QTLs with major effect) conditioning resistance response to the three *C. trifolii* races (i.e., races 1, 2 and 4) (Torregrosa et al. 2004; Mackie et al. 2007). Detailed genetic and physical mapping of the *RCT1* locus described in this paper supports the hypothesis that the resistance to *C. trifolii* race 1, as determined by the stem injection inoculation, is controlled by a single dominant gene in *M. truncatula*.

The *RCT1* locus mapped to a region on the top of the *M. truncatula* linkage group 4 that contains numerous genes related to previous characterized TIR-NBS-LRR type *R* genes. The *RCT1* region is apparently gene rich. Based on annotation of ~570-kb contig, the gene density is about one gene per 5.2 kb, which is much higher than the estimated overall gene density for the genespaces in *M. truncatula* (~7.9 kb/gene) (Cannon et al. 2006). In particular, the 200-kb interval spanning H2-144L3 and H2-152N14, where the *RCT1* gene was predicted to be located, contains three complete TIR-NBS-LRR genes. This observation suggests that the *RCT1* might also encode an NBS-LRR type resistance protein.

The association between NBS-LRR genes and QTLs conferring resistance to *Colletotrichum* species was also reported in other plant species (Ferrier-Cana et al. 2003; Abad et al. 2006). In *Phaseolus vulgaris*, QTLs associated anthracnose resistance against *C. lindemuthianum*, a closely related species of *C. trifolii*, were mapped to a cluster of CC-NBS-LRR genes on one end of the common bean linkage group B4 (Ferrier-Cana et al. 2003). In maize, a gene conferring resistance to anthracnose stalk rot, caused by *C. graminicola*, also encodes a CC-NBS-LRR protein (Abad et al. 2006). Taken together, there is strong evidence that NBS-LRR genes confer gene-for-gene type resistance to *Colletrtrichum* species in diverse plant hosts.

Recently, Mackie et al. (2007) reported the mapping of QTLs responsible for resistance to the three races (races 1, 2 and 4) of *C. trifolii* in alfalfa. Disease reaction assay was performed by both spray and stem injection inoculation. The plants were scored as either resistant or susceptible for the stem injection inoculation, as described in this study, while a disease index of 1–5 were used for spray inoculation. Despite the use of different scoring system, a single locus for the injection assay and the strongest QTL for the spray assay were co-incident on a *M. sativa* group linkage that appeared to be homologous to *M. truncatula* linkage group 8, based on mapping two *M. truncatula* SSR markers 36b12e and 115m15b in alfalfa (Mackie et al. 2007). It is uncertain whether the *RCT1* in *M. truncatula* is orthologous to the locus mapped in *M. sativa*. It was reported that a chromosomal translocation event occurred between chromosomes 4 and 8 in the *M. truncatula* ecotype Jemalong A17 (Kamphuis et al. 2007). Further work is needed to determine the functional and evolutionary relationship between the *RCT1* locus in *M. truncatula* and the locus described by Mackie et al. (2007). This can be done by mapping the candidate *RCT1* gene of *M. truncatula* in alfalfa as well as by mapping more *M. truncatula* markers in alfalfa that are closely linked to the mapped resistance locus.

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