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Prehaustorial resistance against alfalfa rust (*Uromyces striatus*) in *Medicago truncatula*

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Abstract

The legume species *Medicago truncatula* is gaining interests as a plant for structural and functional genomics that can be used to identify agronomically important genes in crop legumes. Resistance to the alfalfa rust (*Uromyces striatus*) was studied in a germplasm collection of *M. truncatula*. Accessions varied in resistance, as expressed by disease severity, but none showed macroscopically visible necrosis. Histological investigations, in selected lines covering the whole range of resistance reactions, revealed little difference in spore germination and none in orientation of germtubes on the leaf surface. However, appressorium formation on the stoma was significantly reduced in some accessions. Differences in resistance among accessions were more evident once the stoma were penetrated by the infection structures. Resistance was mainly due to a restriction of haustorium formation with varying levels of early abortion of the colonies, a reduction in the number of haustoria per colony, and hampered colony growth. In addition, necrosis of the host cells associated with infection hyphae was detectable in some accessions from the beginning of colony development. This information will be useful for eventual mapping and cloning analyses of resistance genes in *M. truncatula* that will in turn be useful for understanding other legume/rust interactions.

Introduction

Barrel medic (*Medicago truncatula*) is an important cover and pasture crop in low rainfall areas, with a number of varieties released (Michalk and Beale, 1976). In addition, *M. truncatula* is being used as a model plant for structural and functional genomics (Cook, 1999). It is a diploid species closely related to the cultivated tetraploid alfalfa (*Medicago sativa*), and to other legumes including pea, lentil, chickpea and faba bean. This close phylogenetic relationship increases the value of *M. truncatula* as a resource for understanding the agronomic traits of importance to grain and forage legumes. As an experimental plant, *M. truncatula* has several key advantages (Cook, 1999; Oldroyd and Geurts, 2001; Dénarié, 2002; Torres et al., 2002). It has a relatively small genome, with tractable genetic properties including its self fertile, annual habit. In addition, *M. truncatula* has proved to be the most easily transformed of all legume species, ensuring its future as a system of choice for analyses of legume gene function.

Alfalfa rust incited by *Uromyces striatus* is an important disease in many areas, being particularly damaging in alfalfa grown for seed (Koepper, 1942). Many other rust species of the genus *Uromyces* are important constraints for grain and forage legumes, such as *Uromyces viciae-fabae* (faba bean rust), *Uromyces pisi* (pea rust), *Uromyces appendiculatus* (common bean rust) and *Uromyces vignae* (cowpea rust). Breeding for rust resistance is the most feasible mean of control. Different resistance mechanisms may be operative at different phases of the infection process, from spore deposition, spore germination, germ tube orientation, appressorium formation, stoma penetration, infection hyphae growth rate, to percentage of successful haustoria formation. In most rust–host pathosystems, spore germination and appressorium formation are independent

of the host plant genotype (Heath, 1974), but once the appressorium and the substomatal vesicle have formed, plant genotypes differ in the extent to which pre- and post-haustorial mechanisms of resistance operate (Niks and Rubiales, 2002). There is no available fungus that causes a natural rust disease in *Arabidopsis thaliana*, the other major model plant, although the *A. thaliana–U. vignae* system has been studied to characterize the nonhost signalling pathway (Mellersh and Heath, 2003). *Medicago truncatula* is susceptible to *U. striatus* (Skinner and Stuteville, 1995). The purpose of this study was to determine the level of resistance against *U. striatus* available in *M. truncatula* and to identify histologically the defence reactions that are operative. This information will be useful for mapping analyses and cloning of the resistance genes, which in turn should be useful for understanding other legume/rust interactions.

Materials and methods

Plant and fungal material

A collection consisting of 231 accessions of *M. truncatula*, kindly provided by the Australian Medicago Genetic Resource (SARDI, Glen Osmond, South Australia), was studied for resistance against alfalfa rust (*U. striatus*) in seedlings under growth chamber conditions. Plants were grown in pots filled with a 1 : 1 mixture of sand and peat. In the first experiment, the whole collection was studied for infection type (IT) and disease severity (DS). Each accession was represented by three pots with five seedlings each. Accessions differing in the level of resistance were selected for the study of the cellular responses to infection by rust. In each of the three consecutive series, inoculation were performed in three runs, each one including a box with five seedlings per genotype.

Inoculation and incubation

Seedlings were inoculated when the third trifoliate leaf was completely expanded. Leaves were pinned to soil in a horizontal position and the adaxial surface of the leaves was inoculated in a spore settling tower by dusting 1 mg of urediopores per plant, diluted in pure talc (1 : 10), resulting in a spore deposition of 280 spores/cm^2.

Plants were incubated for 24 h at 20 °C in complete darkness and 100% relative humidity, and then transferred to a growth chamber at 20 °C under a 14 h light: 10 h dark photoperiod with light intensity of 148 μmol/m^2/s at the leaf canopy.

Screening

Infection type was scored 10–15 days after inoculation (d.a.i.) using the IT scale of Stakman et al. (1962) where 0 = no symptoms, ; = necrotic flecks, 1 = minute pustules barely sporulating, 2 = necrotic halo surrounding small pustules, 3 = well formed pustules surrounded by a chlorotic halo, 4 = well formed pustules with no associated chlorosis or necrosis. DS was estimated as the percentage of host tissue covered by pustules.

Histological observations

Leaves were collected 2 d.a.i. and processed to study the phases of the fungus growth prior to stoma penetration (Sillero and Rubiales, 2002). Three leaf samples per accession per replication were cut. The leaf samples were laid, adaxial surface up, on filter paper dipped in fixative (1 : 1, absolute ethanol/glacial acetic acid, v/v). When the leaf segments had been bleached by several changes of the fixative, they were transferred to filter paper moistened with tap water for at least 2 h, to soften the tissues. Next they were transferred to lactoglycerol (1 : 1 : 1, lactic acid/glycerol/water, v/v/v) for at least 2 h. To stain the samples, a drop of Trypan blue in lactoglycerol (0.1%, w/v) was placed on a cover glass, the sample was carefully laid with the adaxial surface toward the cover glass and then mounted in lactoglycerol on a microscope slide. About 125 urediospores per leaf sample were counted under 200× magnification with a Leica DM LS microscope and grouped into the following categories: germinated urediospores (a spore was considered germinated when a germ tube at least as long as the diameter of the spore was produced); germ tubes growing over stomata but not forming appressoria and germ tubes forming appressoria. Of the germ tubes which formed appressoria, distinction was made whether the appressorium was formed over a stoma or away from the stoma (misplaced).

Three further leaves per accession per replication were cut 2 d.a.i. and stained with Trypan blue (Sillero and Rubiales, 2002). The leaves were fixed in acetic acid : ethanol (1 : 3 v/v) for 30 min; stained by boiling in 0.05% Trypan blue in lactophenol : ethanol (1 : 2 v/v) for 10 min and cleared in a nearly saturated aqueous

solution of chloral hydrate (5 : 2 w/v) to remove Trypan blue from the chloroplast. Early stages of the infection were studied microscopically, using a phase contrast Leica DM LS microscope at 400× magnification. Per leaf, 20 random colonies were studied. The numbers of hyphal tips and haustoria were recorded for each colony, along with the presence or absence of necrosis of the host cells associated to an infection structure. Necrosis was identified by uptake of Trypan blue by the plant cells.

Statistical analysis

Comparison of means (LSD test, $P < 0.05$) was made for all microscopical components of resistance among accessions. When the components of resistance were expressed as percentage, data were angular transformed prior to an analysis of variance.

Results

Disease severity in the collection ranged from 0% to 10% without macroscopically visible necrosis (high IT). Most of the accessions displayed reduced DS whereas only a few displayed higher DS close to that of the alfalfa check cv. Ampurdam.

For most infection stages before stoma penetration (spore germination, germ tube directional growth and stoma recognition) there were no or only small significant differences among accessions (Table 1). The only aspect in which the rust fungus was clearly less successful on some of the accessions than on the susceptible *M. truncatula* check SA19995 was the percentage of germ tubes forming an appressorium over a stoma. This percentage was significantly lower on SA22182, SA9357 and SA21302. SA19995 allowed only an intermediate frequency of appressorium formation.

Differences in the development of the infection process among resistant and susceptible accessions in seedlings were evident 2 d.a.i. (Table 2). There was a significant proportion of infection units on some of the accessions that failed to form any haustoria. Significant differences among accessions were found for the percentage of infection units that failed to produce haustoria, being low (10%) in the alfalfa check, and ranging from moderate to high (37–73%) in the *M. truncatula* accessions. Differences in the rate of haustorium formation were also found among these accessions. Significantly fewer haustoria were formed in all accessions with respect to the alfalfa and SA19995 checks. Haustorium formation was particularly low in accessions SA22182, SA29831, SA28889, SA21302, SA9357 and SA25654. Intercellular growth of the colonies was reduced in all accessions with respect to the alfalfa check, with significant differences among *M. truncatula* accessions. The number of hyphal tips per colony was particularly low in accessions SA28889, SA21302, SA9357 and SA25654. A significant, but moderately low proportion (17–22%) of infection units was associated with host cell necrosis in some of the *M. truncatula* accessions (SA29831, SA28889 and SA25654). Necrosis was visible as a darkening of the cell contents to such an extent that it was usually impossible to determine the presence of a haustorium. No established colony was associated with host cell necrosis.

Table 1. Percentage of occurrence of events in the early development of infection units of *U. striatus* on seedlings of selected *M. truncatula* lines[a]

Accession	% spore germination	% germ tubes not finding a stoma ('lost')	% germ tubes forming a misplaced appressorium (away from the stoma)	% germ tubes overgrowing at least 1 stoma without forming an appressorium	% germ tubes forming an appressorium over a stoma
Alfalfa cv. Ampurdam	62.9 ab	14.8 a	26.2 a	3.7 ab	29.3 abc
SA4327	65.3 ab	24.9 a	46.2 a	4.7 ab	30.9 ab
SA9357	53.6 ab	26.3 a	47.9 a	3.1 b	22.9 bc
SA19995	61.6 ab	20.1 a	31.4 a	3.7 ab	43.5 a
SA21302	51.4 b	18.6 a	32.1 a	1.4 b	15.7 c
SA22182	65.2 ab	20.7 a	44.2 a	1.6 b	23.9 bc
SA25654	53.9 ab	20.8 a	41.0 a	2.9 ab	34.9 ab
SA28889	60.9 ab	21.4 a	39.8 a	3.6 ab	32.4 ab
SA29831	75.4 a	19.2 a	44.5 a	7.5 a	38.0 ab

[a]Values with letters in common in each column are not significantly different ($P < 0.05$, LSD test).

Table 2. Early stages of the infection by *U. striatus* in selected *M. truncatula* lines and final infection type and disease severity[a]

Accession	Infection type	Disease severity (%)	Early abortion (%)	Colonies with necrosis (%)	Number of haustoria/colony	Number of hyphal tips/colony
Alfalfa cv. Ampurdam	4	10 a	10.4 f	0 c	1.4 a	6.3 a
SA4327	0	0 c	41.2 cde	0 c	0.6 bc	4.1 bcd
SA9357	4	0.1 c	71.6 a	0 c	0.1 e	3.1 e
SA19995	4	7.5 a	37.5 de	0 c	1.0 a	4.2 bcd
SA21302	0	0 c	60.6 ab	0 c	0.3 cd	3.2 e
SA22182	0	0 c	53.7 abcd	0 c	0.3 cde	3.5 cde
SA25654	3	2.5 b	73.4 a	17.3 b	0.2 de	2.8 e
SA28889	0	0 c	57.3 abc	17.7 b	0.2 de	3.4 e
SA29831	3	0.5 c	57.9 abc	22.1 a	0.3 cde	3.5 de

[a]Values with letters in common in each column are not significantly different ($P < 0.05$, LSD test).

Discussion

A range of resistance mechanisms against *U. striatus* are operative in *M. truncatula* accessions. After spore germination on a leaf, topographical features the plant surface may be used by the fungus to locate and recognize stomata prior to going through a defined series of morphological stages required to enter the stomatal opening and form the first haustorium within a living host mesophyll cell (Heath, 1974). Some mechanisms of rust exclusion may occur prior to stomatal penetration by *Uromyces*. Such defence mechanisms are due to poor germling adhesion to the leaf surface (Mendgen, 1978; Wynn and Staples, 1981), deviating micromorphology of the epidermal surface that serves as cues in guiding the thigmosensing germ tube towards stomata (Wynn and Staples, 1981), stomatal guard cell morphology (Wynn, 1976) and leaf pubescence (Mmbaga et al., 1994). The limited varietal differences in germination and germtube directional growth presented here support the general view that reduction of urediospore germination and fungal development on the leaf surface are of marginal importance, at best, in reducing infection levels within host species (Niks and Rubiales, 2002).

There is evidence that urediospore germlings of bean rust (*U. appendiculatus*) sense morphological features of host stomata over which they develop appressoria (Wynn, 1976). Additional appressorium-inducing features have been reported on some nonhosts, resulting in a high proportion of appressorium formation away from stomata (Heath, 1974). In the present study, this proportion was relatively high both in alfalfa and in all the studied accessions of *M. truncatula*, and showed little genotypic variations. A proportion of germtubes failing to form appressorium over stomata has commonly been reported on both host and nonhosts, with remarkable examples of high genotypic differences in some cereals (Rubiales and Niks, 1992; Rubiales et al., 1996). However there have been few reports on differences between cultivars in a legume (Sillero and Rubiales, 2002).

Once the fungus has successfully penetrated the stoma, and formed a first haustorium, nutrients are taken from the invaded plant cell to allow further intercellular growth and haustoria formation. Previous studies comparing host and nonhost resistance to rust fungi have shown that nonhost resistance is typically expressed before the formation of the first haustorium (Heath, 1981). In contrast, *R* gene-controlled host resistance is almost invariably expressed after the formation of the first haustorium, often in the form of hypersensitive death of invaded cell. But prehaustorial resistance can also be identified in host interactions, playing a major role in so-called partial resistance, that may be more durable than resistance controlled by *R* genes (Niks, 1986; Niks and Dekens, 1991; Rubiales and Niks, 1995; Niks and Rubiales, 2002). A significant proportion of infection units failed to form any haustoria in certain *M. truncatula* accessions. This high level of prehaustorial resistance to *U. striatus* in *M. truncatula* might successfully be exploited in barrel medic breeding and could be a valuable source of nonhypersensitive (and possibly durable) resistance to alfalfa rust.

The differences identified in *M. truncatula* accessions in levels of both prehaustorial and posthaustorial resistance will allow the study of different

types of resistance which act at different phases of the infection process and are likely to be governed by different genetic systems. High resolution genetic maps of *M. truncatula* which are being constructed (Dénarié, 2002) will facilitate the identification and cloning of genes and quantitative trait loci.

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References

Cook DR (1999) *Medicago truncatula* – a model in the making! Current Opinion in Plant Biology 2: 301–304

Dénairé J (2002) Genomics and genetics of *Medicago truncatula*: Resources for legume crop improvement. Grain Legumes 38: 20–21

Heath MC (1974) Light and electron microscope studies of the interactions of host and nonhost plants with cowpea rust – *Uromyces phaseoli* var. *vignae*. Physiological and Molecular Plant Pathology 4: 403–414

Heath MC (1981) Resistance of plants to rust infection. Phytopathology 71: 971–974

Koepper JM (1942) Relative resistance of alfalfa species and varieties to rust caused by *Uromyces striatus*. Phytopathology 32: 1048–1057

Mellersh DG and Heath MC (2003) An investigation into the involvement of defense signaling pathways in components of nonhost resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. Molecular Plant-Microbe Interactions 16: 398–404

Mendgen K (1978) Attachment of bean rust cell wall material to host and non-host plant tissue. Archives of Microbiology 119: 113–117

Michalk DL and Beale JA (1976) An evaluation of barrel medic (*Medicago truncatula*) as an introduced pasture legume for marginal cropping areas of South-eastern Australia. Journal of Range Management 29: 328–333

Mmbaga MT, Steadman JT and Roberts JJ (1994) Interaction of bean leaf pubescence with rust urediniospore deposition and subsequent infection density. Annals Applied Biology 125: 243–254

Niks RE (1986) Failure of haustorial development as a factor in slow growth and development of *Puccinia hordei* in partially resistant barley seedlings. Physiological and Molecular Plant Pathology 28: 309–322

Niks RE and Dekens RG (1991) Prehaustorial and posthaustorial resistance to wheat leaf rust in diploid wheat seedlings. Phytopathology 81: 847–851

Niks RE and Rubiales D (2002) Detection of potentially durable resistance mechanisms in plants to specialised fungal pathogens. Euphytica 124: 201–216

Oldroyd GED and Geurts R (2001) *Medicago truncatula*, going where no plant has gone before. Trends in Plant Science 6: 552–554

Rubiales D and Niks RE (1992) Low appressorium formation by rust fungi on *Hordeum chilense* lines. Phytopathology 82: 1007–1012

Rubiales D and Niks RE (1995) Characterization of *Lr34*, a mayor gene conferring nonhypersensitive resistance to wheat leaf rust. Plant Disease 79: 1208–1212

Rubiales D, Ramírez MC and Niks RE (1996) Avoidance of leaf rust fungi in wild relatives of cultivated cereals. Euphytica 87: 1–6

Sillero JC and Rubiales D (2002) Histological characterization of the resistance of faba bean to faba bean rust. Phytopathology 92: 294–299

Skinner DZ and Stuteville DL (1995) Host range expansion of the alfalfa rust pathogen. Plant Disease 79: 456–460

Stakman EC, Stewart DM and Loegering WQ (1962) Identification of physiologic races of *Puccinia graminis* var. *tritici.* Agricultural Experimental Station Technical Bulletin. Scientific Journal Series, paper 4691, pp. 1–53

Torres AM, Geffroy V and Rubiales D (2002) *Medicago truncatula* to help progress for resistance against pathogens and pests in legume crops. Grain Legumes 38: 18–19

Wynn WK (1976) Appressorial formation over stomates by bean rust: Response to a surface contact stimulus. Phytopathology 66: 136–146

Wynn WK and Staples RC (1981) Tropisms of fungi in host recognition. In: Staples RC and Toenniessen GH (eds) Plant Disease Control: Resistance and Susceptibility (pp 45–69) Wiley-Interscience, New York