Defence components in *Arabidopsis* against *Leptosphaeria maculans*

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Abstract

The dothideomycete, *Leptosphaeria maculans* is a hemibiotrophic fungal pathogen, causing the blackleg disease of *Brassica* oil crops world-wide. Genetic studies of *L. maculans* resistance in *Brassica* species have been tedious and despite confirmed gene-for-gene interactions, no *Brassica R* genes or *LmAvr* genes have been cloned. *Arabidopsis* has been shown to be a suitable model host system to develop a mechanistic understanding of resistance against this pathogen. 168 *Arabidopsis* accessions and numerous mutants impaired in pathways or key genes important in plant defence have been evaluated for their response to *L. maculans*. It was found that the resistance in *Arabidopsis* was independent of salicylic acid, jasmonic acid and ethylene-induced defences but partly relied on the phytoalexin camalexin. We have utilized two sets of recombinant inbred lines with the aim of identifying genes that control the defence to *L. maculans* resulting in identification of $RLM1_{Col}$ and $RLM2_{Ler}$. In addition, transcriptional analysis has been used to identify *RLM3*, a gene that also governs resistance other necrotrophic pathogens. Ongoing work includes characterisation of *lms* mutants and gene cloning, genetic dissection of defence signalling and assessment of various defence layers and finally *R*-gene interactions on protein level. Updated results on those aspects will be presented and further discussed.

Key words: ABA, blackleg, callose, camalexin, defence signalling, *R*-genes

Leptosphaeria maculans (anamorph: *Phoma lingam*) causes blackleg on *Brassica* crops world-wide. *L. maculans* is a hemibiotrophic fungus, haploid, outcrossing, can be transformed, and has a genome size of about 34Mb (Howlett et al., 2001). *L. maculans* is an ascomycete and a member of the class Dothideomycetes where a number of other specialized genera of plant pathogens belong, like *Stagonospora*, *Mycosphaerella*, *Alternaria* and *Cochliobolus*. Ongoing genome sequence efforts of *L. maculans* are linked to similar progress on other species within the same taxonomic class. This new genome sequence source together with other genome sequencing initiatives will enable us to make comparisons between various taxonomic levels for different gene categories but also generate knowledge on evolutionary aspects. For the joint INRA-PMDV and UNI-Melbourne genome initiative see www.genoscope.cns.fr/externe/English/Projets/Projet_DM/organisme_DM.html.

We have established a system in *Arabidopsis* to study defence mechanisms to *L. maculans* in order to identify resistance genes and enhance our fundamental understanding of the signalling network of importance in this specific plant-pathogen system.

Resistance genes

A vast number of *Arabidopsis* accessions and mutants (>300) impaired in pathways or key genes important in plant defence have been evaluated for their response to *L. maculans*. The natural variation is very limited. Screenings of *Arabidopsis* accessions revealed that all 168 accessions evaluated, except An-1 (Antwerpen-1), displayed a high degree of *L. maculans* resistance. However we observed a segregation ratio close to 15:1 in a F_2 population between Columbia (Col-0) and Landsberg *erecta* (L*er*-0), suggesting that two independent dominant resistance traits reside in each parental accession. Similar results were obtained from crosses between L*er*-0 and Ws-0 (Bohman, 2001). Based on this information, we used the polymorphic data available to genetically map the loss of resistance. We utilized two sets of recombinant inbred lines Col-4×L*er*-0 (Lister & Dean, 1993) and L*er*-2×Cvi-1 (Alonso-Blanco et al. 1998) with the aim of identifying genes that control the defence to *L. maculans* (Staal et al., 2006). We identified a locus responsible for resistance to *L. maculans* in the Col-0 background ($RLM1_{Col}$) comprising seven *R* genes. Disruption with T-DNA insertion in two of the genes found in $RLM1_{Col}$ results in susceptible phenotypes; both genes encode TIR-NB-LRR motifs. Database analysis of sequences in the proximity of the $RLM1_{Col}$ locus indicates an ancient evolutionary linkage to the locus responsible for resistance in the L*er*-0 background ($RLM2_{Ler}$). Interestingly, several *L. maculans* resistance genes in *B. napus* (*LepR1*, *LmR1, CLmR1, Rlm1, Rlm3, Rlm7* and *Rlm9*) are mapped to genomic loci that correspond to the chromosome segment on *Arabidopsis* chromosome 1 that harbours $RLM1_{Col}$ (Delourme et al., 2004; Mayerhofer et al., 2005; Parkin et al., 2005). The sequence of an *R* gene analog (RGA) marker associated to the recessive *B. juncea* derived resistance r_jlm2 in *B. napus* also shows homology to the TIR-NB-LRR sub-family TNL-H of which $RLM1_{Col}$ is a member (Meyers et al., 2003; Saal & Struss, 2005). None of the 9 currently known *AvrLm* genes identified from *Brassica-L. maculans* interactions do however correspond to *Arabidopsis* $RLM1_{Col}$ resistance (Balesdent et al., 2005; Staal et al., 2006).

Furthermore, we have, through a microarray based strategy compared the transcriptomes in pools of Col-0×An-1 progenies, and identified the absence of a locus that causes susceptibility in An-1. The significance of this locus on

chromosome 4 for *L. maculans* resistance was supported by PCR-based mapping and denoted resistance to *Leptosphaeria maculans* 3 ($RLM3_{Col}$) and studies of T-DNA insertion lines. $RLM3_{Col}$ encodes a putative TIR-NB (Toll/interleukin-1 receptor – nucleotide binding) class protein and the analysis implies that a short TIR-X (Toll/interleukin-1 receptor – unknown domain) transcript is responsible for the resistance.

Role of camalexin

The phytoalexin camalexin, present in *Arabidopsis* but absent in *Brassica napus*, was shown to play a partial role in this defence system since *pad3-1* (phytoalexin deficient 3) which is depleted in camalexin production, exhibited a clear susceptibility response, whereas the *L. maculans* susceptible mutant *lms1* expressed wild-type levels of camalexin (Bohman et al., 2004). Further studies have shown that camalexin is a quantitative co-dominant resistance factor of Col origin but it is independent of the *RLM1* locus (Staal et al., 2006). The implications of the effects from camalexin must however be considered in much of our ongoing work in form of crosses with *pad3-1*.

Callose and plant hormones implicated in defence signalling

Synthesis of 1,3-ß-glucans (callose) has earlier been found to be induced by *B. napus – L. maculans* resistance genes. Assessments of *Arabidopsis* genotypes also showed that the *RLM1* locus is required for an efficient callose deposition in response to *L. maculans* (Staal et al., 2006). Studies of physiologically defined *Arabidopsis* mutants, to explore additional key defence factors in the *L. maculans* plant-pathogen system has also been performed, including importance of endogenous ABA levels, and the role of exogenously applied ABA and BABA for resistance responses in *Arabidopsis* (Kaliff et al., 2006). ABA defective mutants of Ler-0 origin (*aba1-3* and *abi1-1*) but not the insensitive mutant *abi2-1*, *abi3-1* or *abi5-1* (Ws-o background) were found to be susceptible to *L. maculans*. The latter trait was also linked to a significantly decreased level of callose depositions. A high degree of susceptibility was also found in the callose synthase mutant *pmr4-1*. Assessment of suppressor mutants of *abi1-1* (Leung et al., 1997) confirmed that the *L. maculans* susceptible response was due to the dominant negative nature of the *abi1-1* mutant. Highly induced camalexin levels made however ABA mutants in Col-0 background (*aba2-1, aba3-1, abi4-1*) appear resistant, but displayed enhanced susceptibility as double mutants with *pad3-1*. Furthermore, β-aminobutyric acid (BABA) pre-treatment of L*er*-0 contributed to an elevated level of endogenous ABA after *L. maculans* inoculation. Comparisons between ($RLM1_{Col}$)*pad3* and $rlm1_{Ler}$*pad3* genotypes also showed that ABA and BABA enhancement of callose deposition require induction from $RLM1_{Col}$. The difference between the *abi1-1* and *abi2-1* was further assessed by global transcription analysis. We interpreted the array data as there being an independent ABI1 feedback mechanism that modulates $RLM1_{Col}$ expression. Genetic analysis showed further that this feedback occurs upstream of *ABI4* and that components downstream of *ABI4* modulate *ABI1* activity. ABA and BABA treatments of the *L. maculans* susceptible callose synthase mutant *pmr4* showed that ABA also induces a callose-independent resistance.

Taken together, we have now identified a number of major key defence factors in *Arabidopsis* to *L. maculans* but we also realize the complexity of the system. Interacting components to these major genes are now being identified. We found earlier that the resistance in *Arabidopsis* was independent of SA, JA and ET-induced defences when single mutants were assessed but gene expression of *PR1* and *PDF1.2* were evident (Bohman et al., 2004). Further genetic dissection of SA, JA and ET responses in an *rlm1pad3* background revealed a complex interaction between the three hormones, and for example EDS1 and PAD4 were found to play a minor role in *RLM1* resistance via suppressing JA responses. Further analysis also shows that *RLM1* is gene-dose dependent and that RAR1 and SGT1b act antagonistically. Additional components influencing the cell death machinery seems however be utilized particularly when the fungal mycelia encompasses necrotrophic growth. Additional plant hormones like for example auxin also seem to play a crucial role in the plant defence signalling network to *L. maculans*. *L. maculans* susceptible mutants impaired in such traits are now under examiation.

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