



Future prospects for ascochyta blight resistance breeding in cool season food legumes

D. Rubiales* and S. Fondevilla

Consejo Superior de Investigaciones Científicas, Institute for Sustainable Agriculture, Córdoba, Spain

Edited by:

David Bryla, United States
Department of Agriculture, USA

Reviewed by:

Maria Carlota Vaz Patto,
Universidade Nova de Lisboa,
Portugal
Weidong Chen, United States
Department of Agriculture, USA

*Correspondence:

D. Rubiales, Consejo Superior de
Investigaciones Científicas, Institute
for Sustainable Agriculture, Apdo.
4084, 14080 Córdoba, Spain.
e-mail: diego.rubiales@ias.csic.es

Legume cultivation is strongly hampered by the occurrence of ascochyta blights. Strategies of control have been developed but only marginal successes achieved. Breeding for disease resistance is regarded the most cost efficient method of control. Significant genetic variation for disease resistance exists in most legume crops with numerous germplasm lines maintained, providing an excellent resource for plant breeders. Fast and reliable screening methods have been adjusted to fulfill breeding program needs. However, the complex inheritance controlled quantitatively by multiple genes, has been difficult to manipulate. Successful application of biotechnology to ascochyta blight resistance breeding in legume crops will facilitate a good biological knowledge both of the crops–pathogen interaction and of the mechanisms underlying resistance. The current focus in applied breeding is leveraging biotechnological tools to develop more and better markers to speed up the delivery of improved cultivars to the farmer. To date, however, progress in marker development and delivery of useful markers has been slow in most legumes. The limited saturation of the genomic regions bearing putative QTLs in legume crops makes difficult to identify the most tightly linked markers and to determine the accurate position of QTLs. The application of next generation sequencing technologies will contribute to the development of new markers and the identification of candidate genes for ascochyta blight resistance.

Keywords: *Ascochyta*, breeding, legumes, resistance, biotechnology

Cool season food legumes (chickpea, faba bean, lentil, and pea) provide an excellent source of high quality plant protein and have a key role in arable crop rotations reducing the need for fertilizer application and acting as break-crops. However, these crops are affected by a number of foliar and root diseases, being ascochyta blights the most important group of diseases worldwide. *Ascochyta* blights are incited by different pathogens in the various legumes, namely *Ascochyta rabiei* (teleomorph *Didymella rabiei*) in chickpea; *A. fabae* (teleomorph *D. fabae*) in faba bean; *A. lentis* (teleomorph *D. lentis*) in lentil; and *A. pisi*, *Phoma medicaginis* var. *pinodella* (previously *A. pinodella*), and *Mycosphaerella pinodes* in pea (Tivoli et al., 2006). A number of control strategies have been developed including cultural practices and chemical control (Pande et al., 2005; Davidson and Kimber, 2007; Fernández-Aparicio et al., 2010; Stoddard et al., 2010). However, only marginal successes have been achieved, most control methods being uneconomical, hard to achieve or resulting in incomplete protection. Breeding for resistance is widely acknowledged as the most economic and environmentally friendly control method. However, resistance breeding is also not an easy task (Muehlbauer and Chen, 2007; Rubiales and Fondevilla, 2010; Sillero et al., 2010). We will review and discuss current achievements in breeding for resistance to ascochyta blights in major cool season food legumes and present future prospects.

Sources of partial resistance have been identified in all of the cool season food legumes and the currently available resistance is being used in breeding programs designed to develop cultivars

with improved resistance. There are some recent reviews on breeding methods, screening procedures, the ascochyta diseases, and biology of the pathogens (Pande et al., 2005; Tivoli et al., 2006). Therefore we will concentrate in this review on different aspects of breeding for resistance to ascochyta blight in legumes. The recent developments in understanding the genetics of host resistance will be summarized and the constraints that hampered deployment of resistances discussed. This review will also provide a perspective on the prospects for utilization of novel biotechnological tools for resistance breeding.

Resistance breeding in legume crops has been slow due to the complex nature of resistance and the relatively low investment on genetics, genomics, and biotechnology of legume crops, but also, mainly because of limited knowledge on the biology of the causal agents and on the plant/disease interactions. Comprehensive studies on host status and virulence of the ascochyta blights are often missing, and in most instances there is little agreement on the existence of races and on their distribution. This is a major limitation for any breeding program. Also, available information on levels of resistance and on the responsible mechanisms is often incomplete. Only after significant input to improve existing knowledge on biology of the causal agents as well as on the plant, resistance breeding will be efficiently accelerated.

Resistances identified so far in the various legume crops against the various ascochyta blights provide only incomplete protection. These resistances are mostly polygenic traits controlled by quantitative trait loci (QTLs). However, in some ascochyta–legumes

interactions both QTLs and major gene have been suggested (Table 1). Molecular markers linked to these genes/QTLs have been reported but practical application of MAS in cool season food legumes has been limited. Although the genomic regions controlling resistance are continuously being saturated with new markers, recombination still occurs between flanking markers and resistance traits hampering an efficient selection. Therefore it is necessary to better define the genomic regions controlling resistance and to increase marker density at these regions in order to identify molecular markers more tightly linked to the genes/QTLs of interest.

The use of the recent biotechnology tools can facilitate both, a better understanding of the resistance mechanism and the development of new molecular markers. Gene expression studies will increase our knowledge on the molecular basis of the resistance and the mechanisms underlying the host–parasite interaction and might represent a source of candidate genes for ascochyta blight resistance. The advent of microarray technology has enabled large-scale surveys leading to a more integrated view of gene expression responses. However, transcript analysis using macroarrays or microarrays requires the accumulation of sequence information on the species involved, which is not yet available for cool season food legumes. Nevertheless, the high degree of sequence homology among legume species, especially for expressed sequences, enables cross-species application of macro- and micro-arrays. A microarray containing 32 ESTs derived from a cDNA library obtained from a resistant grass pea accession inoculated with *M. pinodes* was used to identify 16 ESTs differentially expressed in resistance (Skiba et al., 2005). Other microarray consisting on a set of chickpea unigenes, the grass pea ESTs identified by Skiba et al. (2005), and lentil resistance gene analogs (RGAs) was used to study resistance response in chickpea against *A. rabiei* (Coram and Pang, 2006) allowing identification of 97 differentially expressed genes. This same microarray was also used to identify genes involved in resistance to *A. lentis* in lentil (Mustafa et al., 2009).

More interestingly, advantage can be taken of the knowledge and tools developed in the model legume *Medicago truncatula*. In this model several microarray platforms have been developed including an affymetrix system that can identify genes involved in legumes defense against ascochyta blight. Hybridization of *M. truncatula* microarray chips with RNA from related species such as pea is possible allowing a more comprehensive understanding of pea molecular response to *M. pinodes* (Fondevilla et al., 2011). This approach allowed identification of 346 genes differentially regulated in response to *M. pinodes* in a resistant pea accession including genes involved in cell wall reinforcement, phenylpropanoid and phytoalexins metabolism, pathogenesis-related (PR) proteins, and detoxification processes.

Changes in gene expression, regulated by transcription factors (TFs) underlie many biological processes, including the response against pathogens. For example, the putative transcription factor *PsDof1* has been suggested as a candidate gene for a QTL involved in resistance to *M. pinodes* in pea (Prioul-Gervais et al., 2007). TFs are usually expressed at low levels, and are therefore difficult to identify by low sensitive techniques such as microarrays, but can be efficiently quantitatively assessed by quantitative RT-PCR (Czechowski et al., 2004). A library containing primers suitable

for the amplification of more than 1100 *M. truncatula* TFs by quantitative PCR has recently been developed at the Max-Planck Institute of Molecular Plant Physiology (Potsdam, Germany) and could be used to quantify transcripts of these TFs in the interactions *Medicago–M. pinodes* by real-time RT-PCR as has already been used to study TF expression profiling of the interactions of *M. truncatula* with *Erysiphe pisi* (Curto et al., 2007).

MicroRNAs (miRNA) can be used in addition to the TF profiling to investigate the post-transcriptional regulation of genes complementing previous knowledge on differential gene expression in *M. truncatula* in response to *M. pinodes*. A number of miRNAs have already been described in other plant species having biotic or abiotic stress-inducible homologs that can be selected to analyze their expression in cool season legumes systems (Trindade et al., 2010).

Microarrays, TF platform and miRNA analysis are powerful tools for expression studies. However, these techniques demand previous sequence information knowledge, being limited to known genes. As a consequence, genes specifically involved in a specific ascochyta–legume interaction may not be detected. This limitation can be circumvented by the use of non-targeted techniques such as suppression subtractive hybridization cDNA library (SSH) or cDNA–AFLP that do not require knowledge of sequence data, being an excellent tool to identify novel genes in species such as legumes with limited available genomics sequences. cDNA–AFLP is a highly reproducible differential display method based on restriction enzymes digestions and selective amplifications under high stringent conditions that facilitates the identification of genes specifically expressed in a given situation. By SSH cDNA libraries enriched for genes differentially regulated between two situations are obtained. The cDNA–AFLP technique has already been applied to chickpea to identify candidate genes for resistance to *A. rabiei* (Cho et al., 2005) but have not yet been reported in other legumes.

More powerful transcriptomic techniques are emerging thanks to the next generation sequencing (NGS) technologies. These techniques are enabling the rapid sequencing of millions of sequences at a relative low cost comparing to Sanger Sequencing technology. The called “RNA-seq” technique takes advantage of NGS and are based in sequencing the transcriptome in two different situations and comparing the number of times that a gene is present in these two transcriptomes as an estimation of the level of expression of this gene. One of these derived techniques is called SuperSAGE (Matsumura et al., 2010) that has already been used in chickpea to identify genes involved in different abiotic stresses (Molina et al., 2011) and is currently being used to identify genes involved in ascochyta resistance (Kahl et al., 2011).

Next generation sequencing also allow the “novo” sequencing of the genome or transcriptome of a given organism. The still high cost of these techniques is slowing the generation of a reference genome/transcriptome for legumes species. However, a transcriptome of pea has recently been published (Franssen et al., 2011) and some projects are in progress to sequence the genome of chickpea and pea. Hopefully the improvement of sequencing technologies and the reduction of their cost will make possible the sequencing of all legumes species in a near future. The sequencing of the genomes/transcriptome of legumes will represent a great advance in MAS as it will allow discovering new genes, the massive

Table 1 | Mode of inheritance of resistance to ascochyta blight in legumes.

| Pathogen | Host | Mode of inheritance | Reference |
|-------------------|-----------|---|---|
| <i>A. pisi</i> | Pea | A major gene modified by minor genes or QTLs | Darby et al. (1985), Dirlewanger et al. (1994) |
| <i>M. pinodes</i> | Pea | Major genes QTLs | Clulow et al. (1991), Timmerman-Vaughan et al. (2002), Tar'an et al. (2003), Prioul et al. (2004), Fondevilla et al. (2008) |
| <i>A. lentis</i> | Lentis | Major dominant gene Major recessive gene QTLs | Ford et al. (1999) Chowdhury et al. (2001) Rubeena et al. (2006) |
| <i>A. rabiei</i> | Chickpea | Major genes QTLs | Singh and Reddy (1993), Collard et al. (2001), Chen et al. (2004) Santra et al. (2000), Tekeoglu et al. (2002), Collard et al. (2003), Rakshit et al. (2003), Udupa and Baum (2003), Cho et al. (2004), Iruela et al. (2006) |
| <i>A. fabae</i> | Faba bean | Major genes QTLs | Rashid et al. (1991), Kohpina et al. (2000), Kharrat et al. (2006) Román et al. (2003), Avila et al. (2004) |

identification of new markers as SNPs or SSR, the identifications of miRNA, etc.

Transcriptional profiling does not always reflect resistant phenotypes due to post-transcriptional regulation. Proteomic approaches can be used to characterize proteins differentially expressed in the defense response. Proteins differentially expressed in pea/*M. pinodes* interaction have been identified by MALDI-TOF/TOF or ESI-Q-Trap (Castillejo et al., 2010). Second-generation proteomic techniques such as two-dimensional differential in gel electrophoresis (2D DIGE) can be used for comparative studies. A technology based in stable isotope labeling called iTRAQ can be used to identify posttranslational modifications (PTM) of proteins, such as phosphorylation, S-nitrosylation, and nitration (Kersten et al., 2006; Forrester et al., 2009).

Transcriptomic and proteomic data are important steps in deciphering a complex biological process, but these techniques are still insufficient to understand biological processes fully since most of them are ultimately mediated by cell metabolites, so metabolomic studies are also needed. Although large-scale, comprehensive metabolomic studies are difficult, a number of targeted analyses can be performed to assess the involvement of subsets of metabolites in various stresses (Allwood et al., 2010).

The combined results obtained through the various above described technologies will serve to increase our knowledge about ascochyta–legumes interaction and could also be a source of candidate genes involved in the resistance to ascochyta blight. To discern which candidate genes could be more likely involved in the control of the resistance, these candidate genes could be mapped in populations where QTLs for ascochyta blight have been identified. Those genes located into a QTL will be more likely involved in the control of resistance.

Mapping of genes involved in defense in maps containing QTLs for resistance to ascochyta blight have resulted in the identification of candidate genes for resistance to ascochyta blight. This approach has revealed the co-localization of QTLs for resistance to *M. pinodes* and RGAs, the putative transcription factor *PsDof1* and the pea defensin DRR230-b (Timmerman-Vaughan et al., 2002; Prioul-Gervais et al., 2007). Genes involved in defense co-localizing with resistance QTLs have also been reported in the case of *A. rabiei*-chickpea. Those included RGAs, catalase,

and ribonuclease T2 (Winter et al., 2000; Huettel et al., 2002; Flandez-Galvez et al., 2003; Cho et al., 2004).

Other approaches to identify the genes included in QTLs for ascochyta blight have been attempted. Iruela et al. (2009) used a genome walking method to sequence flanking regions of a sequence characterized amplified region (SCAR) marker linked to a QTL for resistance to *A. rabiei*. They concluded that this region was a putative retrotransposon fragment and no genes clearly related to resistance were identified. Comparative mapping with *M. truncatula* have also been attempted to identify candidate genes located into resistance QTLs. As a first step to identify such genes Rajesh et al. (2008) identified a *M. truncatula* BAC clone syntenic to a chickpea BAC clone containing a molecular marker associated with a QTL for resistance to *A. rabiei*. The synteny between *M. truncatula* and other legumes has been successfully used also in pea to identify the gene sequence that corresponds to the gene *SYM2* involved in symbiosis (Gualtieri et al., 2002).

Although some candidate genes have been postulated, functional analysis are still lacking in most instances. The ability to knockout genes or suppress their expression are powerful methods to determine the function of a gene. This can be done by anti-sense RNA suppression, targeted gene replacement, insertional mutagenesis, gene silencing, and targeted-induced local lesion in genome (TILLING) approaches. TILLING platforms are available for pea (Dalmais et al., 2008), *M. truncatula*, and *L. japonicus* (Perry et al., 2003). These platforms consist in populations of chemically mutagenized plants. Point mutations in specific genes can be detected using mismatch specific endonucleases. In the case of absent of mutants for any desirable gene in TILLING collections these genes could be silenced using different techniques. Virus induced genes silencing (VIGs) have already been successfully used for silencing of several endogenous pea genes (Constantin et al., 2004). It is now possible to transform many grain legumes (Chandra and Pental, 2003; Somers et al., 2003) although in some cases the rate of recovery of transgenic lines is still low. This has facilitated functional studies, but has not resulted in commercial applications due to technical (regeneration recalcitrance of most legumes), social (public concerns), and political (lower rate of investment in legume crops compared to other crops such as rice, wheat, and maize) reasons.

CONCLUSION

Though limited in efficacy in many cases, the control methods available today represent a major progress when compared to the lack of any means for the control of these plants one or two decades ago. Crops can be protected by resistance, by selective fungicides, by biocontrol agents, and by cultural methods that did not exist before.

The current focus in applied breeding is leveraging biotechnological tools to develop more and better markers to allow marker assisted selection with the hope that this will speed up the delivery of improved cultivars to the farmer. To date, however, progress in marker development and delivery of useful markers has been

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