Gene candidates for perception and signal transduction in the disease resistance response of woody species

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Plant disease resistance response. Plant disease resistance response is important for the quality of crops, the protection of natural environments and the safeguarding of some plant species whose diversity may be reduced by the spread of aggressive diseases. Understanding the molecular basis of a resistance response is necessary for the development of effective and environmentally-friendly control strategies.

Several mechanisms are involved in resistance response and depend on the host species, the pathogen and the environmental conditions. Generally plants react to pathogen attacks with pre-existing chemical defences and physical barriers (constitutive resistance), and with inducible responses involving a complex network of cross-talking signal transduction pathways. An inducible resistance response represents a crucial point in the race between plants and pathogenic microbes. From this process host resistance and pathogen virulence have evolved and diversified due largely to reciprocally exerted selection pressures (Jones and Dangl, 2006). Moreover the inducibility of a resistance response is significant in terms of the growth and development of plants because it limits the metabolic cost of any defense activation during the period of pathogen attack (Durrant and Dong 2004).

All inducible resistance responses are based on three phases: (1) recognition of pathogen-derived products through a sensitive perception system; (2) recognition triggers a signal transduction leading to (3) the activation of a battery of defense genes whose products synergistically collaborate to eliminate the invading microbe. The outcome of a resistance response depends on its rapidity, which is guaranteed by an effective perception and signal transduction system. For this reason much research in plant biology is currently engaged in the genetic dissection of these processes.

Inducible resistance responses can be classified into at least four types: non-host resistance, basal resistance, resistance mediated by resistance genes (R genes), and systemic acquired resistance also called induce systemic resistance (SAR, ISR).

Non-host resistance is durable and complete. It is displayed by an entire plant species to all the isolates of a potentially pathogenic microbial species. All plants express this type of resistance towards the majority of microbes. This resistance seems to be under complex genetic control, and involves both preformed and inducible defenses (Heath, 2000; Nürnberger and Lipka, 2005).

Basal resistance is also durable, relies on multiple protective mechanisms and is effective against a broad spectrum of pathogens. From an evolutionary point of view, it is considered as the residue of non-host resistance having been overcome by a pathogen. Therefore in contrast to non-host resistance, basal resistance is not enough to stop infection but is effective in decreasing symptoms. Basal resistance is based on the recognition by the host of pathogen-associated molecular patterns (PAMPs), which are conserved structures typical of whole classes of microbes. PAMPs are recognized by cognate pattern-recognition receptors (PRRs), which are plasma membrane-resident proteins that trigger immediate defense responses. PRRs are also believed to be important for triggering non-host resistance. During evolution, individual races of a given pathogen can develop the ability to evade or suppress PRR-triggered defense mechanisms. As a consequence, non-host resistance or basal resistance is overcome with the establishment of pathogen virulence and host susceptibility (Nürnberger and Lipka, 2005; Altenbach and Robatzek, 2007).

Resistance mediated by R genes is a highly sophisticated resistance system evolved by plants in order to resist specialized pathogens, which can overcome the above-mentioned resistance types. R genes are specific to certain plant genotypes within a species and recognize products of pathogen effector genes which are generally involved in virulence and are specific to certain pathogen races. Recognition leads to resistance which is typically accompanied by a local host cell death, known as hypersensitive response (HR). Therefore the fact that virulence factors can be sensed by the host turns them into avirulence factors. This pathogen race/plant genotype specific disease resistance conforms to the gene-for-gene hypothesis and is genetically determined by complementary pairs of pathogen-encoded avirulence (avr) genes and host R genes. The lack of matching avr/R gene pairs would result in disease. However not all R genes fit this specificity model: in fact some R genes have been undefeated by pathogens for decades, whilst others are known to govern resistance responses against multiple pathogen races or against different pathogen classes. This highlights the biological complexity of R genes, which goes beyond the simplicity of the concept of gene-for-gene (McDowell and Woffenden, 2003; Jones and Dangl, 2006).

Systemic acquired resistance is naturally activated by a local pathogen challenge, thus producing a necrosis, either as a hypersensitive reaction (HR), or as a disease symptom. As a consequence, plants mount a systemic response that confers an increased, long-lasting and broad-spectrum resistance to subsequent pathogen attacks for the whole plant (Sticher et al. 1997; Mètraux, 2001; Durrant and Dong, 2004). Two additional types of systemic resistance are known to be locally induced by the colonization of roots by non-pathogenic rhizobacteria (Van Loon et al. 1998; Pieterse et al. 2001) or by wounding, as the result of mechanical damage or attacks by feeding insects (Pena-Cortés *et al.* 1995**;** Kessler and Baldwin, 2002). The different types of systemic resistance are established through the activation of distinct, complex cross-communicating signal transduction pathways that lead to the expression of distinct and partly overlapping sets of defence-related genes. The three types of systemic resistance are distinguished by the distinct arrays of pathogens that they are effective against and by the signalling molecules that induce resistance. In general, it is well-accepted that salycilic acid (SA) is an essential component of SAR signalling, while jasmonic acid (JA) and ethylene have a role in ISR and wound-induced systemic resistance (Pena-Cortés *et al.* 1995; Pieterse et. al. 2001, Kessler and Baldwin, 2002). A lot of research on signal transduction pathways is currently underway with the focus on the functions of a key regulatory gene of *Arabidopsis thaliana*, *NPR1* (for non-expresser of PR genes). Substantial scientific evidence has identified this gene as a multifunctional component in systemic resistance (Durrant and Dong, 2004; Dong 2004; Pieterse and Van Loon, 2004). NPR1 is also important for basal resistance as well as for known R genes. For these reasons *NPR1* is now considered to be a key component in the whole resistance response (Dong, 2004; Pieterse and Van Loon, 2004).

Genomic research on tree species. The research in our laboratory has focused on the characterization, in some tree species, of gene families related to *NPR1*, PRR genes and R genes. Work has been carried out within the following research projects:

1) Programma triennale di ricerca sul sistema forestale - Biodiversità e produzione di materiale forestale di propagazione - **Tipologie di geni coinvolti nelle risposte di resistenza ai patogeni in selezioni di ciliegio per la produzione di legno - RI.SELV.ITALIA**.
2) Ricerche sul pero finalizzate alla riduzione dell'impatto ambientale e alla valorizzazione della qualità – **Selezione precoce di germoplasma di pero per la resistenza al colpo di fuoco batterico, mediante inoculazione *in vitro* – PRIA**.

3) Ricerche sul nocciolo finalizzate all'ottenimento di produzioni biologiche di qualità - Indagini su aspetti fitopatologici e di resistenza del nocciolo per produzioni biologiche di qualità - **Ricerca di marcatori di resistenza CO.RI.BIO**.
4) Proteine e geni per la protezione delle piante dagli stress biotici e abiotici - **Genomica e proteomica per lo studio della risposta di resistenza in ciliegio e platano - *PROTEO STRESS***

Materials. The characterization of NPR1-like gene families was performed by comparing different species of Rosaceae, belonging to Amygdaloideae and Pomoideae (Pilotti et al., 2008). NPR1-like genes were also searched for in *Platanus* × *acerfolia* Ait. (Willd.). Homologues of R genes and of PRR genes were studied in *Prunus* spp. (cherries), *Corylus avellana* L.and *P.* × *acerfolia*.

The following cherry species/genotypes were collected and studied: 1) *Prunus avium* L.: variety *Mazzard* F12/1, the clones DOUC4 (from Tuscany) and ACW06 (Caucasian origin); 2) *P. serrulata* Lindl.; 3) *P. cerasus* L. var. *Caproniana*. The clones *P. avium* DOUC4 and ACW06 belong to the collection from the Centro di Ricerca per la Selvicoltura di Arezzo (Dott. Fulvio Ducci). Some observations revealed that these clones were highly resistant to a severe leaf fall of uncertain origin, which considerably affected the plantations. Foliar symptoms of attacks by fungal or bacterial pathogens (*Cylindrosporium padi, Wylsonomices carpophilus* and *Pseudomonas syringae* pv *morsprunorum*) were also less severe in comparison with fully susceptible clones. *P. serrulata* was included in the study because of the high resistance showed by young plants in the nursery, to natural infections of *W. carpophilus*. *P. cerasus* was included in the study to make the genomic investigations within the taxonomical group of cherries more complete (gen. *Prunus* subgen. *Cerasus*).

Plane trees were also studied due to diseases that threaten either ornamental plantings or natural stands (Pilotti 2002). Canker stain, caused by the fungus *Ceratocystis platani*, is by far the most destructive disease due to the lethal outcome of the infections and their rapid spread from affected trees to nearby healthy trees (Panconesi 1999). For this reason canker stain was included in the EPPO standards as a regulated pest. After a ten-year selection for resistance to canker stain, we screened twenty-two accessions of *Platanus* spp., which are able to survive *C. platani* inoculations in a stable manner (Pilotti, unpubblished data). Therefore, based on the availability of resistant genotypes, we started a genomic research study aimed at unravelling the molecular basis of resistance to canker stain.

With regard to *C. avellana*, a number of diseases can seriously damage the cultivation worldwide. Some even compromise the viability of the trees. For example “filbert blight” caused by the fungus *Anisogramma anomala* and present in the USA, and bacterial canker and decline caused by *Pseudomonas avellanae* (Pinkerton et al. 1993; Scortichini 2002). The most important commercial cultivars have shown complete susceptibility to these diseases. Some resistance to *P. avellanae* was shown by the local cultivar Tonda Rossa (Scortichini 2006). In our research we conducted molecular analyses on the cv Tonda Rossa and on a wild genotype collected from a natural chestnut grove located in the province of Rome.

Methods. A PCR-based gene candidate approach was used to search for homologues of R genes, PRR genes and *NPR1*. With regard to R genes and PRR genes we focused on the following gene classes: TIR-NB-LRR, nonTIR-NB-LRR, kinase and kinase-LRR. We designed a considerable number of degenerate primers on conserved motifs of these gene classes. Amplifications were performed on genomic DNA and on cDNA retrotranscribed from leaf/bark mRNA (see for example Figure 1). Multiple bands were derived from PCR amplifications. We focused only on expected-size bands. The expected size was assumed as being the length of the regions used for the primer design. Selected bands were excised, eluted from the gel, cloned into a plasmid, and sequenced. Cloning of each band revealed the presence of a wide population of DNA molecules slightly differing in terms of molecular weight and sharing homologous but not identical sequences. Consequently each expected band enabled us to collect different gene types. Low degeneration of the primers led to a highly efficient procedure (fishing of the gene targets) with an absence of or with very few undesired amplifications. In contrast, highly degenerate primers are known from the literature to be less efficient. In fact, it has been reported that, before sequencing, cloned amplicons usually have to be screened with restriction enzymes (RFLP), in order to select RGAs from other aspecifically amplified DNA regions.

Sequence analysis in progress. At the end of the process we collected about 1500 gene fragments with a strong homology with the above gene classes. Cherry sequences were deposited in NCBI GenBanks. The number of cherry gene fragments obtained *per* gene type is detailed in Table 1.

Sequence analysis clearly showed that *NPR1*-like gene fragments from rosaceous species and plane trees were, most probably, orthologues of NPR4 and/or of its closest relative

(At5g45110) (Fig. 2). While *NPR1* has been studied since 1997 (Cao *et al.*; Ryals *et al.*) research on *NPR4* is more recent (Liu *et al*., 2005). NPR4 has a role in the basal resistance to fungal and bacterial pathogens by positively transducing SA and JA signals. This confirms the importance of this gene family.

Research on homologues of R genes showed the existence of wide and highly polymorphic gene families, as previously reported for R gene families from other plant species. The inference of phylogenetic trees also showed the vicinity of some R gene homologues with well-known R genes of arabidopsis and tomato, controlling the resistance to bacterial and fungal pathogens. A preliminary comparison of the different genotypes of cherry or hazelnut trees did not show obvious differences: in general all the phylogenetic groups and sub-groups were present in all the compared genotypes. However there were some gene fragments separately located in the branching pattern of the phylogenetic trees, which were exclusive to some species/genotypes. It remains to be verified whether these differences are at the genomic level or are the result of the random fishing of gene fragments by the cloning technique. PCR amplifications with specific nested primers should be able to solve this uncertainty.

Homologues of some PRR genes were also found, for example, homologues of *FLS2* (for *flagelling sensing 2*) a gene of arabidopsis whose product mediate perception of the bacterial flagellin (a PAMP) which is the main component of the bacterial motility organ (Altenbach and Robatzek, 2007). In this case homologues from the tree species showed only minimum variations within each species (probably an allelic variation) suggesting the presence of an *FLS2*-like gene as a single copy gene. We also examined homologues of *EFR* (EF-Tu receptor) another PRR gene required for the perception of the bacterial elongation factor EF-Tu (a PAMP) (Altenbach and Robatzek, 2007). The results were somewhat ambiguous as *EFR* homologues seemed to belong to a gene family, which was also related to *Xa21* an R gene from rice controlling resistance to the bacterium *Xanthomonas oryzae* pv *oryzae*.

Conclusions. Many studies have been carried out on plant resistance responses in herbaceous and model species. A lot of information is now available relating to perception and signal transduction. On the other hand, much less information is available on tree species due to their greater biological complexity and the difficulties in experimental procedures.

Knowledge derived from model species is considered to be a good starting point to begin genomic and proteomic research on non-model species. Also, information derived from non-model and biologically complex species will increase our future knowledge of plant biology.

In this study, a gene candidate approach enabled us to extensively characterize gene families related to R genes, PRR genes and *NPR1*.

Partial sequences obtained from the study will be used in the future as valuable markers aimed at assisting resistance selection procedures and map-based cloning of resistance genes. Monitoring the expression of these gene families through DNA microarrays, will also facilitate the identification of genes with crucial roles in resistance responses.

In conclusion, this research provided the necessary knowledge for the future development of procedures that are able to select effective and broad spectrum resistance to pathogens. It is also worth highlighting that in disease control, the use of resistant genotypes is generally much more preferable to the heavy application of chemicals (which should be reduced as much as possible). The gene sequences obtained in this research and published in the NCBI GenBank, represent a source of useful information for researchers starting this type of study on other phylogenetically related tree species.

Table 1 – Gene fragments isolated from the different species/genotypes of cherry trees through a PCR-based gene candidate approach (research project: RISELVITALIA, PROTEOSTRESS)

Gene classes	TIR-NB-LRR	nonTIR-NB-LRR	Kinase and Kinase-LRR	NPR1-like	Total
Total	180	109	170	11	470

Fig. 1 Structure of NPR1 protein and length of gene fragments obtained from rosaceous tree species and plane tree

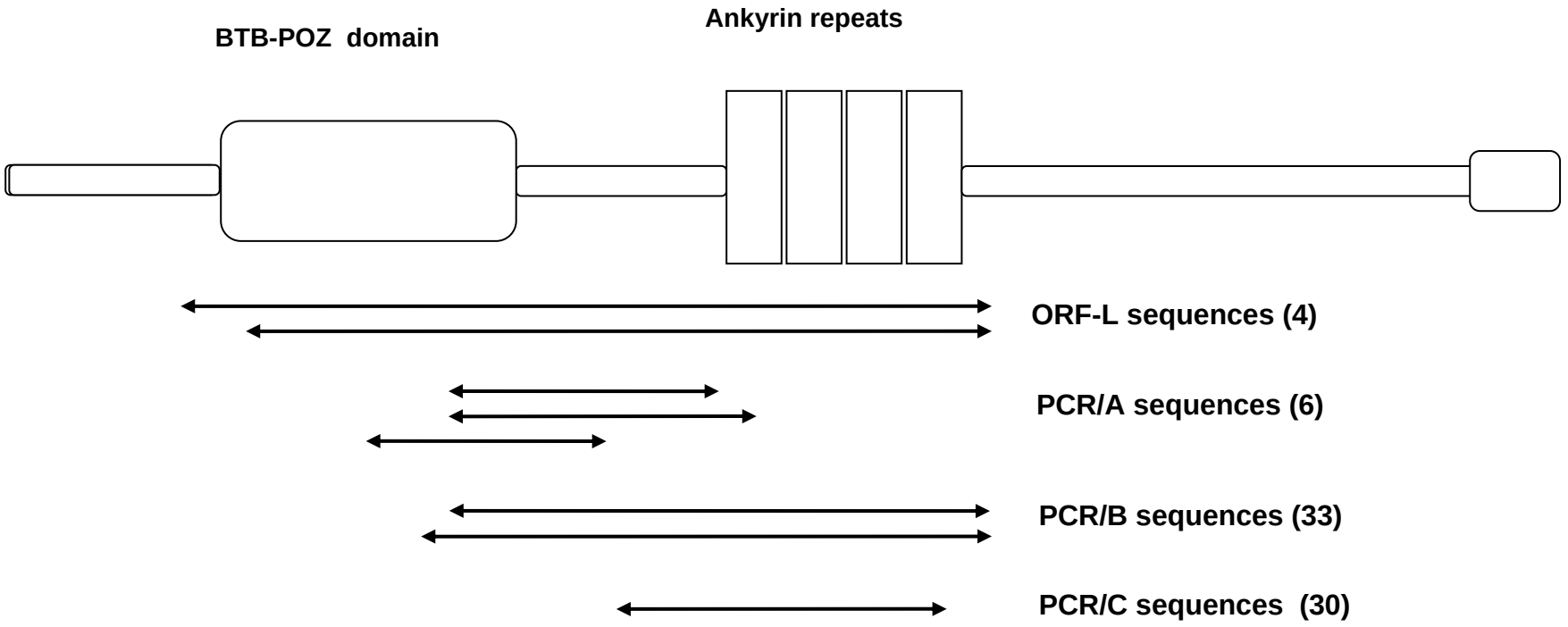
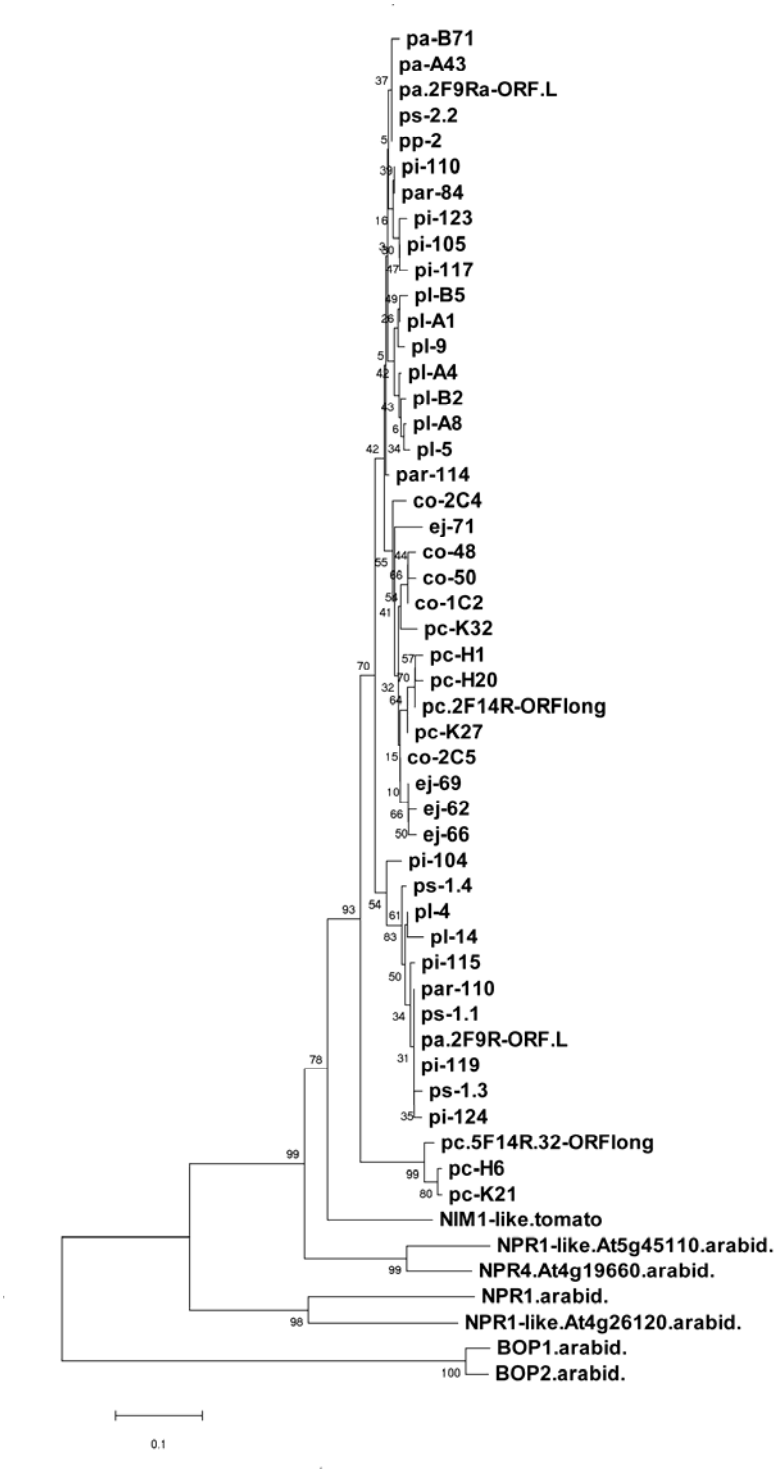


Fig. 2 Neighbour-joining tree based on the ClustalW alignment of amino acid deduced sequences of gene fragments from Rosaceous species under study. *P. communis* (pc), *C. oblonga* (co), *E. japonica* (ej), *P. avium* (pa), *P. serrulata* (ps), *P. laurocerasus* (pl), *P. domestica* subsp. *insititia* (pi), *P. armeniaca* (par). NPR1 family from arabidopsis and NPR1-like from tomato are also included as reference sequences. The numbers refer to bootstrap values (1000 replicates). The scale at the bottom indicates genetic distance proportional to the number of substitutions *per site*



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