Molecular Mechanisms for Resistance to Biotic Stresses

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Abstract

Diverse pathogens including viruses, viroids, fungi, and bacteria are responsible of diseases on Citrus. Some of them in addition represent a treath to Citrus industry in some specific areas, others are either worldwide spread or have a restricted distribution. Breeding program searching for resistance to a given pathogen must take into consideration the nature of the interaction being studied. In addition a large number of data generated by sequencing projects will contribute to the identification of individual genes or groups of genes potentially associated with resistance to biotic and abiotic factors. This chapter introduce the molecular basis of plant resistance to innate immune response elicited by non-specific elicitors and how successful pathogens have evolved to evade them or trigger them later in the infection so that they become infective. The other paragraphs are dedicated to illustrating three important disease model studies caused by a fungus (Alternaria brown rot), an oomycete (Phythophthora root rot) and a virus (Citrus Tristeza).

16.1 Plant Diseases that Pose a Threat to Citrus Industry

Cultivated citrus species are susceptible to many diverse pathogens including viruses, viroids, fungi, and bacteria. Some of them are responsible of diseases considered the most limiting factors for the development of citrus industry in some specific areas, others are either worldwide spread or have a restricted distribution. Historically, *Phytophthora* and 'tristeza' diseases, are known since long time and have changed the citrus production systems to budding on rootstocks by the beginning of the nineteen-century (Moreno et al. 2008). Since then many diseases and pests exclusively associated with the canopy (scion) or the root system (rootstock) have developed, along with those resulting from the interaction between them.

The progressive specialization of cultivation on a regional base and the use of monoclonal types of citrus were favorable conditions for the

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pathogens in different areas, and their spread increased by the huge increase of transportation and globalization. Nowadays climate changes are contributing in a great measure to further expand the world list of economically important and destructive citrus diseases. Amongst the most threaten are: Citrus variegated chlorosis (CVC); Citrus leprosis (CiL); Huanglonbing (HLB, previously known as greening); Citrus sudden death (CSD); Citrus bacterial canker (CBC); Citrus black spot and Alternaria brown spot (Timmer et al. 2000).

The narrow genetic base used by the citrus industry, the high genetic plasticity of citrus that allow them to adapt to different conditions, and vegetative propagation by buds and use of rootstocks from nucellar embryos are among the factors associated to severity of pests and diseases (Machado et al. 2011). Therefore, any breeding program searching for resistance to a given pathogen must take into consideration the nature of the interaction being studied. Several of them are of relevance due to the productivity losses they cause and the high cost of their control. The work on breeding for resistance and genetic mapping in different countries has been focused on different target according to regional relevance of citrus and specific problems. The largest interest has been devoted to destructive pest and pathogens, frequently included in the quarantine list of the Regional Plant Protection Services worldwide. The large number of data generated by sequencing projects has stimulated the use genotyping arrays to study the expression of thousands of genes, contributing to the identification of individual genes or groups of genes potentially associated with several metabolic pathways, resistance biotic and abiotic factors as well expression of QTL (Quantitative Trait Loci) at different environmental conditions. The integration of the database with the construction of arrays for gene expression studies is helpful to understand host-pathogen interactions and other traits of interest (Machado et al. 2011; Gmitter et al. 2012).

This chapter first introduces the molecular basis of plant resistance to innate immune response elicited by non-specific elicitors and how successful pathogens have evolved to evade them or trigger them later in the infection so that they become infective. The other paragraphs are dedicated to illustrating three important disease model studies caused by a fungus (Alternaria brown rot), an oomycete (Phythophthora root rot) and a virus (Citrus Tristeza). An extensive literature review on the pathogen- citrus interaction was recently published by Dalio et al. (2017). Two important disease model studies caused by bacteria, namely Huanglongbing and CBC, will be deepen in separate chapters (Chaps. 14 and 15).

16.2 Plant Resistance

16.2.1 Plant Innate Immunity—PRRs and PTI

The perception of environmental signals and the ability to respond accordingly are essential for all organisms in nature to survive. The innate immune system provides such abilities and protects organisms like plants and animals. Mammals have evolved a sophisticated adaptive immune system that relies on creation and selection of somatic diversity for recognition of pathogen-derived molecules. In contrast, plants rely on cell-autonomous innate immunity, activated upon pathogen detection by either cell surface or intracellular receptors. Plasma membrane located Pattern Recognition Receptors (PRRs) have evolved to detect specific molecular patterns that are interpreted by the plant cell as danger signals. These danger signals can be either infectious non-self determinants, such as microbe- or Pathogen Associated Molecular Patterns (PAMPs/MAMPs), or self-molecules, Damage Associated Molecular Patterns (DAMPs) that are released upon pathogen perception or pathogen-induced cell damage, and activate PAMP Triggered Immunity (PTI) (Macho and Zipfel 2014; Choi and Klessig 2016; Duxbury et al. 2016). PTI regulates a wide array of responses, including regulation at a cell to organism level, aimed at hampering pathogen growth and disease progression. Early PTI events include the rapid generation of Reactive Oxygen

Species (ROS), the activation of Mitogen-Activated Protein Kinases (MAPKs), and the expression of immune-related genes (Macho and Zipfel 2014). While plants defective in PTI signaling appear to be more susceptible to adapted but also non-adapted pathogens. This indicates that PTI is sufficient against the majority of the plant pathogenic microbes, while the best demonstration regarding its biological relevance is the necessity for adapted successful pathogens to interfere by the active suppression of this first layer of plant defence in order to cause disease (Macho and Zipfel 2014). These are the so called Successful pathogens.

16.2.2 Plant Innate Immunity—NLRs and ETI

The successful pathogens have developed pathogenicity components (largely known as effector proteins) to block PTI. Plants from their side, carry a repertoire of intracellular Nucleotide Binding-Leucine Rich Repeat (NB-LRR or NBS-LRR or NLRs) receptor proteins, which structurally and functionally resemble mammalian Nod-like Receptors (NLRs) to recognise pathogen effectors (Jones and Dangl 2006). Plant genes that code NLR receptors are generally known as disease resistance (*R*) genes and their products directly or indirectly intracellularly detect pathogen effector proteins (Jones and Dangl 2006; Duxbury et al. 2016; Jones et al. 2016). The importance of NLRs to plant defence is illustrated by the expanded complement of NLRs in plants compared to NLRs in most animals. For example, *Arabidopsis thaliana* carries ~120 full length NLRs, rice carries ~595 NLRs and wheat (*Triticum aestivum*) ~1224 NLRs, while most mammals have ~20 NLRs (Jacob et al. 2013; Sarris et al. 2016). NLR-mediated defence is termed Effector-Triggered Immunity (ETI) and often concludes to the induction of the hypersensitive response (HR), a form of programmed cell death that restricts pathogen's spreading to neighbouring host tissues and biotrophic pathogen growth. For historical reasons, recognized effectors that trigger ETI are often referred to as avirulence (Avr) proteins. The main difference between plant NLRs and mammalian NLRs is that plant NLRs detect pathogen effector proteins, while mammalian NLRs recognize DAMPs or relatively conserved pathogen molecules such as flagellin or peptidoglycan (Eitas and Dangl 2010; Maekawa et al. 2012; Duxbury et al. 2016; Mermigka et al. 2020; Mermigka and Sarris, 2019). The activation of plant and animal NLRs relies on a signal transduction ATPase with numerous domains (STAND) domain function, which is under strict control to prevent auto-activation (Leipe et al. 2004; Duxbury et al. 2016). The NB domain of plant NLRs belongs to nucleotide-binding, Apaf-1, R-protein and CED-4 (NB-ARC) class (Williams et al. 2014), and in the auto-inhibited or 'inactive' state, is proposed to be ADP-bound, while the exchange of ADP to ATP allows the NB-ARC domain to adopt an activated or 'active' state (Williams et al. 2014). A strict regulation mechanism, which usually includes intra-molecular interaction with the LRR domain and other domains, keeps the NB-ARC domain in the 'inactive' state in the absence of microbial ligands, preventing auto-immunity (Maekawa et al. 2012; Duxbury et al. 2016).

In plants there have been characterized two main groups of NLRs based on the N-terminal part of the protein. There are NLRs that either carry a Toll/Interleukin-1 receptor/Resistance protein domain (TIR) or a coiled-coil (CC) protein domain in their amino-terminal end. In mammalian NLRs the amino-terminal domain usually harbours a caspase-activation and recruitment domain (CARD), a pyrin domain (Pyr) or baculovirus inhibitor-of-apoptosis repeats (BIRs) (Maekawa et al. 2012; Duxbury et al. 2016). Both groups of plant NLRs are involved in pathogen recognition, but the two subfamilies have distinct genetic requirements and cluster separately in phylogenetic comparisons of their NB-ARC domains (Jones and Dangl 2006; McHale et al. 2006; Andolfo et al. 2014; Sarris et al. 2016). It is noteworthy that TIR-NLR-triggered immunity is often attenuated or abolished at or above 28 °C (Dinesh-Kumar et al. 1995; Zhu et al. 2010; Heidrich et al. 2013).

16.2.3 Plant Innate Immunity—Effectors Recognition 'Ligand and Guard/Decoy Models'

Although many NLR/effector protein pairs have been studied, the molecular mechanisms of effector perception by plant NLRs, such as precise knowledge of the interactions of protein domains, and mechanisms linking NLR/effector interaction with the activation of downstream resistance signalling pathways, remain unclear. In some cases, plant and animal NLRs function in pairs to mediate immune recognition (Narusaka et al. 2009; Eitas and Dangl 2010; Williams et al. 2014; Sarris et al. 2015; Saucet et al. 2015). Activation of ETI can occur via direct physical interaction of an effector with a plant NLR receptor, ('the ligand-receptor model'), followed by defence activation, resulting in transcriptional re-programing (Keen 1990; Ravensdale et al. 2012). Few cases of direct NLR/effector interactions have been reported so far (Jia et al. 2000; Dodds et al. 2006; Krasileva et al. 2010; Catanzariti et al. 2010; Cesari et al. 2013). The 'guard' and 'decoy' models provide plausible explanations for recognition of multiple effectors. According to those models, some plant NLRs monitor the integrity of host proteins with which they associate. These NLRs 'guard' host proteins that are targets of effectors, or host proteins that resemble those targets (Duxbury et al. 2016; Ntoukakis et al. 2014).

16.2.4 Plant Innate Immunity—Effectors Recognition 'the Integrated Decoy Model'

Interestingly, several plant NLRs reveal fusions with unusual protein domains (e.g. the WRKY domain of RRS1 in Arabidopsis, the heavy-metal associated domain of RGA5 in rice, mitogen-activation protein kinase domain of AT4G12020 and Lim domain of CHS3 in Arabidopsis, and many more), while the roles of these domains in defence activation remain largely unknown (Meyers et al. 2003; Nishimura and Dangl 2014; Sarris et al. 2016). The rice CC-NLR pair RGA4/RGA5 carries out recognition of two microbial effectors, AVR-Pia and AVR1-CO39, by direct interaction with RGA5 through a small unusual protein domain located at the C-terminal region, related to heavy metal–associated domains (Cesari et al. 2013). However, the exact mechanism of perception and defence activation remains unknown. Notably, some of these extraneous protein domains have evolved by duplication of pathogen effector target proteins and have been incorporated into plant NLR immune receptors (Sarris et al. 2016). Such an NLR is the Arabidopsis RRS1 that contains a WRKY domain fused to its C-terminus (Narusaka et al. 2009; Sarris et al. 2015; Saucet et al. 2015). An early hypothesis relating to RRS1 is that it represents a protein that has the capability to recognize, signal and activate defense through the binding of its WRKY domain to DNA (Narusaka et al. 2009). PopP2, an acetyltransferase effector from *Ralstonia solanacearum*, interacts with this C-terminal domain (Deslandes et al. 2003). *RRS1* is encoded in the genome in a head-to-head orientation with *RPS4*, which encodes another phylogenetically distinct TIR-NLR required for RRS1-dependent effectors' recognition. This raises the question of whether *RRS1* acquired the WRKY domain exclusively as a decoy against interference with other WRKY transcription factors that are involved in defence (Eulgem and Somssich 2007). The gene pair *RPS4/RRS1* also confers recognition of the *Pseudomonas* effector AvrRps4 and an unknown factor from *Colletotrichum higginsianum* (Birker et al. 2009). This is consistent with the theory that limited number of host components is differentially targeted by different pathogens (Sarris et al. 2016).

Two breakthrough studies concerning NLRs with integrated domains have led to new conjecture regarding the role of these domains (Sarris et al. 2015; Le Roux et al. 2015). These works together show that PopP2 can bind and acetylate the WRKY domain of RRS1, reducing its DNA binding activity and triggering HR.

Furthermore, Sarris et al. (2015) show that the *P. syringae* effector AvrRps4 can also bind to RRS1's WRKY domain and trigger HR. These studies also show how these effector proteins can associate with multiple WRKY transcription factors. A remaining puzzle in the field of plant NLRs with integrated domains (NLR-IDs) is whether or not the IDs are true decoys or participates as functional domains (e.g. real WRKY transcription factor in RRS1) in the plant physiology that occurs subsequent to activation. However, there are scientific opinions suggesting that this must be considered the endgame of the guard model whereby the guard and guardee become linked genetically to ensure adaptive co-evolution and eventually fuse to become one unit.

16.2.5 Plant Innate Immunity—Citrus spp NLRsomes

Citrus species are amongst the most important fruit trees and have been cultivated for more than 4000 years (Barrett and Rhodes 1976; Scora 1975; Wang et al. 2015). Molecular markers analysis and phylogeny showed that cultivated *Citrus* species (sweet orange, grapefruit, and lemon) are derived from three original cultivated *Citrus* species: *C. medica* (citron), *C. reticulata* (mandarin) and *C. maxima* (pummelo) (Wang et al. 2015). A recent work deals with the identification and comparison of the NB-ARC domain-containing genes from three *Citrus* genomes: *C. clementina*, *C. sinensis* from USA and *C. sinensis* from China. The authors describe the identification of similar numbers of NBS domain-containing genes amongst these three genomes. The authors describe the identification of 618, 650 and 508 NLR genes from *C. clementina*, *C. sinensis* China and *C. sinensis* USA genomes respectively (Wang et al. 2015). While, a recent and yet unpublished genome analysis performed by this chapter's author (Sarris P. F. and Pavlidis P. et al. unpublished data) based on the NCBI deposited *Citrus* genomes, reveal the presence of 623 NLRs in *Citrus clementina* cv Clemenules (NCBI BioProjects: PRJNA232045, PRJNA223006); 813 NLRs in Valencia sweet orange (NCBI BioProjects: PRJNA225998, PRJNA86123) and 491 NLRs in Miyagawa wase satsuma (NCBI BioProject: PRJDB5882). Further studies are on-going regarding the identification and the phylogenetic analysis of *Citrus* NLRs that carry integrated domains (Citrus NLR-IDs).

16.3 Disease Case Studies

16.3.1 Phythophthora Diseases

Phythophthora spp. are responsible of serious soil borne diseases of citrus, including damping off in the seedbed, root and crown rot in nurseries, foot rot and brown rot of fruits, causing significant economic losses to citrus industry. After the severe outbreak of the nineteen-century citrus propagation system moved to the use of the resistant rootstock sour orange. The most prevalent species include: *P. boehmeriae*, *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. drechsleri*, *P. hibernalis*, *P. megasperma*, *P. palmivora*, *P. nicotianae* (Panabieres et al. 2016).

P. nicotianae and *P. citrophthora* cause severe damage in citrus nurseries and orchards worldwide. *P. citrophthora* is mostly associated to trunk gummosis, whereas *P. nicotianae* is associated to root rot (Panabieres et al. 2016). Root rot and crown rot are critical for the grove survival (Graham and Menge 2000). Citrus rootstocks show a different level of sensitivity to *Phytophthora* spp. Almost all varieties are susceptible to gummosis, from highly susceptible (*C. sunki*) to resistant (*P. trifoliata*). Some hybrids of *C. sinensis* x *P. trifoliata* are resistant to *P. citrophthora* and less resistant to *P. nicotianae*.

Several studies investigated the hemibiotroph behavior of *P. nicotianae* that establishes itself in host tissues as a biotroph and once inside switch to a necrotrophic phase of growth. Recently reviewed by Dalio et al. (2018) there are old and new evidences of the mechanisms that are involved in the resistance to *Phytophthora*

spp. In different species of citrus rootstocks resistance appears related to phenolic compounds concentration. In both stem gummosis and root rot infections, total phenol content is higher in the leaves of resistant varieties than in those susceptible.

A correlation was found between phytoalexin production by the pathogen and the ability of *P. trifoliata* and Swingle citrumelo rootstocks to regenerate roots from the tip of the infected roots themselves. The phytoalexins escoparone and esculin have been detected in the bark and roots of citrus in response to infection by *P. citrophthora* and *P. nicotianae* associated to the inhibition of the pathogens. Escoparone accumulation was faster in the resistant cultivars.

Studies of *P. nicotianae* transcriptome in order to understand the basis of citrus gummosis, have identified genes that encode cell wall degrading proteins, such as phospholipases, glucanases and endopolygalacturonases, elicitins (ELI), effectors that induce necrosis in plants, such as crinkling and necrosis-inducers (CRN) and necrosis inducing proteins (NIP) (Rosa et al. 2007). Ten different elicitin classes of parasiticein, differently expressed, have been identified in vitro in *P. parasitica* (Panabières et al. 2005). In *P. parasitica*—citrus interaction in susceptible cultivars elicitins are up-regulated at the later stages of infection, associated to tissue necrosis (Boava et al. 2011a, b).

An increase in expression of effectors was detected in the late stage of infection in the susceptible *C. sunki* rootstock (susceptible) (Dalio et al. 2018). *Phytophthora* species are able to secrete two types of effectors related to their localization in plant tissues: the apoplastic or extracellular effectors, such as elicitins and NPP-like effectors; and cytoplasmic effectors, such RxRL and Crinkler effectors (CRNs), which possess special amino acid motifs in their structure enabling their entry inside cells independent of the presence of the pathogen (Hogenhout et al. 2009; Kamoun 2009).

Researches involving the model *P. trifoliata* (resistant) and *C. sunki* (susceptible) rootstocks, showed that defense genes, such as pathogenicity-related genes with anti-oomycete properties, and genes that act in plant water conductivity, are activated in the host in response to infection by the pathogen through signal transduction chains. These genes are also regulated by plant hormones, including salicylic acid (SA) and abscisic acid (ABA) (Boava et al. 2011a, b). In deep, the studies have reported the changes in global gene expression profiles and have shown differentially expressed genes involved in several processes, such as cell defense, photosynthesis and carbohydrate metabolism (Boava et al. 2011a, b). An increase in expression of above-mentioned effectors was observed in late stages of infection by *Phytophthora* spp., when pathogens enter the necrotrophic stage and promote hypersensitive response (HR) and necrosis in tissues of susceptible plant varieties, including citrus (Boava et al. 2011a; Oßwald et al. 2014) and more specifically *C. sunki* rootstock (susceptible) (Dalio et al. 2018).

Pathogenesis-related proteins (PRs) PR1, PR2 and PR5, which are responsive to salicylic acid (SA), have anti-oomycete properties, contributing directly to the defense against *Phytophthora* spp. (Dalio et al. 2017). The evaluation of the response to *P. nicotianae* infection in the susceptible (*C. sunki*) and resistant (*P. trifoliata*) genotypes of citrus showed that PR genes such as PR1, PR2, PR3 and PR5 were more up-regulated in *P. trifoliata* than in Sunki tangerine. This result suggests the involvement of these transcripts in mechanisms of resistance to citrus gummosis (Boava et al. 2011b). In addition, Boava et al. (2011b) showed that the expression of POX genes, others related to PR, and lipoxygenase (LOX), a gene that has been widely associated with plant defense against pathogens and both were over expressed in the resistant rootstock at later stages of infection, compared to the susceptible ones. ESTs from citrus species such as *C. sinensis*, *C. clementina* and *P. trifoliata* under various conditions, including biotic stresses in the CitEST project (Guidetti-Gonzalez and Carrer 2007) helped to identify a gene of the type TIRNBS-LRR, named RPS protein 4 (RPS4), and another gene named late embryogenesis abundant 5 (LEA5), both responsive to abscisic acid (ABA), had an increase in gene expression in resistant hybrids of *P. trifoliata*

(resistant parent) and *C. sunki* (susceptible parent) after infection by the hemibiotrophic pathogen *P. nicotianae* and that are differentially expressed between the resistant and susceptible parent (Boava et al. 2011a).

16.3.2 Alternaria Brown Spot of Tangerines

Two different pathotypes of fungus *Alternaria alternata*, the 'tangerine pathotype' and the 'rough lemon pathotype' are causal agents of Alternaria Brown Spot (ABS) an important disease of tangerines and their hybrids and of a similar disease that affects only rough lemon and Rangpur lime, respectively (Timmer et al. 2000, 2003). ABS affecting leaves, twigs and fruit have a large economic impact on tangerines and hybrids cultivation. Symptoms on young leaves and young shoots appear as brown to black spots and could be surrounded in the leaves by a yellow halo; affected leaves often abscise. Brown to black lesions tiny to large crater-like lesions develop on fruits.

The disease cycle is simple since there is no teleomorph known for *A. alternata* (Timmer et al. 2003). Conidia are produced primarily on the surface of lesions on mature or senescent leaves and on blighted twigs when lightly moistened or at high humidity and also wind dispersed.

The host specificity of the tangerine and rough lemon pathotypes of *A. alternata* depends upon the production of host-specific toxins (HSTs) that are also responsible for necrotrophic colonization and that possess the same selectivity as the pathogens (reviewed by Tsuge et al. 2013). Most HSTs are considered to be pathogenicity factors, required to invade tissue and induce disease and determines the host range of toxin-producing pathogens. *Alternaria* pathotypes produce HSTs which are diverse ranged chemical compounds, from low molecular weighted peptides to cyclic peptides. The toxin from the rough lemon pathotype was named ACR or ACRL-toxin, and that from the tangerine pathotype was named ACT-toxin (Miyamomto et al. 2010). The toxin is released during the germination of conidia, and rapidly affects the plasma membrane integrity of susceptible host cells. ACT-toxin causes veinal necrosis and electrolyte leakage from susceptible leaves but not on resistant leaves (Kohmoto et al. 1993). The mode of action of ACT toxin is still uncertain, but a rapid loss of electrolytes from leaf tissues and ultrastructural changes of cells treated with the toxin indicated that the primary action site of ACT-toxin was likely the plasma-membrane.

Although HSTs are responsible for pathogenicity a number of studies have investigated the role of other potential virulence factors. A mutant of Citrus Alternaria depleted in endopolygalacturonase (endoPG) production, important for fungal penetration, endopolygalacturonase showed reduced ABS symptoms (Isshiki et al. 2001). Fruits of Fortune mandarin, *Citrus limon* and *Citrus paradisi*, inoculated with *A. alternata* showed a degradative metabolism of flavonoids (flavanones, flavones and polymethoxyflavones) and *de novo* synthesis of the phytoalexin caused by an extracellular fungus laccase. Study of the substrate specificity of this enzyme revealed that flavonoids are substrates of *A. alternata* laccase suggesting its role in pathogenesis.

Citrus genotypes have been tested in order to evaluate the resistance to the fungus. In the overall more studies defined that clementine, Willowleaf and satsuma mandarins are resistant whereas genotypes Dancy and Fortune mandarins, Orlando, Minneola and Nova tangelos and the Murcott tangor are susceptible. Diploid progeny analysis suggested that inheritance of ABS resistance in citrus is controlled by a single recessive allele. Therefore, resistant cultivars are considered to be recessive homozygous for this locus, whereas susceptible cultivars could be heterozygous or homozygous dominant. Since the single locus inheritance of resistance, segregation is expected therefore markers for assisted selection were investigated.

In a recent study, a region containing the so-called ABSr locus, near the centromere on chromosome III using bulked-segregant and half-tetrad analyses from triploid populations was

located (Cuenca et al. 2013). This region was flanked by a Simple Sequence Repeat (SSR) marker (TTC8) and a Single Nucleotide Polymorphism (SNP) marker (CiC3248-06), found at 3.77 and 1.71 cM from the ABSr locus, respectively, delimiting a 3.3 Mb genome region. Moreover, no recombination was observed between another SSR marker (AT21) and the ABSr locus. This locus seemed to be included in a genomic region very rich in disease resistance homologous genes.

In a further study Cuenca et al. (2016) fine mapped the ABSr locus on LG III of the clementine's genetic map, using a 268-diploid progeny arising from a heterozygous susceptible × resistant hybridization, and identified candidate genes for resistance. This study also allowed to develop SNP molecular markers for efficient Marker Assisted Selection in citrus breeding programs.

Cuenca et al. (2016) limited the candidate region containing the ABSr locus to 1.5 cM flanked by two SNP markers at 1.1 and 0.4 cM, corresponding to 366 kb in the clementine reference genome (between positions 25.496.094 and 25.862.085 in the chromosome III). This region contained eight genes harboring NBS-LRR repeats and, candidate as genes for ABS resistance. Among the identified resistance genes, Ciclev10018637 and Ciclev10023511 encode for a Leucine-Rich Repeat (LRR) receptor-like protein with serine/threonine kinase domain. LRR receptor-like kinases (LRR-RLK) have a central role in signaling during pathogen recognition and activation of plant defense mechanisms, and developmental control. Both genes were mapped very close to the most significant SNP related to ABS resistance (SNP08). Another strong candidate for ABS resistance found within the region of interest and close to the SNP08 was the gene Ciclev10024361, encoding for an S-adenosyl-L-methionine dependent methyltransferases superfamily protein, with thiopurine S-methyltransferase superfamily protein. This gene is a good target for achieving resistance against necrotrophic pathogens, and therefore, for resistance to ABS.

Numerous genes involved in resistance to necrotrophs studied in other pathosystems were blasted against the citrus genome identifying many putative hortologues many of which located in chromosome III, although outside the region of interest, which deserve further attention (Cuenca et al. 2016). However, the study of the strongest candidate genes for ABS resistance in Citrus, Ciclev10018637, Ciclev10023511, and Ciclev10024361 could allow a good starting point to determine whether these genes are really involved in the Alternaria-citrus interaction.

In the same study it was determined that a single SNP marker, SNP08 flanking the ABSr locus, could discriminate the susceptible from the resistant genotypes. The SNP marker linked with the dominant susceptible allele of the ABSr locus is *G*.

16.3.3 Citrus Tristeza

Citrus tristeza is caused by the Citrus Tristeza Virus (CTV, Closteroviridae): a pathogen that changed the course of the citrus industry (Moreno et al. 2008). CTV can cause any of four distinct syndromes in citrus plants, depending on the virus isolate and the scion/rootstock combination. Decline (D-CTV) is a bud union disease that develops only in susceptible scion/rootstock combinations, when grafted on sour orange rootstock. The observed decline can be extremely rapid ('quick decline'), with wilting and death of trees occurring within a few days or weeks, or it can be a slower process, occurring over months or even years. Stem pitting (SP-CTV) is induced by an aberrant cambium development, resulting in pits in the wood, which reduces the plant growth, the size of the fruit and productivity, irrespective of the rootstock, and can affect both rootstock and grafted varieties. Seedling yellows (CTV-SY), characterized by stunting and leaf chlorosis, affects only sour orange, lemon and grapefruit. A fourth form of disease is associated to a complete lack of symptoms on most hosts, except for vein clearing and stem pitting, when the virus multiplies to high titers (Dawson et al. 2013). CTV decline is kept under control by

using resistant rootstocks, while stem pitting is mitigated by cross protection using weak strains of the virus.

CTV is a positive-sense single-stranded RNA (ssRNA) virus, member of the genus Closterovirus, with a complex genome (19.3 kb RNA divided into 12 ORFs and two UTRs), causing serious economic losses to citrus industry worldwide. CTV is a phloem-associated virus. It replicates in the cytoplasm of companion or phloem parenchyma cells of its hosts. It is graft-transmitted through the vegetative multiplication of infected host plants and by aphids in a semi-persistent manner (Moreno et al. 2008). Following the most recent review of current knowledge on CTV, the large number of isolates reported have been grouped into strains (Harper 2013). Their characterization is a prerequisite for a reliable control, breeding and surveillance program. Sequencing and biological assays demonstrates that CTV isolates assigned to a strain can differ remarkably in their phenotypes and infected plants may contain a pool of sequence variants from a single strain or several strains, resulting in isolates with mixed virus populations (Harper 2013). As stated by EFSA Opinion on CTV (EFSA PLH Panel 2017) 'a combination of biological, molecular and, possibly, serological data are needed for a conclusive characterization of the genetic and pathogenic features of a CTV isolate'.

Natural resistance

Host interference in CTV infection mechanisms is dependent on the citrus genotype. The virus systemically infects its hosts using only the long-distance movement from source-to-sink, while cell-to-cell movement is absent or limited to only small clusters of adjacent cells (Folimonova et al. 2008), likely related to the interaction of virus gene products with specific hosts (Dawson et al. 2013). Citrus species counteract the attack of CTV by RNA silencing, a central host defense reducing viral degradation to contain replication and restrict the virus to phloem cells. The p20 and CP proteins overwhelm intercellular silencing while p20 and p23 suppress intracellular silencing (Lu et al. 2004).

The constitutive expression of p23 appears to be responsible of the CTV titer in sour orange, allowing the virus to escape from the phloem in sour orange and sweet orange (Fagoaga et al. 2011). Nevertheless, it has been observed that viral replication and infection are not completely blocked, and the virus and its hosts reach a balance so that the virus remains in the host without causing severe symptoms or plant death. Other non-conserved genes-p33, p18 and p13-are necessary in different combinations for movement and overcoming host resistance (Dawson et al. 2013). The balance between expression of the effectors induces substantial changes in the severity of symptoms (Tatineni and Dawson 2012), or in the accumulation of miRNAs (Ruiz-Ruiz et al. 2011) and alters the plant small RNA regulatory pathway, resulting in symptom expression.

In a broad sense most Citrus species as seedlings are tolerant to the disease, despite some low symptoms. A variable degree of natural CTV resistance had been found in some citrus and related genotypes, including *C. maxima*, *C. aurantium*, *Atlantia ceylanica*, *Fortunella crassifolia*, *Poncirus trifoliata*, *Severinia buxifolia*, and *Swinglea glutinosa* (Garnsey et al. 1987, 1997; Ghosh et al. 2014; Bernet et al. 2004, 2008; Yoshida et al. 1983; Mestre et al. 1997). However, their resistance is not absolute, and depend on the CTV isolates tested (Garnsey et al. 1997; Dawson and Mooney 2000). *P. trifoliata* is resistant to most CTV isolates, and used as rootstock to effectively control the damage caused by seeding-yellow and decline-inducing CTV strains, but is susceptible to stem-pitting and resistant breaking isolates that can overcome this resistance, and are able to replicate and systemically invade resistant plants (Harper 2013). In citrus production area with *P. trifoliata* as rootstock, it is necessary to be on guard against occurrence of stem-pitting strain.

Being only *P. trifoliata* sexually compatible with citrus, many *Citrus*× *Poncirus* hybrids have been developed by conventional breeding. Some hybrids showing resistance to CTV have been widely used as rootstocks for citrus tree, such as Swingle citrumelo (*C. paradisi* × *P. trifoliata*)

(Castle et al. 1993), Carrizo citrange (*C. sinensis* × *P. trifoliata*) (Castle and Tucker 1998), US-812 (*C. reticulata* × *P. trifoliata*) (Bowman and Rouse 2006). While have no way to be used as scion cultivar because the coincident introgression of some undesirable traits from *P. trifoliata*. Moreover, conventional breeding of citrus has many problems including inbreeding depression, polyembryony, and long juvenility period, indicating its application in scion improvement for CTV resistance seems practically impossible.

The upsurge of genetic transformation of resistant gene has raised hope to overcome the shortcomings of conventional breeding in scion improvement. Since the resistance of *P. trifoliata* was conferred by a single dominant Mendelian gene designated Ctv (Gmitter et al. 1996; Fang et al. 1998), which induces broad-spectrum resistance to most CTV isolates. Apparently, it works by confining the movement of the virus to the root cells. Sequence analysis of the Ctv genomic region show CTV resistance is in a 121-kb region comprising ten genes (Rai 2006) and another different linkage group (Mestre et al. 1997). Grapefruit plants transformed with some of these ten candidate Ctv-R genes showed different levels of resistance: lack of infection, slow spread or initial infection followed by its abortion (Rai 2006). Despite a CC-NB-LRR R protein has been characterized the corresponding Avr CTV gene is still unknown (Rai 2006) and some of the viral proteins recognized by NB-LRR are not VSRs (de Ronde et al. 2014). Recently, the researches of Sahin-Cevik et al. (2014) and Gomez-Munoz et al. (2017) revealed WRKY transcription factors, the RNA silencing and salicylic acid defense pathways may play role in CTV resistance of citrus, which provide reference for identification of the key resistance gene in future.

Virus-driven resistance

Citrus plants can obtain virus resistance by the infection of a mild strain of the same virus in advance. This virus-derived resistance is called cross protection, which has been applied in CTV control. Several citrus producer countries have successfully isolated mild strains protective against severe SP-CTV isolates, including Brazil, South Africa, Australia, Japan and China (Costa and Muller 1980; Van Vuuren et al. 1993; Broadbent et al. 1991; Zhou et al. 2008). In Brazil, the protective CTV isolates developed by Muller and Costa have proven highly effective for over 40 years, and have been established in more than 80 million Pera trees as pre-immunization (Zanutto et al. 2013). Zhou et al. (2002) found CTV cross protection is caused by post-transcriptional gene silencing (PTGS), and its efficiency depend on the similarity between mild and severe virus strains in genetic background. A super infection exclusion mechanism has been shown in homologous genotypes inside the strain T36 (Folimonova 2013). It is possible that cross protection could be influenced and even broken by the change of host and field environment (Powell et al. 1992; Scott et al. 2013), which limit the development of this control strategy.

Another virus-driven resistance just like a simplified cross protection, is conferred by viral genes or sequences which are transformed into plant genome by genetic engineering. Some transgenic citrus lines with CTV major coat protein gene (p25), exhibited protection against CTV, but some others were susceptible (Dominguez et al. 2002). Similar results were also observed in transgenic citrus lines with other CTV genes. This resistance difference among transgenic lines have relationship with the accumulation of transgene-derived siRNA (Fagoaga et al. 2006; Lopez et al. 2010), indicating the second virus-derived resistance is also caused by PTGS. To directly utilize PTGS as means to impart CTV resistance into citrus plants, transformed vectors with stem-loop structure were designed to attenuate or block virus gene expression. Soler et al. (2012) found the stem-loop structure targeting simultaneously the three viral silencing suppressors (p20, p25 and p23) may achieve full resistance to CTV in citrus.

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