Tropical Plant Pathology (2018) 43:279–288
https://doi.org/10.1007/s40858-018-0229-x

REVIEW ARTICLE

Genetic tools and strategies for citrus breeding aiming at resistant rootstocks to gummosis disease

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Received: 27 July 2017 / Accepted: 18 May 2018 / Published online: 8 June 2018

Abstract

Phytophthora nicotianae Breda de Haan (syn. *Phytophthora parasitica* Dastur), causal agent of citrus gummosis disease, has caused great damage to citrus orchards throughout the world. While chemical and horticultural measures do not guarantee the preventive or curative control of citrus gummosis, the use of resistant rootstocks is the most reliable management strategy against the disease. Aiming at the development of citrus rootstocks resistant to gummosis and to better elucidate the *Phytophthora*-citrus pathosystem, citrus breeding programs have been ongoing worldwide, mostly employing directed crosses. These studies have succeeded in identifying differences in symptom development between resistant and susceptible rootstocks, as well as in the progeny of their crosses. In addition, differentially expressed genes were assessed, which ultimately should lead to the identification of markers involved in resistance to *P. nicotianae*. In this review we summarize the current knowledge of the molecular basis of citrus gummosis and the main strategies employed to obtain genetically resistant rootstocks.

Keywords *Citrus sunki* · *Phytophthora parasitica* · *Poncirus trifoliata* · Citrus · Disease management · Induced defense · Plant breeding

Introduction

Citriculture is one of the most important agricultural and agroindustrial activities worldwide. Citrus fruits are widely used to produce beverages, consumed *in natura* and are source of many essential oils and compounds used in the pharmaceutical industry. In 2015, the world production of citrus was 130.9 million tons, with China (29%), Brazil (14%) and the USA (6%) as the main producers. Brazil (24%) is the largest sweet orange producer, followed by China (10%) and India (9.9%) (FAO 2016).

One of the oldest and most important citrus disease in the world is citrus gummosis, caused by the pseudofungi *Phytophthora* spp. (Stramenopila, Oomycetes). The most important causal agent species, *P. nicotianae* Breda de Haan (syn. *P. parasitica* Dast.) and *P. citrophthora* (Sm. & Sm.) cause severe damage to citrus nurseries and orchards worldwide (Graham and Feichtenberger 2015). *P. nicotianae* is the predominant species associated with this disease in Brazilian citrus crops, being able to cause infection in both the roots and in the stem (Panabières et al. 2016).

The resistance mechanisms to *Phytophthora* are still not well understood in citrus. Due to differences in type of affected tissues and different responses of various species of citrus to infection, it is known that more than one mechanism of resistance and/or tolerance is involved. Many studies using quantitative evaluations of citrus genotypes for gummosis susceptibility indicate *Poncirus trifoliata* and Swingle citrumelo, a hybrid of trifoliate with grapefruit, as tolerant to either foot or root rot. Both restrain the lesion caused by infection, and are thus promising sources of resistance for citrus breeding

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Section Editor: Silvaldo Silveira

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programs aiming to develop resistant varieties and rootstocks to gummosis (Graham and Timmer 2003; Graham and Feichtenberger 2015).

Recent studies attempted to explain the molecular basis of citrus response to gummosis, and to report the *P. nicotianae* transcriptome. These studies have reported the changes in global gene expression profiles and have shown differentially expressed genes involved in a number of processes, such as cell defense, photosynthesis and carbohydrate metabolism (Boava et al. 2011a; Boava et al. 2011b). 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) (Fernandes et al. 2009; Boava et al. 2011a; Boava et al. 2011b). In addition, genes associated with pathogenicity, including effectors, host colonization and defense in the host, were reported in *P. nicotianae* (Rosa et al. 2007).

Planting different varieties in orchards is of extreme importance for the citrus industry, since the annual production is better distributed, and productivity is more secure and stable, being beneficial from a pest management perspective. Additionally, traditional and biotechnological breeding strategies have been combined to diversify citrus crops varieties and develop citrus rootstock resistant to gummosis disease (Zulian et al. 2013; Boava et al. 2003; Medina Filho et al. 2003). In parallel, it is known that there is a strong interaction between *P. nicotianae*, the bacteria belonging to the genus '*Candidatus* Liberibacter spp.' that cause HLB (huanglongbing or greening), the most damaging citrus disease worldwide, and global climatic changes. In this context, it is necessary to have better management of citrus gummosis disease, based not only on the application of chemicals, but also on the sustainability of the environment created by the use of both resistant rootstocks and newly developed hybrids (Graham and Feichtenberger 2015; Panabières et al. 2016).

Citrus gummosis disease and its control

Considering the *Phytophthora*-citrus complex, gummosis disease stands out with its various synonyms, such as foot rot, stem rot and stem gummosis disease. Despite being known since the tenth century in the Iberian peninsula, and with large outbreaks in countries such as Portugal, Spain, France, Italy and Greece as well as in the American continent during the nineteenth century, citrus gummosis was only reported in Brazil in 1917. Since then, this disease has plagued commercial nurseries and orchards distributed throughout the country, with significant losses mainly in the state of São Paulo, where the Brazilian citrus industry is concentrated (Feichtenberger 2001; Medina Filho et al. 2003).

Phytophthora spp. belongs to the Oomycota, which is a diversified group of achlorophyllous and filamentous algae whose growth habit and reproductive structures (mainly sporophore) are related to filamentous (true) fungi. Due to a number of structural, biochemical, physiological and molecular characteristics, they are currently classified in the kingdom Stramenopila, phylum Oomycota, class Oomycetes, order Peronosporales and family Pythiaceae (Alexopoulos et al. 1996; Gallegly and Hong 2008; Shivankar et al. 2015). Unlike fungi, which possess a cell wall composed of chitin, Stramenopila and oomycetes in general have cell walls composed of β1–3 and β1–6 glucans as well as cellulose (Hawksworth et al. 1995).

The species of the genus *Phytophthora* infect several plant species worldwide. They are soil-borne pathogens and causal agents of many diseases that affect the radicles, roots, stem, leaves, tips and even fruits on susceptible rootstocks. Twelve *Phytophthora* species can infect citrus: *P. nicotianae*, *P. citrophthora*, *P. palmivora*, *P. boehmeria*, *P. cactorum*, *P. capsici*, *P. citricola*, *P. cinnamomi*, *P. drechsleri*, *P. hibernales*, *P. megasperma* and *P. syringae*. Gummosis is caused mainly by *P. nicotianae* (syn. *P. parasitica*) and *P. citrophthora* (Sm. & Sm.) (Siviero et al. 2002a; Caixeta et al. 2013).

In Brazil, *P. nicotianae* is solely responsible for causing gummosis disease in more than 90% of the cases recorded in citrus orchards and nurseries. Infected plants present a large variety of symptoms, such as darkening and death of the bark and wood, yellowing and fall of leaves, which may cause foliar and tip necrosis, post-harvest brown rot, darkening and death of roots, reduction of radicles, low development, wilt and even death of the plant (Boava et al. 2003; Medina Filho et al. 2003). According to Feichtenberger (2001), the most recurrent symptoms of gummosis correspond to gum exudation in citrus stem lesions and the tipping of the plant with longitudinal cracking of the bark, which is more evident in susceptible varieties. In the internal area of the stem, the color of the affected bark and wood tissues is yellow at first, and then becomes brownish. With a wide range of factors that can intensify the incidence of the disease, including climate and susceptibility of the variety, the development of the lesion occurs longitudinally, with variable height, and laterally, being able to cover a large part of the circumference of the affected stem, causing the plant to die (Gasparotto et al. 1998) (Fig. 1).

Species of the genus *Phytophthora* survive well in the soil, usually have non-septate hyphae and well developed mycelial stalk, producing sexual and asexual spores, as well as resistance structures known as chlamydospores that survive under unfavorable conditions in the soil or within infected plant tissues. During prolonged periods of rain with high humidity, *P. nicotianae* can form oospores, which are sexual spores originated from the fertilization of the oogonium (female

Fig. 1 Symptoms of citrus gummosis disease. **a** Yellowing of leaves and death of an lemon tree. **b** Stem rot lesion in an lemon tree

gametangium) by the antheridium (male gametangium). These two types of spores can act as the primary route of infection, and under ideal conditions of temperature and humidity, they germinate forming hyphae on the soil surface or in affected plant tissues, developing the persistent, papillate sporangia (zoospore-producing structures). The zoospores, which are asexual biflagellate spores with mobility, are released in abundance by the sporangia, and when in contact with the host they undergo differentiation and form cysts that germinate, giving rise to hyphae that infect the roots and stem. By means of splashes, these hyphae can also infect branches, leaves and fruits of the scion. With the colonization of tissues and the appearance of gummosis symptoms, new sporangia may be formed, and a new cycle of the disease begins (Feichtenberger 2001; Tyler 2002).

The use of sanitation methods is important for healthy production of citrus seedlings and rootstocks. Protected nursery regulations in Brazil triggered the development of several control measures that can be employed focusing on the prevention of *Phytophthora* associated with citrus gummosis disease in the field (Table 1). Protected nurseries produce citrus seedlings under certified screen houses, in which artificial pathogen-free substrates are used to grow plants. Nonetheless, the gummosis-resistant or tolerant combinations of citrus varieties and rootstocks (genetic resistance) constitute the most efficient way of combating the disease, since it is less

Table 1 Control measures for the prevention of *Phytophthora*-associated citrus gummosis disease

Type	Actions	References
Horticultural control	Selection of areas for planting; crop rotation; practices of soil conservation; organic and mineral fertilization in adequate quantity; use of healthy seedlings with good genetic, agricultural and phytosanitary characteristics; windbreaks; management of weeds	Feichtenberger 2001; Graham and Feichtenberger 2015
Chemical control	Fungicides and resistance inducers made with calcium and potassium phosphite	Boer et al. 1990; Feichtenberger 2001
Physical control	Fumigation or steam for thermal treatment of the lesions	Feichtenberger 2001; Feichtenberger 2003
Biological control	Use of natural enemies of the pathogens	Feichtenberger 2003; Graham and Feichtenberger 2015
Genetic control	Selection of tolerant/resistant citrus rootstock combinations by breeding programs	Graham and Timmer 2003; Graham and Feichtenberger 2015

costly compared to horticultural, physical and biological controls, and efficiently reduces the impact of the use of chemical compounds in the environment (Feichtenberger 2001; Feichtenberger 2003).

Phytophthora-citrus interaction and mechanisms of pathogenicity and resistance

It has already been demonstrated that plant hosts, when interacting with *Phytophthora*, may respond to infection by callose deposition (O'Donnell et al. 2003; Giesbrecht et al. 2011). In resistant plant species, layers of callose may encapsulate hyphae, a phenomenon carried out by the plasma membrane of the plant cell. In addition, changes in the concentration of phenolic compounds in the phloem, such as gallic acid and tyrosol, may be related to induction of resistance against *Phytophthora* spp. (Ockels et al. 2007). Araújo et al. (2008) verified the changes in phenolic compounds concentration in the leaves of different species of citrus rootstocks when infected by *P. nicotianae*, and found that there is a higher total phenol content in resistant varieties than in those susceptible, in both stem (gummosis) and root infections. Thus, it was assumed that there is an early accumulation of phenolic compounds at the infection sites of resistant plants, which attempt to block the spread of the pathogen to other tissues.

P. nicotianae is a broad pathogen, which can be used in both intra- and interspecific comparative genomic analyzes in order to better characterize its pathogenicity, and biochemical and molecular interactions with citrus. This characterization includes the identification of effector proteins: how they are organized and distributed in the genome, and their role in recognition, infection and defense induction in plants (Kamoun et al. 2014). The *P. nicotianae*/Citrus Expressed Sequence Tags (PP/CitEST) database was constructed based on the *P. nicotianae* transcriptome in an attempt to understand citrus gummosis disease, according to Rosa et al. (2007). In this study, similarity analyses of massive sequencing of transcripts from *P. nicotianae* showed homologies with gene sequences of pathogenic fungi and pathogenic oomycetes related to pathogenicity and defense factors. Genes that encode cell wall degrading proteins were identified, including phospholipases, glucanases and endopolygalacturonases, enzymes generally involved in the first steps of a plant disease. In addition, elicitins (ELI) and another group of effectors that induce necrosis in plants, such as crinkling and necrosis-inducers (CRN) and necrosis-inducing proteins (NIP), were also identified and phylogenetically grouped (Rosa et al. 2007). An increase in expression of these effectors has been 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; Dalio et al. 2011; Oßwald et al. 2014) and more specifically *Citrus sunki* rootstock (susceptible) (Dalio et al. 2018a).

P. trifoliata and Swingle citrumelo rootstocks have the ability to regenerate roots in the presence of *P. nicotianae* from the tip of the infected roots themselves. It is not known yet which resistance factors limit the infection to the root tip, but it is believed that there may be a relationship with phytoalexins, which are phenolic compounds found in the woody tissues of citrus plants infected with *Phytophthora* (Graham and Timmer 2003). Two phytoalexins, named escoparone and seselin, have been reported as being synthesized in response to infection by *P. citrophthora* and *P. nicotianae* in the bark and roots of citrus, respectively, inhibiting the growth and development of these pathogens (Vernenghi et al. 1987). Erwin and Ribeiro (1996) obtained a positive correlation between the concentration of escoparone in the roots and stems of citrus seedlings and the resistance factor, as there was a faster production of this phytoalexin in the resistant cultivar compared to the susceptible one.

In plant-pathogen interactions, there is often the occurrence of processes resulting from gene-to-gene immunity in the host, which restrict the damage caused by the invader pathogen. This happens through a coordinated system of signals that results in the activation of transcription factors, such as the ones belonging to the WRKY family; and in the expression of a group of resistance proteins called pathogenesis-related proteins (PRs), such as many classes of chitinases (encoded by PR3, PR4, PR8 and PR11 genes) and glucanases (encoded by the PR2 gene), which are cell wall degrading enzymes already identified in citrus presenting antifungal properties. Among the 17 proteins (PR1 to PR17) described in the PR family, it is known from *in vitro* and *in planta* experiments that PR1, PR2 and PR5, which are responsive to salicylic acid (SA), have anti-oomycete properties, contributing directly to the defense against *Phytophthora* spp. (Porat et al. 2000; Christensen et al. 2002; Meyers et al. 2003; Dalio et al. 2014). In a gene expression study that sought to evaluate the response of susceptible (*C. sunki*) and resistant (*P. trifoliata*) genotypes of citrus to infection caused by *P. nicotianae*, 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).

SA is part of a large family of secondary metabolites produced by plants. It is a phenolic compound that regulates several vital functions, including flowering, thermogenesis and resistance against diseases caused by biotrophic and hemibiotrophic pathogens. The main regulator of the downstream pathway of SA is the NPR1 (non-expressor of PR genes 1) protein. Its interaction with transcription factors such as those belonging to the TGA and WRKY gene families results in the induction of systemic acquired resistance (SAR), an integrated immune response which provides long-lasting resistance, and in the induction of resistance (R) genes (Glazebrook 2001; Vlot et al. 2009; Dempsey et al. 2011; Diaz-Puentes 2012). The overexpression of phenylalanine ammonia lyase (PAL), involved in SA biosynthesis, produces high levels of this phytohormone and, consequently, the expression of PR genes, which provide greater resistance (Vlot et al. 2009). It is known that the PAL protein, besides participating in the synthesis of lignin and suberin, also regulates the formation of phenolic compounds such as phytoalexins, a frequent phenomenon in the *Phytophthora*-citrus interaction (Erwin and Ribeiro 1996; Taiz and Zeiger 2002). In addition, the tissue lignification process, actively induced after infection, constitutes a physical barrier against pathogens by promoting lignin polymerization, a reaction that requires the enzymatic activity of peroxidase (POX) proteins (Resende et al. 2003).

Boava et al. (2011b) evaluated *P. trifoliata* and *C. sunki* rootstocks in response to *P. nicotianae* infection, as well as the expression of POX genes, others related to PR, and lipoxygenase (LOX), a gene that has been widely associated with plant defense against pathogens. The results showed an overexpression of POX and LOX in the resistant rootstock at later stages of infection, compared to the susceptible ones. This suggests that these genes may be suitable candidates in citrus resistance breeding to *P. nicotianae*. LOX genes are involved in the biosynthesis of jasmonic acid (JA), production of reactive oxygen species (ROS), development of the hypersensitive response (HR), including cell death, and formation of antimicrobial compounds (Jalloul et al. 2002; Pieterse et al. 2009; Lyons et al. 2013).

The CitEST project consitutes a large database obtained by ESTs from citrus species such as *C. sinensis*, *C. clementina* and *P. trifoliata* under various conditions, including biotic stresses. This is an important genomic tool for the identification of genes that encode resistance proteins against the main pathogens that infect citrus crops. A number of *in silico* studies analyzed CitEST and identified several genes with similarity to R genes, which could belong to different categories, including TIR-NBS-LRR and CC-NBS-LRR (Guidetti-Gonzalez and Carrer 2007; Reis et al. 2007; Targon et al. 2007). Other research has used global gene expression to demonstrate the involvement of R genes in the defense response of citrus to pathogens. In a pathosystem comprised of tangerine and the bacterium *Xylella fastidiosa*, a gene of the type CC-NBS-LRR was up-regulated one day after infection (Rodrigues et al. 2013). In parallel, a gene of the type TIR-NBS-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*. This demonstrates that such genes are possibly related to the defense process of the plant against this pathogen. In addition, these two genes were also differentially expressed between the resistant and susceptible parent, being promising candidates for the identification of transcripts involved in resistance to *P. nicotianae* (Boava et al. 2011a). Infection by *Phytophthora* spp. triggers a series of dysfunctions related to plant water conductivity because of root destruction, which may lead to ABA imbalance (Oßwald et al. 2014).

A molecular model comprised of three resistance mechanisms can be proposed from these studies based on changes in global gene expression profiles in *Phytophthora*-citrus interactions. At first, effector molecules secreted by the oomycete interact with resistance R proteins, triggering the interaction with the NPR1 protein, which in turn activates the accumulation of SA and generates defence responses. SA in the cytoplasm causes a change in the redox potential of the cell, triggering the induction of pathogenicity-related genes with anti-oomycete properties such as PR1, PR2 and PR5, which encode cell wall and plasma membrane degrading enzymes. In another mechanism similar to the first, additional effectors can be recognized by other cytoplasmic R proteins responsive to accumulation of ABA, such as those encoded by LEA5 and RPS4, generating defence responses from a change in the redox potential of the cell. Finally, there is an alternative mechanism in which the proteins PAL, cinnamyl alcohol dehydrogenase 2 (CAD2) and POX are involved in the synthesis of phenolic compounds which are precursors of lignin and suberin, resulting in a significant defense response in which the last stage catalyzed by POX is dependent on hydrogen peroxide (Fig. 2).

Genetic breeding of citrus rootstocks

A number of genetic breeding programs for citrus rootstocks are ongoing throughout the world, such as the ones at Centro APTA Citros Sylvio Moreira (which possesses one of the largest citrus germplasm banks in the world) and Embrapa Mandioca & Fruticultura Tropical in Brazil, Huazhong Agricultural University in China, the University of California-Riverside in the USA, and Instituto Valenciano de Investigaciones Agrarias in Spain. In these programs, controlled hybridizations have been conducted and as a result several rootstocks that are currently used in citriculture have been obtained, including mandarins, citranges and citrandarins, among others. This allowed the creation of new varieties, while maintaining the diversity of the citrus crop, which generates increased productivity, tolerance to diseases

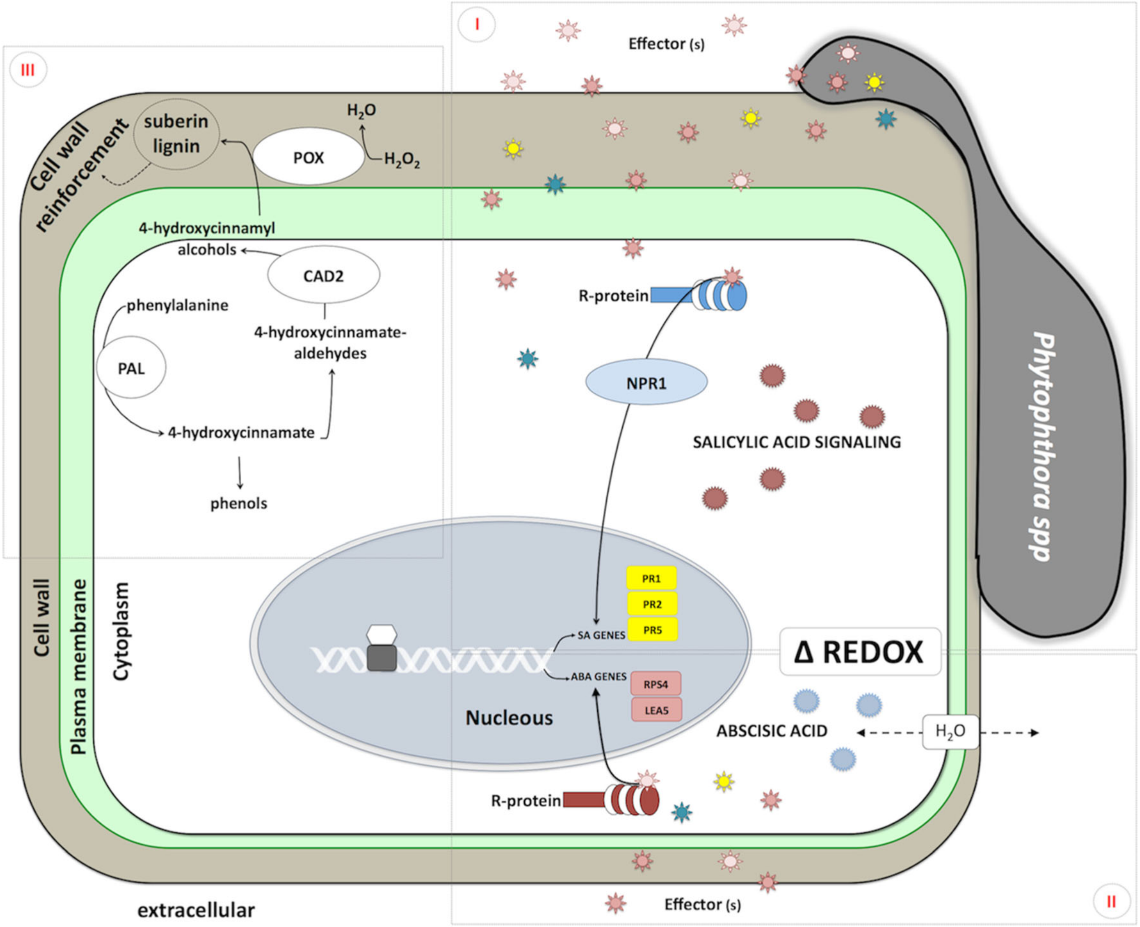

Fig. 2 Proposed molecular model of the plant defense mechanisms in the *Phytophthora*-citrus interaction. **I.** Effectors interact with R proteins encoded by PR1, PR2 and PR5, triggering the interaction with NPR1 protein which activates the accumulation of salicylic acid (SA) in the cytoplasm, causing a change in the redox potential of the cell. This generates defence responses related to the expression of cell wall and plasma membrane degrading enzymes. **II.** Other effectors interact with R proteins encoded by RPS4 and LEA5, which are responsive to the accumulation of abscisic acid (ABA) in the cytoplasm, which also causes a change in the redox potential of the cell. This generates defence responses related to the expression of proteins that act in plant water conductivity. **III.** PAL, CAD2 and POX are enzymes involved in the synthesis of phenolic compounds which are precursors of lignin and suberin, with the last stage, catalyzed by POX, being dependent on hydrogen peroxide (H_2O_2)

and the development of new genetic resources for use and conservation (Kahn et al. 2001; Oliveira 2006; Liu and Deng 2007; Oliveira et al. 2008).

Using rootstocks for citrus cultivation brings several benefits to citriculture, contributing to the breeding of a number of physico-chemical characteristics (absorption, synthesis and utilization of nutrients, transpiration and chemical composition of leaves, response to products of leaf and fruit abscission, color of the bark and juice, as well as acidity and sugar content of the juice), biological characteristics (size, precocity of production and longevity of plants, maturation and durability of fruit in the plant, tolerance to agricultural diseases and pests), and also those characteristics related to abiotic factors (cold, salinity, drought, post-harvest conservation, productivity and fruit quality) (Pompeu Júnior 1991). The adequate combination of rootstock and scion can guarantee to the grower advantages for planting certain species of citrus which are better adapted to the climate and soil of a certain area (Pompeu Júnior 2005).

The goals of genetic breeding programs of citrus rootstocks have been to associate the fitness of the plant with a higher, stable and good quality production, as well as to combine resistance to diseases with tolerance to several abiotic stresses, such as drought. As an example, *Citrus limonia* Osbeck (rangpur lime) has been the main rootstock used by the Brazilian citrus industry. It has precocity and allows high productivity of the scion, yields good harvest from three-year old plants, is well adapted to periods of drought and is tolerant to *Citrus tristeza virus* (CTV). However, *C. limonia* is susceptible to diseases such as gummosis and citrus sudden death (CSD), which exemplifies the need for the development of new citrus rootstocks that may thrive under different conditions (Kahn et al. 2001; Cristofani-Yaly et al. 2005; Liu and Deng 2007).

New strategies and genetic tools for citrus rootstock breeding aiming at resistance to *Phytophthora* diseases

A large variety of citrus rootstocks are susceptible to *Phytophthora*, including rangpur lime which, as mentioned in the previous section, is the main rootstock in Brazilian citrus orchards. Modern plant biotechnology techniques could help to accelerate the selection and breeding of citrus rootstock resistant to *Phytophthora* diseases.

The application of molecular technologies has provided additional gains in the efficiency of citrus rootstock breeding programs (Fig. 3). The use of molecular markers reduced the time for the characterization of large numbers of genotypes (Bered et al. 1997; Souza et al. 2012). Thus, the identification and localization of quantitative trait loci (QTLs) in genetic linkage maps are targets of study in several citrus breeding programs. For instance, the use of genetic mapping to detect QTLs linked to resistance to various diseases has resulted in the construction of two linkage maps, one for the *P. trifoliata* parent and one for *C. sunki* (Cristofani-Yaly et al. 1999). *P. trifoliata* is an important genotype due to its advantageous agronomic characteristics, including resistance to *Phytophthora*, CTV and the citrus nematode *Tylenchulus semipenetrans*. Also, using the same F1 progeny of *Citrus sunki x Poncirus trifoliata* from Cristofani-Yaly et al. (1999), QTLs associated with *Phytophthora* resistance in *P. trifoliata* and *C. sunki* were detected, with a total of two QTLs being detected in the map of the resistant *P. trifoliata* parent and only one in the map of *C. sunki*, which is susceptible to the pathogen (Siviero et al. 2006). The authors were able to select F1 hybrids with similar or greater levels of resistance compared to *P. trifoliata*. Hybrids of microtangerines (such as *C. sunki*) with *P. trifoliata* are called citrandarins. They are part of a new generation of citrus rootstocks which combine the advantageous characteristics of tangerines, such as tolerance to decline and CSD, with the qualities of the trifoliates, such as resistance to *Phytophthora*, CTV and *T. semipenetrans*, besides the great capacity to form compact and productive plants (Schinor et al. 2013). Many types of citrandarins have already been produced in the USA and evaluated in Brazil, with promising results regarding resistance to *Phytophthora* (Blumer and Pompeu Júnior 2005).

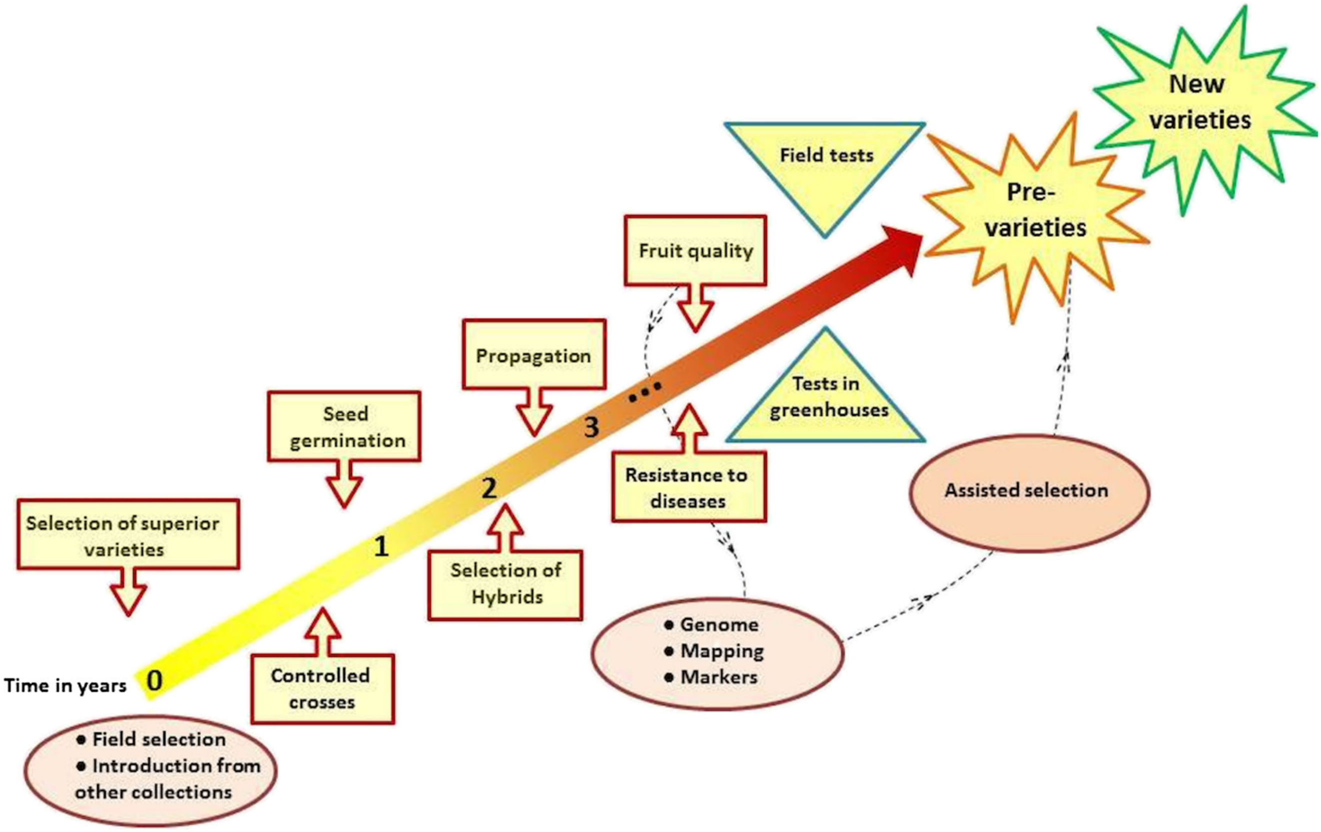

Fig. 3 Gain in years on the efficiency of genetic breeding of citrus rootstocks (modified according to Souza et al. 2012). **0–1.** Introduction and selection of superior varieties in the field to be submitted to controlled crosses and later seed germination. **1–2.** Selection and propagation of highly segregating hybrids. **2–3.** Elaboration of tests under field and greenhouse conditions to evaluate characteristics such as fruit quality and resistance to diseases. The selected progeny is assessed by molecular techniques, including marker-assisted selection and genetic mapping, which results in genetically characterized pre-varieties that reflect the acquisition of new potential varieties for greater quality and phytosanitary control

Table 2 Inoculation methods to evaluate resistance to *Phytophthora* associated with citrus gummosis disease

Method	Description	References
In vitro evaluation of plantlets	Insertion of a needle infested with the pathogen's mycelium in the region of the stem	Siviero et al. 2002a; Siviero et al. 2002b
Detached bark method	Detached bark infected with a disc of the culture medium containing *Phytophthora* is used for inoculation	Siviero et al. 2002a; Siviero et al. 2002b
Disc method	Insertion of mycelial discs originated from culture media colonized by the pathogen	Siviero et al. 2002a; Siviero et al. 2002b
Method of insertion under the bark	Contact of the pathogen with the plant without causing injury	Siviero et al. 2002a; Siviero et al. 2002b
Toothpick method	Introduction of toothpicks infested with *Phytophthora* on the stem of citrus plants	Siviero et al. 2002a; Siviero et al. 2002b
Needle method	Use of a metal needle or a spike of citrus previously contaminated with *P. parasitica*	Siviero et al. 2002a; Siviero et al. 2002b
Injection of zoospores	Use of a suspension of *Phytophthora* zoospores present in the soil for inoculation in incisions in the basal part of plants	Siviero et al. 2002a; Siviero et al. 2002b

As an innovative perspective, the isolation of new genes related to defense and the genetic transformation of citrus with these resistance genes consist in a combined strategy to obtain resistant plants (Cordeiro and Sá 1999; Talon and Gmitter Junior 2008). In addition, strategies can be elaborated using transgenic citrus plants for one or more introduced R genes, which are involved in the SA pathway that regulate (NPR1, TGA and WRKY) or act directly (PR genes) against *P. nicotianae*. The overexpression of these genes in response to the disease may lead to new varieties of citrus resistant to gummosis and other *Phytophthora*-related diseases. Using this strategy, Fagoaga et al. (2001) obtained transgenic citrus plants resistant to *P. citrophthora* (the other gummosis-triggering agent), which had an overexpression in the gene encoding a pathogenesis-related 5 (PR5) protein. PR5 encodes proteins called osmotins, which have several physiological functions, acting on plant development and in response to osmotic stress, cold tolerance and also biotic stress. These proteins have antifungal and anti-oomycete properties, as they make the plasma membrane of these microorganisms permeable, inhibiting hyphal growth and spore germination (Guzzo 2003).

On the other hand, the study of susceptible plants can generate the identification of susceptibility genes, which should then be avoided in any parent or hybrid. Recently, Dalio et al. (2018b) found that the effector PpRxLR2 is fundamental for the aggressiveness and virulence of *P. nicotianae*. The authors stated that very likely the PpRxLR2 effector targets a specific protein of the susceptible rootstock *C. sunki*. The gene encoding this protein may be considered to be a susceptibility gene and plants possessing this gene should not participate in any breeding program.

In relation to inoculation methods to evaluate resistance to *Phytophthora* associated with citrus gummosis, several techniques have been described in the literature (Table 2). The evaluation of resistance to the disease can be carried out based on incidence, in which the number of plants displaying gummosis symptoms are counted, as well as on severity, by measuring the area or length of the lesion on the stem of infected plants (Siviero et al. 2002b; Lima 2016). Siviero et al. (2002a) found that the most efficient methods to inoculate *P. nicotianae* correspond to the *in vitro* evaluation of plantlets, the disc method and the insertion under the bark in young plants. They also demonstrated that the measure of the total area of the lesion caused by the oomycete, as well as its length, are ideal variables for evaluation of the disease in plantlets, young plants and in the field (Fig. 4).

Concluding remarks

The identification of genes responsible for vital processes in recombinant systems is an important step in plant-pathogen interaction studies, allowing tracing gene expression profile of contrasting phenotypes (susceptible x resistant) and their variable hybrids in response to a disease. In order to develop new management strategies, it is necessary to have the knowledge

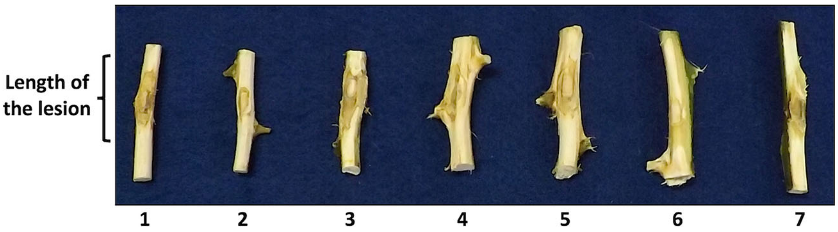

Fig. 4 Lesions caused by the inoculation of *Phytophthora parasitica* at 15 cm from the grafting region in *Citrus sunki* **(1)**, *Poncirus trifoliata* **(2)**, *Citrus limonia* **(3)** and four hybrids of *Poncirus trifoliata* x *Citrus sunki* **(4–7)** (modified according to Lima 2016)

about which is the best type of control measure, as well as about the resistance mechanisms that govern the molecular interaction of citrus with the pathogen.

The use of resistant rootstocks remains the most efficient control method of *Phytophthora* diseases in citrus plants, minimizing costs and reducing the impact caused by chemicals in the environment. As many rootstocks are moderately resistant to *P. nicotianae*, new rootstocks based on controlled crosses for the generation of hybrids are already being made in breeding programs, such as hybrids of microtangerines with trifoliate orange (citrandarins), and should continue to be established. Breeding programs should be based on resistance to *P. nicotianae* and at the same time on tolerance to various abiotic and other biotic stresses for the selection of new rootstocks.

Transgenic-based approaches, genome editing and effector-gene silencing are currently being performed in parallel with *Phytophthora* and citrus genome data analysis. In the near future, all information generated in these studies will provide a better understanding of the molecular basis of the interaction, unraveling more efficient targets for breeding programs aiming to control *P. nicotianae* diseases in citrus.

Acknowledgments This study was carried out with the financial support of Fapesp and the National Research Institute for Science and Technology on Citrus Genomics and Breeding. RPML was the recipient of a CNPq scholarship.

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