Biotechnology Research and Innovation (2020) 3, 95–109

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

REVIEW ARTICLE

Citrus biotechnology: What has been done to improve disease resistance in such an important crop?

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Received 16 August 2019; accepted 9 December 2019
Available online 3 January 2020

KEYWORDS
Transgenic plants;
GMO;
New breeding techniques

Abstract Citrus cropping is widely distributed as an important economic activity worldwide. Amongst the major producers are Brazil, China, United States, Mexico and some European countries. Brazil is the largest sweet orange producer accounting for more than three-quarters of the orange juice exports around the world, followed by China and the United States (Foreign Agricultural Service/USDA, 2019). Although juice is the main commodity in many countries producing citrus, by-products like essential oils, molasses, dried pulp, pectin, blend syrup and others are also part of the citrus trade chain. Since citriculture is threatened by several pathogens and its control is mainly based on regular chemical applications both during plant development and post-harvest, management becomes a non-environmentally friendly strategy. Meanwhile, consumers are searching for sustainable products of great quality and high aggregated value pressuring the agricultural industry to crop in a sustainable manner. In this scenario, citriculture also needs innovative solutions to meet such demand. Although classic breeding programs succeeded over time, citrus narrow genetic base and long evaluation periods turns it difficult to demand faster solutions for emerging problems. Biotechnology rises as a source of innovative solutions since new varieties can be developed for specific problems. In this context, the use of biotechnology approaches involving genetic engineering that allow the development of more resistant varieties are the focus of many research groups. Here we show how biotechnology has been used to develop citrus plants more resistant to the main phytopathogens that impact citrus production.

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https://doi.org/10.1016/j.biori.2019.12.004

Introduction

Citrus crops are widely distributed, representing an important economic activity worldwide. Amongst the major producers are Brazil, China, the United States, Mexico, and a number of European countries. Brazil is the largest sweet orange producer, accounting for more than three-quarters of the orange juice exports globally, followed by China and the United States (Foreign Agricultural Service/USDA, 2019). Although fruit juice is the main commodity in many countries producing citrus, by-products like essential oils, molasses, dried pulp, pectin, blend syrup, and others are also important components of the citrus trade chain.

Since citriculture is threatened by several pathogens, with control mainly based on regular chemical applications both during plant development and post-harvest, management becomes a non-environmental friendly strategy. Meanwhile, consumers are searching for sustainable products of great quality and high aggregated value, with pressure on the agricultural industry to produce in a sustainable manner. In this scenario, citriculture requires innovative solutions to meet such demands. Although classic breeding programs have succeeded over time, the narrow genetic base and long evaluation periods limit faster solutions for emerging problems. Biotechnology, by contrast, offers a source of innovative solutions for the development of new varieties for specific problems.

In this context, biotechnology approaches that involve genetic engineering through new breeding techniques (NBTs or Innovative Techniques for Precision Improvement) that enable the development of disease resistant varieties is the focus of many research groups. Here we show how biotechnology has been applied to develop citrus plants more resistant to the main phytopathogens that impact citrus production (Fig. 1).

Viral disease resistance: a matter of silencing

The two major viral diseases that affect citrus are leprosis and tristeza. The first was originally reported in the state of Florida, United States, in the 1990s. Later, Central and South American countries also reported the disease (Fawcett, 1911; Rodrigues, Kitajima, Childers & Chagas, 2003; NAPPO Phytosanitary Alert System, 2005; Castillo et al., 2011). Citrus leprosis can be caused by two types of non-systemic viruses, Citrus leprosis virus C (CiLV-C), which is the cytoplasmic type and predominant form, and the Citrus leprosis nuclear type (CiLV-N) (Colariccio et al., 1995; Rodrigues, Kitajima, Childers & Chagas, 2003). Both are transmitted by mites (*Brevipalpus* spp). Symptoms caused by both viruses are similar, showing necrotic and chlorotic lesions that can be present on leaves, fruits, twigs or branches, although CiLV-N tends to cause smaller lesions. Fruit drop can also be associated with characteristics of Citrus leprosis (Bastianel, Freitas-Astúa, Kitajima, & Machado, 2006; Bastianel et al., 2010; Castillo et al., 2011; Rossetti et al., 1990). CiLV-C genome sequences were reported by Locali-Fabris et al. (2006) revealing candidate genes for silencing in breeding programs.

Citrus tristeza virus (CTV) epidemics have occurred in different regions around the world. Brazil, Argentina and California fought the disease early in the 1930s. Later, Florida, Spain, Israel, and Venezuela reported epidemics, with outbreaks also occurring in Cyprus, Cuba, Mexico, the Dominican Republic and Italy, in 2002. The wide distribution of this pathogen might have originated from transporting of citrus plants or other propagative tissues (Moreno, Ambrós, Albiach-Martí, Guerri, & Peña, 2008). Together with virus dispersal, the use of the susceptible *Citrus aurantium* L. (sour orange) as rootstock created favorable conditions for CTV epidemics (Moreno et al., 2008).

Severe and rapid decline caused by tristeza is the result of CTV interactions and commercial hosts propagated on sour orange rootstocks (Moreno et al., 2008). Grafting onto sour orange requires review and new rootstock varieties for testing. If in one hand this big change means losses to agronomic traits, on the other hand it represents gains in terms of development of a modern citrus industry based on sanitation, quarantine and budwood certification procedures (Timmer, Garnsey, & Graham, 2000).

CTV replicates in phloem vessels and is transmitted by the aphid vector *Toxoptera citricida*. It causes three different syndromes depending on the species or scion−rootstock combinations, namely quick decline, stem pitting and seedling yellows. All syndromes cause cumulative economic losses due to decline, unthrifty or complete cessation of growth, with yield reductions over time (Timmer et al., 2000). Disease management practices involve quarantine and budwood certification to avoid CTV introduction in free areas where sour orange is the orchard rootstock. Also, eradication of infected trees when incidence is low is conducted to prevent or delay epidemics, and in areas where eradication is not possible due to efficient CTV dispersal by vectors, the use of tristeza-tolerant rootstocks is mandatory to prevent orchard decline (Gottwald, Polek, & Riley, 2002; Moreno et al., 2008; Navarro et al., 2002). For highly susceptible scion cultivars, such as sweet orange Pera, the use of a mild CTV strain is well succeeded example of cross-protection.

Since very few sources of resistance to CTV occur in citrus, transgenic breeding programs have been developed, mostly based on virus coat-protein mediated resistance (Beachy, Loesch-Fries, & Tumer, 1990). Results in developing a fully resistant plant evolved according to the understanding of silencing mechanisms through interfering RNA. Thus, the first studies reported a range of transgenic plants varying from susceptible to fully resistant to CTV, with resistance, in some cases, linked to accumulation of siRNA.

Transgenic Mexican lime plants were developed carrying the coat protein p25 from a mild and a severe strain of CTV. Resistance was observed in 33% of the transgenic plants with no symptom development and no viral detection (Domínguez et al., 2002). It was demonstrated that post-transcriptional gene silencing (PTGS) was activated in transgenic plants, leading to the accumulation of small interfering RNA (siRNA) and resulting in the resistant phenotype of the transgenic lines (Fagoaga et al., 2006). Grapefruit has also been used to evaluate transgene-mediated resistance to CTV, with plants transformed with constructions carrying the virus coat protein of four different isolates, the last 400 bp including part of the p23 gene and the 3ÚTR in sense and non-sense orientations, and the full gene encoding an RNA-dependent RNA polymerase. A delay in virus detection was shown for dif-

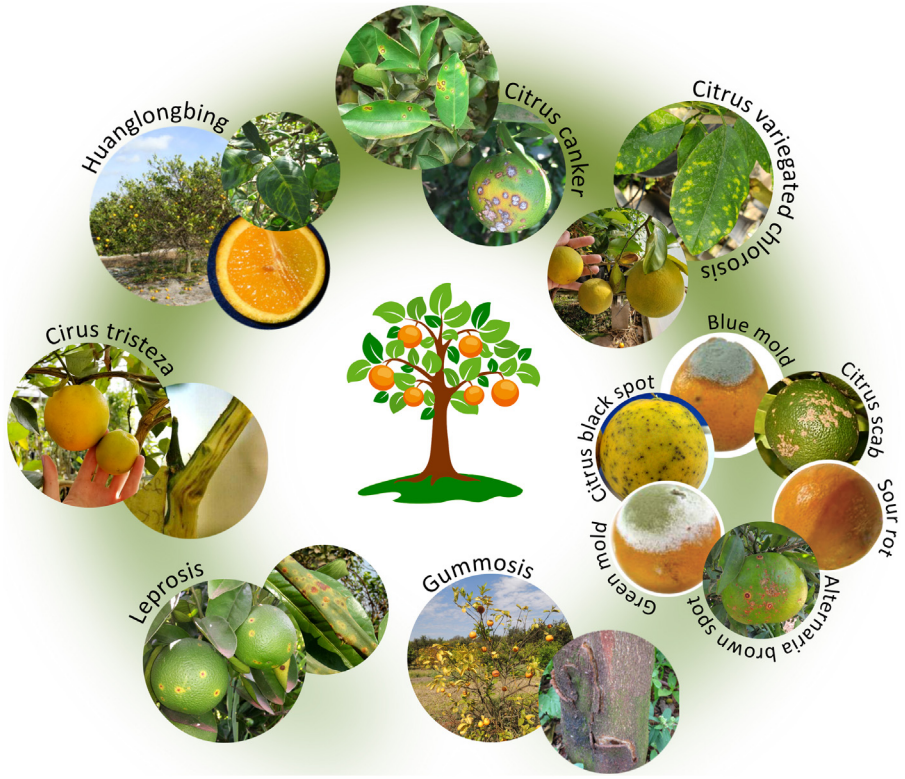

Figure 1 The main citrus diseases targeted by genetic engineering for pathogen resistance. Diseases caused by bacteria (huanglongbing or greening, citrus canker, citrus variegated chlorosis), fungi (blue old, sour rot, alternaria brown spot, green mold, and citrus black spot), oomycetes (gummosis) and viruses (leprosis and tristeza).

ferent transgenic lines after one year of inoculation and full resistance to CTV was reported for at least one line after three years of evaluation (Febres, Lee, & Moore, 2008).

Proof of concept investigation of hairpin structures to induce silencing through siRNA and enhanced resistance was conducted using Mexican lime. Here, the authors obtained three transgenic lines completely resistant to CTV that were both symptomless and with no virus detected even in non-transgenic rootstocks in which they were grafted (Soler et al., 2012).

Comprehensive strategies in the control of diseases caused by fungi and oomycetes

Citrus is affected by several diseases caused by fungi and oomycetes. These diseases occur in different parts of the plant and stages of development, as well as in fruits after harvest and during storage.

The two major post-harvest diseases caused by fungi in citrus are green mold, caused by *Penicillium digitatum* (Pitt & Hocking, 2009), and blue mold caused by *Penicillium italicum* (Ismail & Zhang, 2004), causing postharvest losses of up to 30 and 80%, respectively (El-Otmani, Ait-Oubahou, & Zacarías, 2011). The third most important disease is sour rot caused by the fungus *Geotrichum citri-aurantii* (Eckert & Brown, 1986). The economic losses caused by post-harvest diseases represent one of the main challenges to be overcome by the citrus industry (Fischer, Toffano, Lourenço, & Amorim, 2007). The main control of these diseases is currently based on application of fungicides (Berk, 2016). In lower proportions, alternatives such as the use of antagonistic microorganisms (Droby, Wisniewski, Macarisin, & Wilson, 2009), sanitizing products (Mari, Bertolini, & Pratella, 2003) and natural antimicrobial substances (Ippolito & Nigro, 2000) are also employed. Given the limitations of current control methods, resistant transgenic plant development represents a promising strategy for fungal disease control.

In this context, transgenic sweet orange plants (*Citrus sinensis* L. Osb. cv. Navelina and Pineapple), presenting down-regulation of D-limonene synthase activity, were resistant to *P. digitatum* and to other specialized fungi (Rodriguez et al., 2014, 2015). This transgenic plant down-regulates the expression of a limonene synthase by introducing an antisense construct with the *CitMTSE*1 gene (Rodríguez, Cervera, Peris, & Peña, 2008). The downregulation of D-limonene synthase is associated with a constitutive upregulation of genes involved in the plant innate immune response to pathogens and increased accumulation of jasmonic acid, leading to fungal disease resistance (Rodríguez et al., 2015).

Gummosis disease, wherein lesions around the base of the tree exude sap, together with root rot, are caused by different species of the genus *Phytophthora* (Feichtenberger, 1990). The species *P. citrophthora* (Smith & Smith), *P. hibernales* Carne, *P. palmivora* (Butler) and *P. parasitica* Dastur (=*P. nicotianae* Breda de Haan) (Graham & Feichtenberger, 2015) have been reported as citrus pathogens. Among them, *P. parasitica* is the main citrus pathogen due to its geo-

graphical distribution and severity (Panabieres et al., 2016), and along with *P. citrophthora*, these caused severe damage in *Citrus* nurseries and orchards worldwide (Boava et al., 2011). The availability of resistant citrus is very limited and although this disease mainly affects varieties used as rootstock, combinations with scions is the most efficient method to prevent this disease. This became important for citriculture in Brazil after replacing sour orange, susceptible to citrus tristeza virus (CTV), by the *Citrus limonia* Osb. (Rangpur lime) susceptible to Phytophthora.

Transgenic Rangpur lime rootstock expressing the bacterio-opsin gene (*bO*) showed greater tolerance to *P. nicotianae* (= *P. parasitica*) (Azevedo et al., 2006). The expression of this gene induced a HR-lesion phenotype in transgenic plants in the absence of the pathogen. Besides, transgenic plants expressing *bO* presented high expression of defense-related genes associated with HR, including chitinase, glucanase, and salicylic acid (Mittler, Shulaev, & Lam, 2007). Transgenic plants inoculated with *P. nicotianae*, showed greater tolerance to the oomycete, in correlation with a significantly higher levels of transgene expression (Azevedo et al., 2006).

Pathogenesis-related proteins (PRs) belong to the largest class of antimicrobial proteins, produced by plants as a downstream defense mechanism (Borad & Sriram, 2008). Osmotin and thaumatin-like proteins, for instance, which belong to the PR-5-type proteins family, are known to exhibit activity against fungi and oomycetes (van Loon & van Strien, 1999). Transgenic citrus expressing a PR-5 from tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) showed increased tolerance to *P. citrophthora*, with high survival rates in presence of the pathogen even after one year (Fagoaga et al., 2001).

Citrus scab is a fungal disease, caused by *Elsinoë fawcettii* Bitancourt & Jenkins, that affects many citrus varieties, such as lemons, grapefruit, mandarins, tangors, rough lemon, sour orange, Rangpur lime, and sweet oranges (Chung, 2011). This disease is one of the most important foliage fungal pathogens affecting fruit, leaves and branches. The symptoms are lesions that emerge as tiny spots, often with an irregular and rough appearance. As the host tissues expand, the affected areas become elevated and form erumpent scab pustules comprising fungal and host tissues (Timmer, Mondal, Peres, & Bhatia, 2004). These symptoms severely depreciate fruit value, reducing marketability. In addition, this disease is also of great importance as it contributes to an increased incidence of citrus leprosis, a viral disease transmitted by the mite *Brevipalpus phoenicis* (Feichtenberger, 1990).

Transgenic Duncan grapefruit (*Citrus paradisi* Macf.) plants were developed for enhanced resistance against citrus scab by insertion of the *attE* gene, which encodes an antimicrobial peptide (Mondal, Dutt, Grosser, & Dewdney, 2012). Several transgenic lines showed significantly lower susceptibility to *E. fawcettii* compared to non-transformed plants (Mondal et al., 2012).

Citrus black spot, caused by *Phyllosticta citricarpa*; Alternaria brown spot, caused by *A. alternata*, and citrus post-bloom drop, caused by *Colletotrichum abscissum* are also among the major fungal diseases of citrus. Due to the lack of genetic information, there are no targets for development of transgenic plants resistant to these fungi. Nevertheless, they are object of transcriptome studies for understanding how these diseases occur and for identification of targets for control for inclusion in breeding programs.

Bacterial diseases and a myriad of biotech-engineering solutions

Bacterial diseases are also responsible for considerable losses in the citrus economy. In the United States, particularly in Florida, citrus production has been devastated by *Huanglongbing* (HLB), which is also known as Greening. HLB reduced production from 240 million boxes of fresh orange, prior to an outbreak in 2005, to about 72 million boxes in 2019, according to USDA estimates (USDA Citrus Forecast, 2019).

Besides Greening, citrus canker is another disease that contributes to losses in citrus production. The state of São Paulo, the main citrus producer in Brazil, is facing the return of this disease, which was once eradicated due to strict laws. Citrus variegated chlorosis (CVC), an endemic problem in the country, remains an issue in a number of producing states. These three major bacterial diseases are the most studied due to their impact to the citrus agribusiness (Neves et al., 2010).

Due to the possibility of obtaining plants with broad-spectrum resistance with a single trait insertion, the introduction of genes aiming at increasing systemic acquired resistance (SAR) in citrus plants is a widely used biotechnological approach. In addition to this, overexpression of genes producing antimicrobial peptides (AMPs) is also a widely used strategy attempting to obtain plants more resistant to bacterial infections. These and other approaches will be further described, according to what has been developed for the three main bacterial diseases affecting citriculture.

The biggest challenge*: Huanglongbing*

HLB is the most devastating citrus disease worldwide (Bové et al., 2006). Perhaps the most striking example is that occurring in Florida, which historically leaded the orange juice trade for years, but over the last decade has being suffering with orchards becoming unproductive (Foreign Agricultural Service/USDA, 2019). This disease is associated to three species of phloem-restricted Gram-negative bacteria: *Candidatus* Liberibacter asiaticus (*C*Las), *Candidatus* Liberibacter africanus (*C*Laf) (Coletta-Filho et al., 2004; Jagoueix, Bové, & Garnier, 1994) and *Candidatus* Liberibacter americanus (*C*Lam) (Texeira et al., 2005). *C*Lam and *C*Las are found in Brazil and are transmitted by the insect vector *Diaphorina citri* (Hall, Richardson, Ammar, & Halbert, 2013; Tabachnick, 2015), as well as by infected budwoods (Bové, 2006). Symptoms include yellow shoots, blotchy mottling leaves, corky veins, twig dieback, smaller or lopsided fruits with aborted seeds, root degradation and tree decline (Bové, 2006; da Graça et al., 2016; Wang et al., 2017).

HLB affects all commercial citrus varieties and a source of genetically resistant germplasm remains unknow (da Graça et al., 2016; Ghosh, Motghare, & Gowda, 2018). Preventive measures such as healthy seedlings production under protected greenhouses, elimination of diseased plants and chemical control of the vector are adopted to

decrease the spread of the disease (Bassanezi et al., 2010). Despite these efforts, the most effective way to control HLB would be the replacement of susceptible citrus varieties by HLB-resistant plants. However, no resistant trees or scion-rootstock combinations have been identified so far. Citrus genetic transformation via the introduction of a single trait is an opportunity for improvement of citrus varieties, maintaining their genotypic and phenotypic characteristics.

Different genetic strategies have been tested to obtain HLB-resistant citrus varieties and decrease citrus susceptibility to the pathogen, including the expression of antimicrobial genes from plants, animals, fungal or bacterial origin, as well as the use of host disease-response pathway components (Grosser, Dutt, Omar, Orbovic, & Barthe, 2011; Zou et al., 2017).

Phloem-specific promoters have been used to drive the expression of antimicrobial peptides (AMPs) in transgenic citrus plants for controlling HLB. AMPs are important components of the innate immune defense system against microbial pathogens. Modes of action involve interactions between peptides and microbial membranes, followed by pore formation, which may lead to bacterial membrane disruption, cytoplasmic leakage, and interference with intracellular macromolecules (Nawrocki, Crispell, & McBride, 2014). One example is the synthetic peptide *D4E1* that induces pores in the cell membrane of both Gram-negative and Gram-positive bacteria (de Lucca et al., 1998). The expression of the *D4E1* gene, driven by the *At*PP2 promoter, was used to produce transgenic Pera and Valencia sweet orange plants expressing peptides specifically in the phloem as an attempt of HLB control (Attílio et al., 2013).

Overexpression of cecropin was also a strategy using AMP tested to confer resistance to HLB. Tarocco blood orange transformed with Cecropin B genes, also driven by phloem-specific promoters, showed enhanced resistance and reduction in *C*Las bacterial population even after one year of inoculation. Also, absence of symptoms were reported in two transgenic events even after two years of inoculation. These results showed that the overexpression of the synthesized *cecropin B* gene in the phloem can significantly enhance resistance to HLB disease (Zou et al., 2017).

Plants can produce AMPs, including lipid transfer proteins, defensins and thionins, which act as the first line of defense against invading plant pathogens. Thionins are cysteine-rich small peptides showing antibacterial, antifungal, anticancer and cytotoxic activities (Guzmán-Rodríguez, Ochoa-Zarzosa, López-Gómez, & López-Meza, 2015). These peptides have dual functions, recognizing and binding to bacterial outer membrane, as well as creating numerous pores in the membrane (Pelegrini & Franco, 2005). Although high levels of endogenous thionin are produced by citrus they are not enough to prevent HLB. The modified Mthionin was overexpressed under the control of double 35S promoter in the rootstock Carrizo citrange, and lower *C*Las titer was detected in roots of transgenic plants suggesting that Mthionin inhibited *C*Las growth. Moreover, some new rough lemon leaves grafted on transgenic rootstock have non-detectable *C*Las titer (Hao et al., 2016 Hao, Stover, & Gupta, 2016). Chimeric proteins Thionin1-D4E1 and Thionin-LBP also have been used to develop Carrizo and Hamlin transgenic plants. Challenging assays under greenhouse conditions demonstrated the elimination of *C*Las and reduction of symptoms development (Nguyen, Hao, Stover, & Gupta, 2016).

Another work using the AMP approach, reports the transformation of sweet orange plants with *attacin A* (attA) gene isolated from *Tricloplusia ni*. Transgenic events bearing constructions with the target gene driven by the *At*Suc2 phloem-specific promoter and a signal peptide showed higher transcription levels of *attA* (Tavano, Vieira, Mourão Filho, Harakava, & Mendes, 2015). Besides the *At*Suc2 phloem promoter from *A. thaliana*, PP2 promoters from *A. thaliana* (AtPP2) and from *C. sinensis* (Cs PP2) driven the expression of *attA* were evaluated. Higher efficiency transformation in Hamlin sweet orange was observed when constructions containing PP2 promoters were used, and CsPP2 Valencia transgenic events showed increased *attA* transcription levels. Despite increased effectiveness of *Cs*PP2 promoter in efficiency of transformation and gene expression, which indicates a high potential value for generation of *Citrus* cisgenic plants, no difference in titer of *CLas* was detected between transgenic and non-transgenic plants (Tavano et al., 2015).

As previously pointed, attempts to induce plant inherent immune system to combat disease development through genetic engineering have been adopted as a strategy against HLB. In this way, approaches aiming at enhancing plant Systemic Acquired Resistance (SAR) were also reported. The master positive regulator of SAR, *NPR1*, was overexpressed in transgenic sweet orange varieties of Hamlin and Valencia using the constitutive CaMV 35S promoter or the phloem specific *At*SUC2 promoter. In greenhouse, under continuously inoculation of *CLas* by infected psyllid, or field trials with a pressure of more than 90% infection rate of HLB, around 30% of the plants evaluated remained HLB negative for more than one year. In addition, the expression of several genes like PR1, PR2 and WRKY 70, involved in plant defense signaling, were induced by overexpression of *At*NPR1. All tested transgenic lines had enhanced PR1 and PR2 genes expression. One of the three transgenic lines in which bacteria were not detected during the study had insertion of a single copy of the transgene and exhibited a four-fold level of PR1 gene expression compared to control plants (Dutt, Barthe, Irey, & Grosser, 2015). In another work, transgenic events of Duncan grapefruit and Hamlin sweet orange overexpressing *At*NPR1 with already increased resistance to citrus canker (Zhang et al., 2010) were challenged against HLB. Reduced leaf symptoms and the same levels of disease tolerance were maintained in vegetative propagated plants in both varieties. Therefore, it was demonstrated that overexpression of *At*NPR1 in transgenic citrus can provide tolerance to HLB under strong disease pressure (Robertson et al., 2018).

The development of transgenic citrus plants with increased resistance to HLB represents an important and more environmentally sustainable strategy to fight the disease, which currently is basically dependent on controlling the insect vectors through many applications of insecticide. However, transgenic method takes a long time, especially for citrus, which have a long juvenile stage. An innovative approach, which has been applied to provide HLB resistance more quickly than GMOs is the delivery of transgenes using a mild strain CTV vector in citrus matures trees. Even though this method is non-stable and not inherited to the next

generation, the expression of the target sequence into the phloem where the *CLas* is present is a promising tool against HLB (Levy & El-Mohtar, 2018).

Once again, the delivery of AMPs into the phloem tissue, or knockdown of specific genes in psyllids to shorten their life span through CTV-based/RNAi vectors is a biotechnology possibility beyond transgeny (Folimonov, Folimonova, Bar-Joseph, & Dawson, 2007; Hajeri, Killiny, El-Mohtar, Dawson, & Gowda, 2014).

The use of citrus gene silencing through overexpression of self-complementary hpRNA and genomic editing via the CRISPR/Cas technology are promising alternatives to help fighting HLB. Attempts based on overexpression of new genes to help recognition of bacterial effectors, silencing of target genes related to HLB symptoms or developing plants with genes that alter the vector life cycle. Nevertheless, many questions about HLB remain elusive. Plant genes potentially targeted by the pathogen need to be identified aiming at edition and different strategies should be used attempting to reduce losses due to HLB in a faster and more efficient way.

Citrus variegated chlorosis a Brazilian problem disease

Citrus Variegated Chlorosis (CVC) was the first bacterial disease transmitted by buds and insect vector in which Brazilian citriculture fought in the late 1980s (Coletta-Filho & de Souza, 2014; Rossetti et al., 1990). Although bacteria from the same genus is known to cause disease in other plant species around the world and CVC incidence is reported in some South America countries (Ayres, Gimenes-Fernandes, & Barbosa, 2001), it is an endemic issue in Brazil. CVC is characterized by symptoms resembling zinc nutritional deficiency, chlorotic spots with a necrotic center in leaves, water stress and a drastic reduction in fruit sizes, with subsequent reduction in juice production (Machado et al., 2007; Machado, Quaggio, Lagôa, Ticelli, & Furlani, 1994). All sweet orange varieties are susceptible and although death of infected plants is rare, vigor and production declines over the years (Bové & Ayres, 2007). The CVC causal agent is *Xylella fastidiosa* subsp. *pauca*, a Gram-negative bacterium that lives restricted to the xylem vessels and sharpshooter foreguts (Parra, Lopes, Zucchi, & Guedes, 2005). Before CVC, source plants were cultivated in open field conditions, therefore, unprotected from being potentially infected by infective insect vectors. Once citrus production is based on grafting buds from source plants to rootstocks, after 2002 all nursery system related to this practice in Sao Paulo state, Brazil, had to be grown under a vector protected system, to overcame CVC (de Souza, Takita, Amaral, Coletta-Filho, & Machado, 2009). Nowadays, all production of Brazilian citrus trees undergoes federal and state laws, whose restrictions are based on the expressiveness of citriculture to the local trade. In São Paulo state, propagation of citrus is based on source plants that must be protected in greenhouses and annually undergoing to phytosanitary certification, as determined by the normative instruction #48 from September 24th, 2013 (CDA, 2019). Also, nurseries must be registered and need a phytosanitary report issued by competent governmental agencies for commercialization of new plants. Brazilian scenario of citrus seedlings production changed after CVC to a more controlled certification system.

Management practices adopted against CVC in orchards involve the application of insecticide to control insect vectors and pruning of branches with severe symptoms (Coletta-Filho et al., 2000). Although effective to lower CVC incidence, these practices do not directly control bacteria since, in Brazil, *X. fastidiosa* is not only restricted to citrus, but also affects plum, coffee and more recently olives (Coletta-Filho, Francisco, Lopes, Muller, & Almeida, 2017), making it difficult to be eradicated from the fields. Even if CVC symptomatic trees are present in less than 1.5% in São Paulo citrus growing regions (https://www.fundecitrus.com.br/levantamentos/cvc), bacteria are still present in orchards and an outbreak is hard to predict.

Although all sweet orange varieties are susceptible, mandarins are tolerant to *X. fastidiosa* due to its genetic bases (de Souza et al., 2007; Rodrigues, de Souza, Takita, Kishi, & Machado, 2013). It was reported before that resistance in mandarins is the result of a set of genetic responses involved in activation of different defense pathways throughout the period of infection (de Souza et al., 2007; Rodrigues et al., 2013). Although several genes have been identified as related to defense against *X. fastidiosa*, it is difficult to determine the key ones to be used in breeding programs (Mauricio et al., 2019). Besides genes from the resistant host, genes from the bacteria itself can be used in transgenic approaches (Lindow et al., 2014). It is known that *X. fastidiosa* forms multilayers of cell aggregates enabling the regulation of virulence by intercellular communication through the exchange of signal molecules called diffusible signal factor (DSF), small fatty acid chains that when detected by bacterial receptors can modulate the activity of specific genes Newman, Almeida, Purcell, and Lindow (2004). DSF is considered an avirulence signal, since when disrupted, bacteria spread faster through the plant causing worse disease symptoms (Newman et al., 2004). Once DSF is used in communication between bacteria it could also be used to confuse them, a strategy termed pathogen confusion that worked in transgenic sweet orange and grape plants (Caserta, Souza-Neto, Takita, Lindow, & De Souza, 2017; Lindow et al., 2014).

Sweet orange Hamlin and Pineapple varieties were transformed with the *rpfF* gene from *X. fastidiosa* subsp. *pauca* (Caserta et al., 2017). The *rpfF* gene encodes an enzyme called DSF synthase, that is flexible in producing different species of 2-enoic acids with different signaling activity according to the molecules that are available in the substrate (Ionescu et al., 2016; Newman et al., 2004). Citrus plants producing DSF were challenged with *X. fastidiosa* and evaluated in initial and advanced stages of infection, respectively at 9 months and 18 months after inoculation. The severity of symptoms was lower in transgenic plants even in advanced stages of symptoms. Accordingly, there was a reduction in progression of disease severity of 31% for Pineapple transgenic plants and 28.1% for Hamlin transgenic plants in comparison with wild type, which are very promising results since severity is close related to the reduction of fruit size in field conditions (Caserta et al., 2017). It was the first transgenic citrus resistant to *X. fastidiosa*, which are under field conditions testes since December 2018.

Due to its ability to infect a wide range of host *X. fastidiosa* has recently been considered the most dangerous plant bacterium worldwide (Almeida et al., 2019). The development of transgenic plants producing molecules able to alter bacterial behavior reducing its pathogenicity is a strategy that can be used in different susceptible species to *X. fastidiosa* as a new technology, mainly in the direct management of this pathogen.

The ancient problem remains worrying - *Citrus Canker*

Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is one of the most economically important citrus diseases. Leaves, stems, and fruits are affected by the pathogen and the most prominent symptoms consist of roughly circular water-soaked lesions, causing defoliation, dieback, and fruit drop (Brunings & Gabriel, 2003). No phytosanitary measures capable of eradicating the disease from orchards are available to date. However, control measures can be effective in regions where the disease is not endemic attempting to manage disease severity and spreading. Among these are the control of Asian citrus leafminer (*Phyllocnistis citrella*), construction of windbreaks around the plots, copper spray and equipment decontamination (Ference et al., 2018). All the commercial varieties are susceptible to citrus canker disease; thus, genetic engineering might be a relevant strategy in supporting breeding programs aiming at an increasing in citrus canker resistance.

Biotechnology approaches based on the use of plant receptors that recognize pathogen molecules and trigger plant immune response are strategies adopted to confer broad-spectrum resistance (Afroz et al., 2011; Boutrot & Zipfel, 2017; Dalio et al., 2018; Gómez-Gómez & Boller, 2000; Hao, Pitino, Duan, & Stover, 2015; Lacombe et al., 2010; Lu et al., 2015; Song et al., 1995; Tripathi, Lorenzen, Bahar, Ronald, & Tripathi, 2014; Zipfel et al., 2006). The FLS2 receptor from *A. thaliana* recognizes the conserved peptide flg22 from bacterial flagellin (Gómez-Gómez & Boller, 2000) and although citrus harbors FLS2 orthologs, there is weak interaction with *X. citri* flagellin or even insensitiveness to flg22 from *X. citri* (flg_{Xcc}) in some canker susceptible genotypes (Shi, Febres, Jones, & Moore, 2015). Since *Nicotiana benthamiana* FLS2 (*Nb*FLS2) recognizes $flg22_{Xcc}$ and activates defense responses (Chinchilla, Bauer, Regenass, Boller, & Felix, 2006; Zou et al., 2012), Hamlin sweet orange and Carrizo citrange overexpressing *NbFLS2* displayed ROS overproduction and activation of *PR1* and *WRKY22* marker genes in response to $flg22_{Xcc}$ with also a reduction in citrus canker susceptibility (Hao et al., 2015). These results indicate that the use of flagellin receptors from different species strengthen basal defenses increasing citrus canker resistance in susceptible genotypes.

Afterwards, two FLS2 receptors (FLS2-1 and FLS2-2) were identified and characterized in three citrus species with variable canker resistant/-susceptible levels. Protein sequence analysis and gene expression profile were associated with the phenotypic variation and responsiveness to $flg22_{Xcc}$ (Shi, Febres, Jones, & Moore, 2016). Based on that, transient expression of *Fm*FLS2-1 from the resistant 'Nagami' kumquat *Fortunella margarita* and *Cr*FLS2-2 from 'Sun Chu Sha' mandarin *C. reticulata* in highly susceptible 'Dunkan' grapefruit (*C. paradise*) showed less citrus canker symptoms after challenge with *X. citri*. These PRR receptors showed great potential in developing cisgenic citrus plants aiming at more resistant genotypes to citrus canker disease.

The Xa21 receptor from rice mediates the recognition of the protein produced by *X. oryzae* pv. *oryzae*, the causal agent of bacterial blight disease (Pruitt et al., 2015; Ronald et al., 1992; Song et al., 1995; Wang, Song, Ruan, Sideris, & Ronald, 1996). In view of the potential use of this type of PRR against bacterial diseases caused by *Xanthomonas*, such as citrus canker, Mendes et al. (2010) developed sweet orange transgenic lines of four varieties Hamlin, Natal, Pera and Valencia overexpressing *Xa21* to evaluate citrus canker resistance. The effect of the Xa21 receptor in controlling disease severity varied between varieties and respective events. Except for Valencia, the sweet orange transgenic plants showed a significant reduction in disease severity after inoculation. Using the same strategy, (Li, Xiao, & Guo, 2014) transformed the highly susceptible Anliucheng sweet orange with *Xa21* under the control of its own promoter and the majority of the transgenic events showed lower disease severity, and hypersensitivity response (HR) in inoculation sites was reported in one of the events. Similarly, Omar, Murata, El-Shamy, Graham, & Grosser (2018) generated W. Murcott mandarin, one of the most important citrus variety in the world, overexpressing *Xa21*. After nine days post inoculation, water-soaked lesions were lacking, hyperplastic lesions occurred slowly compared to non-transformed plants, and bacterial population showed significantly reduction.

CsMAPK1 is another gene associated to plant immunity and it was preferentially induced in response to *X. aurantifolii* pathotype C, causal agent of B and C types of canker. This pathotype is limited to Mexican lime and triggers HR in sweet orange (Brunings & Gabriel, 2003; Cernadas, Camillo, & Benedetti, 2008). Under the control of a *Xanthomonas*-inducible promoter (PR5), *CsMAPK1* was expressed in Troyer citrange plants to enhance citrus canker against *X. citri*. *CsMAPK1* expression increased ROS production, decreased *X. citri* growth and reduced citrus canker symptoms (de Oliveira et al., 2013).

Regarding the second line of the plant innate immune system orchestrated by the R-genes, *Capsicum chacoense*-derived Bs2 gene was stably expressed in Pineapple sweet orange using either a constitutive promoter (2x CaMV 35S) or the pathogen-inducible promoter from the potato glutathione S-transferase. The use of the constitutive promoter was not effective, but a 70% disease symptom reduction and the production of downstream immune signaling, such as ROS, it was observed in transgenic lines with pathogen-inducible promoter (Sendín et al., 2017). Such decrease was attributed to the recognition of the cognate virulence factor AvrBs2 delivered by *X. citri*.

Attempts of increase SAR responses, as reported for HLB, were prior evaluated against citrus canker. As a major regulator, NPR1 gene was a target evaluated in different studies with Duncan grapefruit and Hamlin sweet orange transgenic plants overexpressing *AtNPR1*. In both transgenic varieties, respectively, a remarkably reduction of 10 to 100-fold in bacterial population was reported (Boscariol-Camargo, Takita, & Machado, 2016; Zhang et al., 2010). Moreover, in trans-

genic plants showing the highest transgene expressing levels the decrease in bacterial population culminated in significant fewer lesions and stronger expression of *EDS1* and *PR2* genes. Interestingly, Chen et al. (2013) identified a NPR1 ortholog from Pumelo (*C. maxima*) and transferred to the Duncan grapefruit background. *Citrus* NPR1 homolog 1 (*Ct*NH1) enhanced resistance to *Xcc* and led to modulation of chitinase 1, a well-known PR gene.

Besides PRRs and R-genes, citrus transformation with hairpin protein was also evaluated aiming at enhanced citrus canker resistance. Sweet oranges Hamlin and Valencia expressing *Erwinia amylovora hrpN* gene driven by a pathogen inducible GST1 promoter showed high resistance by reducing about 79% of disease severity by possibly eliciting systemic acquired resistance. *hrpN* expressing transgenes also exhibited normal vegetative growth and development (Barbosa-Mendes et al., 2009). Other pathogen-derived genes were also used aiming at citrus canker control. The *Xanthomonas* pathogenesis protein PthA is essential for causing the hyperplasia symptom of canker and its nuclear localization signal (NLS) is responsible for leading the protein to the nucleus of the plant cells. Sweet orange expressing the PthA-NLS region showed increased resistant to citrus canker by avoiding the binding of PthA to importin α of the host and consequently its import to the nucleus (Yang et al., 2010). Moreover, transgenic lines of sweet orange and Carrizo citrange expressing RpfF from *X. fastidiosa* encoding a quorum sensing factor, showed higher resistance to *X. citri* by disrupting bacterial communication and reducing activation of virulence factors (Caserta et al., 2014).

As also showed for HLB, the use of genes coding for antimicrobial peptides (AMP) has been shown a promising strategy in genetically engineering citrus plants. Similarly to what was previously shown for HLB resistance induction, Boscariol et al. (2006) and Cardoso et al. (2009) developed sweet orange varieties (Hamlin, Valencia, Pera and Natal) expressing the *attacin A* gene (*attA*). All varieties, but Pera, produced transgenic lines showing a variable, yet significant reduction in citrus susceptibility to *X. citri*. The authors suggested strong influence from the genetic background regarding the natural level of susceptibility from the varieties. The AMP encoded by cecropin B and shiva A belonging to the *Cecropin*-family were isolated from *Hyalophora cecropia* and optimized for plants. Coding regions were introduced into the genome of sweet oranges Jincheng and Newhall navel and the expression of both genes showed enhanced resistance to citrus canker disease in 11 transgenic events (He et al., 2011). Interestingly, agronomic traits such as total soluble solids, total acidity and reduced sugar content remained stable (He et al., 2011). Furman et al. (2013) showed that the constitutive expression of dermaseptin, an AMP derived from *Phyllomedusa spp.*, in sweet orange Pineapple strongly reduced the frequency and intensity of citrus canker symptoms.

Kobayashi et al. (2017) aimed at delivering of AMP sarcotoxin IA isolated from the flesh fly *Sarcophaga peregrina* in the host intercellular space by fusion to a signal peptide derived from *N. tabacum PR1a* gene. Transgenic events showed reduced *X. citri* population and lower incidence of canker lesions, without causing any deleterious effects on the growth and development of the plants. Moreover, the presence of sarcotoxin in transgenic plants delayed the modulation of precursors of secondary and signaling metabolites leading to a more favorable immune response (do Prado Apparecido, Carlos, Lião, Vieira, & Alcantara, 2017). A synthetic antimicrobial peptide known as D2A21 has been studied for its capacity to improve resistance to HLB and citrus canker diseases (Stover et al., 2013). Although remarkable results could be achieved after transferring D2A21 to Carrizo citrange regarding *X. citri* infection, HLB resistance was not observed (Hao, Zhang, & Stover, 2017). Also, the previously showed work regarding the overexpression of methionine in Carrizo citrus plants reports increased citrus canker resistance and inhibition of pathogen growth when compared to non-transgenic plants (Hao et al., 2016).

Hydrogen peroxide (H_2O_2) is produced during normal cellular processes but also plays a major role in plant defense responses to pathogen (Heller & Tudzynski, 2011; Yoda, Hiroi, & Sano, 2006). H_2O_2 is predominantly produced through the polyamine degradation mediated by flavine-containing polyamine oxidases (PAO) or copper-containing amine oxidases (CuAO) (Rea, Metoui, Infantino, Federico, & Angelini, 2002; Yoda et al., 2009). The spermidine synthase gene (*MdSPDS1*) from apple (*Malus sylvestris* var. *domestica*), which encodes one of the enzymes involved in polyamine biosynthesis, was used to produce transgenic sweet orange. Two transgenic lines were less susceptible to *X. citri*. This result was correlated with H_2O_2 production and/or up-regulation of transcripts involved with defense-related proteins and jasmonic acid metabolism (Fu, Chen, Wang, Liu, & Moriguchi, 2011).

Transgene production has been shown to be effective against *X. citri* in different approaches using several species and citrus varieties. Moreover, citrus canker resistance is being evaluated in plants developed using new breeding techniques (NBT), such as CRISPR-Cas9. Citrus canker management is majorly based on copper applications in orchards, which leads to environmental damages and potential bacterial resistance. Strategies like the NBTs are of great importance since it culminates in more sustainable management practices.

CRISPR technology as a tool to improve citrus disease resistance: challenges to overcome

The use of CRISPR-Cas9 to improve crop resistance to biotic stress has been a major concern in the scientific community. As a break-through technology, CRISPR/Cas9 have been extensively exploited in many studies in citrus plants (Jia & Nian, 2014; Jia, Orbovic, Jones, & Wang, 2016; Jia, Orbović, & Wang, 2019; Jia et al., 2017; LeBlanc et al., 2018; Peng et al., 2017; Zhang, LeBlanc, Irish, & Jacob, 2017). Nevertheless, most works are associated with description and improved methodologies to verify if CRISPR/Cas9 is indeed able to edit citrus plants.

Until now, few studies using CRISPR/Cas9 as a tool for genome editing aiming at citrus disease resistance were published (Jia et al., 2016, 2017; Peng et al., 2017; Wang et al., 2019). Most of the studies were performed to target the *LATERAL ORGAN BOUNDERIES 1* (*LOB1*) gene, described as a citrus susceptibility gene for *X. citri*. It was shown

that *LOB1* is upregulated by PthA4, a *X. citri* effector (Hu et al., 2014; Abe & Benedetti, 2016). This effector binds a specific region on the *LOB1* promoter, called Effector Binding Element (EBE), which was the target sequence for editing the *LOB1* promoter in grapefruit (Jia et al., 2016). The authors showed the addition of a single nucleotide in EBE region, however, no effects on increasing resistance to *X. citri* in the edited plants was observed. When PthA4 recognition was tested by GUS assay, the low activation of the reporter demonstrated that PthA4 might still identify EBE regions with some level of modifications. The authors also suggested that both alleles of *LOB1* should be edited to confer resistance to *X. citri* (Jia et al., 2016). Complementarily, the first exon from *LOB1* gene was edited in grapefruit and different percentage of editing among transgenic events were obtained (Jia et al., 2017). This was the first report of edited citrus plants resistant to pathogens.

Using a different approach, sgRNAs for different regions along the EBE sequence were used, generating a great diversity of edited positions, with up to 182 nucleotides deleted from Wanjincheng sweet orange LOB1 promoter (Peng et al., 2017). The most resistant events to *X. citri* presented a deletion of the whole sequence of EBE or at least an edition in the putative TATA-box. Authors also demonstrated that the resistance was enhanced when all alleles were edited with high percentage (Peng et al., 2017).

Recently, the transcription factor CsWRKY22, likely to be negatively related to citrus canker resistance (Zhou et al., 2017) was silenced by CRISPR/Cas9-targeted knockout in Wanjincheng orange background (Wang et al. 2019). Transgenic events submitted to *X. citri* infection showed delayed symptoms development, although pustule formation was not prevented. The authors attested that the delayed phenotype might be due to the chimerism, and to obtain stronger resistance, 100% mutation rates are mandatory. Thus, CRISPR/Cas9 was shown to be a powerful tool for citrus improvement for disease resistance, although 100% mutation rates could be challenging depending on the target gene.

Although CRISPR/Cas9 is a promising technology for development of citrus resistance to pathogens, there are several challenges to overcome. The bottleneck in developing new disease resistant varieties mostly relies on finding new target genes for editing. To do so, the knowledge of plant-pathogen interaction mechanisms is critical to find such target genes. Moreover, even with the integrative CRISPR/Cas9 system being widely used for generating important agronomic traits in crops, the use of transgenesis can still rely on GMO legislation. Specially for perennial species like citrus this process can be even longer, meaning that the target gene of choice needs to be carefully selected. Facing this issue, many efforts have been done to produce crop edited genotypes based on non-integrative techniques such as purified CRISPR/Cas9 ribonucleoproteins to edit plant protoplasts. To date, the use of this strategy to generate citrus edited plants are lacking. However, it would be of great importance to assess the efficiency rates in editing and regenerating citrus protoplast material.

The release of genetically modified citrus for cropping

In this review, regulatory approaches to genetically modified organisms (GMO) in field trials of citrus will focus in the United States (U.S.) and Brazil. The regulatory systems can reflect policies that are more precautionary or more permissive toward GMO. In the United States the basis for regulation of GE crops and derived products is the pre-existing product-regulation laws. Three agencies can be involved in GMO in U.S. regulation: the U.S. Food and Drug Administration (FDA) for food safety, the U.S. Department of Agriculture (USDA) for plant-pest characteristics and other adverse environment effects, and the U.S. Environmental Protection Agency (EPA) to ensure the absence of environment or human health threats (National Academies of Sciences, Engineering, and Medicine, 2016). In Brazil, the National Technical Commission of Biosafety (CTNBio) is responsible for all biotechnology issues, following the biosafety law nº 11.105, from March 24, 2005. CTNBio conducts the assessment of food-safety and environmental risks, and also authorizes projects, laboratory activities, field trials and commercial activities. Certificate of Quality in Biosafety (CQB) is mandatory to institutions or companies which intend to work with GMO. There are other agencies responsible for supervising field trials, such as: Ministry of Agriculture, Livestock, and Food Supply (MAPA); Brazilian Institute of Environment and Renewable Natural Resources (IBAMA); National Health Surveillance Agency (ANVISA).

The planned release of GM plants into the environment (LPMA) should follow CTNBio Normative Resolutions nº 1, 2, 6 or 8, which establishes the rules for requesting, evaluating and monitoring the genetic modified organism in the field. The RN8/2009 describes simplified rules to planned releases GMOs belonging to risk class 1. These Normative Resolutions determine a deadline of 90 days to analyze the requests, but sometimes it can be delayed. In the case of citrus, there is a specific Normative Resolution for cultivation of transgenic sweet orange in the field (RN10/2013), which has been followed by the institutions and companies that are working with transgenic citrus. Field trials take at least 4–5 years to be concluded.

The first citrus trees field-tested in Brazil were produced by Alellyx, a biotechnology company belonging to the Votorantim Group. The evaluated plants contained several genetic constructions aiming at the development of plants resistant to Citrus Leprosis and CVC. The company was incorporated in 2010 by Monsanto and ended their research with citrus after that.

Fund for Citrus Protection (Fundecitrus) also started to grow genetically modified orange in 2014, after approval of the RN10 in 2013, expecting citrus resistance to black spot and citrus canker. The 650 transgenic seedlings planted in the countryside of São Paulo state are derived from sweet orange imported from Spain (Estado de S. Paulo - 26/02/14). Other researches are underway, seeking to evaluate genetically modified citrus plants that produce volatile compounds repellent to *Diaphorina citri*, insect vector of the HLB (Huanglongbing) (http://ctnbio.mcti.gov.br, 218ª Reunião Ordinária).

Recently, the Citrus Center Sylvio Moreira, from the Agronomic Institute, also started the planting of genetically modified citrus in the field aiming at evaluating transgenic citrus plants developed with traits for increased tolerance to CVC, citrus canker, HLB, and higher carotenoids content in fruits. All Brazilian field trial can be found at: http://ctnbio.mcti.gov.br/liberacao-planejada#/liberacao-planejada/consultar-processo.

In the U.S., the Southern Gardens Citrus — a large citrus growing company — has been genetically engineering plants to express genes isolated from spinach that defend against the bacteria agent causal of HLB. Some years after of field trials results suggest some degree of resistance in transgenic trees (ISAAA, 2016). Lately, the company is using engineered CTV (a harmless strain) that can express the spinach defense gene directly at the phloem. The goal of the researchers is to transmit the modified virus onto trees by grafting. This approach does not involve genetically modified fruits and reduces public concern about transgenic oranges (Ledford, 2017).

Transgenic sweet orange expressing an *A. thaliana* NPR1 gene under the control of constitutive or phloem specific promoters were also evaluated in field (Dutt et al., 2015). Transgenic trees exhibited reduced diseased severity and a few lines remained disease-free.

Despite these field trials, until now, there is no transgenic citrus approved commercially. Brazilian commercial approval process of a genetically modified organism must follow the recommendations of Normative Resolution CTNBio nº 18 (RN18/2018) that recently reviewed the RN5/2008. The company responsible for the development of technology and the GMO must submit application and documentation as established in the Article 10 of RN5.

Regardless of the country, the process of commercial release of a GMO is very elaborate, costly, and time-consuming. This process involves environmental impact studies and risk assessment for human and animal health. There is no transgenic citrus commercially launched until now. New breeding technologies (NBTs or Innovative Techniques for Precision Improvement) like CRISPR, RNAi, viral vector, could facilitate genetic engineering for the next generation of crops and maybe reduce barriers for commercialization processes. NBTs can be considered non-GMO if they result in the absence of recombinant DNA / RNA in the final product. In Brazil, there is a special resolution to analyze NBTs (RN16/2018) which states that, products or plants derived from them are analyzed case by case, depending on the technology applied or the genetic modification generated.

Final considerations

As summarized in this review, citriculture is a commercial activity of great importance in several countries. The ability to maintain economic competitivity, however, requires strategies beyond commercial issues, given that trade value is heavily influenced by costs related to pest and disease management. The effective control of diseases such as leprosis, HLB, citrus canker and multiple fungal diseases are challenges shared by all producing countries. In most cases, the resources employed against such diseases are based principally on chemicals, which, due to continuous application, can result in harm to the environment and select for emergence of resistant microorganisms. In this scenario, technology is of fundamental importance for the development of resistant citrus varieties. The generation of engineered plants using transgenic and genomic editing techniques, aiming at reducing susceptibility in orchards to specific diseases, is an alternative that can enable a more sustainable citriculture with less environmental impact. It is worth mentioning that transgenics as a technique to develop new plants is safe for both the environment and for food production. New breeding approaches endorse the use of technology as a tool for the generation of high quality and productive plants for the food supply.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by research grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (2013/10957-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-INCT Citros 465440/2014-2 FAPESP 2014/50880-0). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. R. Caserta; N. S. Teixeira-Silva and L. M Granato are post-doctoral fellows granted by FAPESP (2017/16142-0; 2019/01447-5 and 2019/01901-8). L. K. Mitre; C. A. Nascimento; S. O. Dorta and R. R. Souza-Neto are Ph.D. candidates (CNPq142014/2015-0; 88882.329498/2019-01 and 380040/2019-0; and FAPESP2017/13885-1, respectively). M. A. Takita, M. A. Machado and A. A. De Souza are recipients of research fellowships from CNPq.

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