CHAPTER 1. General introduction

Molecular Basis of Resistance to Fusarium Ear Rot in Maize

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Abstract

The impact of climate change has been identified as an emerging issue for food security and safety, and the increased incidence of mycotoxin contamination in maize over the last two decades is considered a potential emerging hazard. Disease control by chemical and agronomic approaches is often ineffective and increases the cost of production; for this reason the exploitation of genetic resistance is the most sustainable method for reducing contamination. The review focuses on the significant advances that have been made in the development of transcriptomic, genetic and genomic information for maize, *Fusarium verticillioides* molds, and their interactions, over recent years.

Findings from transcriptomic studies have been used to outline a specific model for the intracellular signaling cascade occurring in maize cells against *F. verticillioides* infection. Several recognition receptors, such as receptor-like kinases and *R* genes, are involved in pathogen perception, and trigger down-stream signaling networks mediated by mitogen-associated protein kinases. These signals could be orchestrated primarily by hormones, including salicylic acid, auxin, abscisic acid, ethylene and jasmonic acid, in association with calcium signaling, targeting multiple transcription factors that in turn promote the down-stream activation of defensive response genes, such as those related to detoxification processes, phenylpropanoid and oxylipin metabolic pathways.

At the genetic and genomic levels, several quantitative trait loci (QTL) and SNP markers for resistance to Fusarium ear rot deriving from QTL mapping and genome-wide association studies are

described, indicating the complexity of this polygenic trait. All these findings will contribute to identifying candidate genes for resistance and to applying genomic technologies for selecting resistant maize genotypes and speeding up a strategy of breeding to contrast disease, through plants resistant to mycotoxin-producing pathogens.

Introduction

A large number of fungi can attack and invade developing maize ears and kernels, causing numerous diseases classified as ear rots. Many ear rot fungi produce mycotoxins that can affect the quality and marketability of grains. *Fusarium verticillioides* (Sacc.) Nirenberg (synonym *F. monoliforme* Sheldon, teleomorph *Gibberella monoliformis* Wineland) causes stalk rot and ear rot in maize, and is endemic in maize fields at harvest (Bottalico, 1998; Battilani et al., 2008). *F. verticillioides* is the main causal agent of Fusarium ear rot (FER) (Logrieco et al., 2002; Folcher et al., 2009). Interest in *F. verticillioides* has been renewed by the discovery that the fungus can produce the secondary metabolite fumonisins (Gelderblom et al., 1988).

Breeding for resistance to FER and fumonisin contamination is considered the environmentally safest and most economical strategy (Munkvold, 2003a; Eller et al., 2008a), and many studies have focused on the search for resistance (Clements et al., 2004; Lanubile et al., 2011; Maschietto et al., 2017). These studies have demonstrated genetic variation for resistance to FER and fumonisin contamination, but no evidence of complete resistance to the pathogen has been observed. Quantitative Trait Loci (QTL) mapping studies in maize have indicated that resistance is a quantitative trait determined by polygenes having small effect (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Ding et al., 2008; Chen et al., 2012; Maschietto et al., 2017). Large genetic bases and the strong influence of the environment have slowed progress in accurate QTL localization, therefore reducing the efficiency of marker-assisted selection (MAS) (Robertson-Hoyt et al., 2006). Increasing population size and the number of markers used, improving ear rot phenotyping protocols and integrating data from multiple environments, will overcome such limitations (Robertson et al., 2005).

Transcriptomic and genome-wide association studies (GWAS) are useful tools for identifying candidate genes, especially when combined with QTL mapping in order to map and validate loci for quantitative traits (Korte and Farlow, 2013). The combination of these methods has overcome the limitations of either method performed alone (Brachi et al., 2010). Two recent GWAS were performed in maize to detect SNP associated with increased resistance to FER, resulting in ten SNP markers with significant effects on several chromosomes (Zila et al., 2013; 2014).

As an alternative to plant breeding techniques, next-generation precision genome engineering relying on genome editing technologies can play a key role in accessing genetic resources and using them to increase plant disease resistance, by targeting suitable plant defense mechanisms. Such approaches, however, require efficient transformation protocols as well as extensive genomic resources and accurate knowledge, before they can be efficiently exploited in practical breeding programs.

In this review, we provide an extensive overview of recent developments related to basic research and breeding efforts aimed at improving resistance to FER and fumonisin contamination in one of the most important grain food crops, i.e. maize.

Importance of Fusarium ear rot disease

F. verticillioides, often in association with *F. subglutinans* and *F. proliferatum* (Logrieco et al., 2002), causes FER or pink ear rot, typically occurring on random groups of kernels or on physically injured kernels (White, 1999; Munkvold et al., 2003a; Lanubile et al., 2014a). FER prevails in drier and warmer climates, like those common in southern Europe and the USA (Logrieco et al., 2002; Eller et al., 2008a). FER strongly affects grain production, with yield reduction often estimated between 10 and 30% (Bottalico, 1998; Logrieco et al., 2002).

The interest in this fungus has arisen from mycotoxin accumulation in pre-harvest infected plants or in stored grains. *F. verticillioides* mycotoxins, including fumonisins, have been associated with chronic or acute mycotoxicoses in livestock. Feeds contaminated with FB1 caused leukoencephalomacia in horses and pulmonary edema and hepatic syndrome in swine (Ross et al., 1990). FB1 carcinogenic activity in rats (Gelderblom et al., 1996) and its relation with neural tube birth defects in humans (Missmer et al., 2006) has led to the classification of FB1 as carcinogenic for animals and humans. It has been estimated that 25% of world food crops are affected by mycotoxins, but for fumonisins the percentage could be even higher (Bottalico, 1998; Logrieco et al., 2002; Pietri et al., 2004; Eller et al., 2008a).

Regulations for permitted mycotoxin limits in food and feed have been set in most countries (Ferrigo et al., 2016). The European Commission has indicated maximum tolerable levels for fumonisins as 4000 ppb in unprocessed maize, 1000 ppb in maize intended for direct human consumption and 800 ppb in maize-based breakfast cereals and snacks. Outside Europe, in the main maize producing countries, the U.S. Food and Drug Administration (FDA) has recommended that fumonisin levels in dry milled corn products and cleaned maize used for popcorn should not exceed 2000 and 3000 ppb, respectively. The Health Surveillance Agency for Brazil (ANVISA) has

established maximum tolerable limits of 1500 and 1000 ppb in maize meal and other maize-based products, respectively. Furthermore, the permissible levels of fumonisins in maize flour are not more than 200 ppb for the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor). The different regulations on mycotoxin levels are due to a global market, and since European regulations appear stringent, a common strategy would seem to be the best way forward to ensure food safety.

***Fusarium verticillioides* infection in maize kernels**

F. verticillioides has been shown to behave as an endophytic fungus that tends to be symptomless in the kernels and can be systematic in the maize plant (Munkvold et al., 1997). Whitish pink fungal growth on kernels and/or silks is typical. Infected kernels may also exhibit a "starburst" symptom, i.e. white streaks radiating from the point of the silk attachment at the cap or from the base of the kernel (Figure 1).

Figure 1. Fusarium ear rot (FER) symptoms. A. Different degrees of FER on ears of resistant (right) to highly susceptible maize lines (left). B. Starburst showing white streaks radiating from the point of silk attachment at the cap of the kernel or from the base.

There are three main access pathways for the fungus into the ear: (i) fungal spores germinating on the silks and then fungal mycelia growing down the silks to infect the kernels and the cob (rachis); (ii) through wounds on the ear generated by insects, birds or hail damage; (iii) systemic infection of the ear through infected stalks that generate infected seeds (Munkvold et al., 1997; Munkvold, 2003a). Kernel infection develops most efficiently from strains that are inoculated into the silks (Munkvold et al., 1997), but the prevalence of one or the other pathway depends on the insect pressure in the area. Only recently the biology of maize kernel infection was investigated using a fluorescent-protein expressing transformant of *F. verticillioides* (Duncan and Howard, 2010). After the introduction of a conidial suspension through the silk channel, the fungus penetrated kernels via

the stylar canal and spread within the pericarp, colonizing adjacent cells through pits. Starburst symptoms were observed only at the later times of inoculation, indicating the destruction of the pericarp cell wall (Duncan and Howard, 2010). Early reports focused on germinating seeds revealed that *F. verticillioides* penetrated directly by hyphae through the epidermal cells of the seedling and colonized the host tissue by inter- and intracellular modes of growth (Murillo et al., 1999; Oren et al., 2003). Scutellum colonization occurred earlier with branched hyphae growing into the parenchyma cells, and produced pronounced cell alterations and collapsed protoplasts. Pathogen ingress into the infected tissue induced defense-related ultrastructural modifications, such as appositions on the outer host cell wall surface, the occlusion of intercellular spaces, and the formation of papillae. Pathogenesis-related proteins from maize (PRms) represent the first barrier for fungal penetration and accumulated at very high levels in the aleurone layer and scutellar epithelial cells, as well as within the papillae. This suggests that signaling mechanisms that lead to their accumulation can operate at a distance from the infection point (Murillo et al., 1999).

Maize-*Fusarium verticillioides* molecular interaction

Next Generation Sequencing (NGS) and microarray approaches have been used to identify molecular mechanisms connected with *F. verticillioides* infection in resistant and susceptible maize genotypes (Lanubile et al., 2010; 2012a; 2014b; Campos-Bermudez et al., 2013; Wang et al., 2016). All these studies compared the response of resistant and susceptible lines to infection, considering early (12-48 hours post inoculation-hpi) and late (from 72 to 120 hpi) stages of infection. Microarray hybridization studies were performed in the earliest published works (Lanubile et al., 2010, 2012a; Campos-Bermudez et al., 2013), whereas RNASeq technology has been employed in the more recent references (Lanubile et al., 2014b; Wang et al., 2016). Most of the information about differentially expressed genes has been obtained from infected maize kernels (Lanubile et al., 2010; 2012a; 2014b; Wang et al., 2016), whereas only two experiments have focused on infected silks (Lanubile et al., 2010; Campos-Bermudez et al., 2013). RNASeq has allowed for the identification of several thousands of differentially expressed genes and led to the possibility of detecting new expressed genes (Lanubile et al., 2014b; Wang et al., 2016).

A specific model for the intracellular signaling cascade against *F. verticillioides* infection occurring in maize cells is proposed by the integration of transcriptomic results deriving from Campos-Bermudez et al. (2013), Lanubile et al. (2014b), and Wang et al. (2016).

The first line of defense in plants is the recognition of conserved molecules characteristic of many microbes. These elicitors are also known as microbe-associated molecular patterns (MAMPs).

Fungal enzymes breaching the plant cell wall produce oligogalacturonides that are typical MAMPs and elicit defense responses (Ridley et al., 2001; Sanabria et al., 2008; Boller and Felix, 2009). In maize the well-characterized β-1,3-glucanases and chitinases (Lanubile et al., 2012a) may be involved in the degradation of cell walls of *F. verticillioides*, releasing MAMPs-derived cell wall fragments. Recognition of MAMPs by pattern recognition receptors (PRRs) that are plasma membrane localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs; Boutrot and Zipfel, 2017; Zhang et al., 2017) triggers MAMP-triggered immunity (MTI), thereby reinforcing the host defenses. Several PRRs, including cysteine-rich receptor-like kinase (CRRK), leucine- rich receptor-like kinase (LRRK), RLK, serine threonine kinase (STK), and BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) were identified in transcriptomic studies (Lanubile et al., 2014b; Wang et al., 2016).

A second line of the plants' defense is recognition of a given effector through a set of plant resistance (*R*) gene products resulting in effector-triggered immunity (ETI) (Jones and Dangl, 2006; Pel and Pieterse, 2013). *R* genes have been found in the interaction maize–*F. verticillioides* belonging to coiled coil- nucleotide binding site-leucine rich receptors (CC-NBS-LRR), NBS-LRR, and nucleotide-binding adaptors shared by APAF-1, R proteins, and CED-4 (NB-ARC) families (Lanubile et al., 2014b; Wang et al., 2016).

Both MTI and ETI triggered down-stream signaling networks in coordination with mitogen-associated protein kinase (MAPK) cascades, as reported in Figure 2.

In parallel, Ca^{2+} signaling through the cell membrane could be due to the induction of a specific calcium-dependent protein kinase (CDPK) gene expression after infection (Lanubile et al., 2014b). In turn, several CDPKs also activated respiratory burst oxidase homolog (RBOH) protein to induce early ROS production. The rapidly produced ROS affected the cellular oxidation state, inducing ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutaredoxin (GRX), thioredoxin (TRX), peroxidase (PRX), and glutathione-*S*-transferase (GST) gene expression, involved in plant cell wall reinforcement (Campo et al., 2004; Mohammadi et al., 2011). It has been shown that in resistant maize seedlings, before infection, APX and superoxide dismutase (SOD) enzymatic activities were higher than in the susceptible ones, while 5 days after inoculum, they remained unchanged. On the other hand, in the susceptible seedlings all enzymes assayed were activated only after *F. verticillioides* infection (Lanubile et al., 2012b).

These signals are primarily orchestrated by hormones until they reach the nucleus (Berens et al., 2017). The involvement of hormone-signaling genes, including salicylic acid (SA), auxin (AUX),

abscisic acid (ABA), ethylene (ET), and jasmonic acid (JA), has been observed (Figure 2). The targets of the hormone-signaling transduction pathways have been found to be multiple transcriptional factor families, such as WRKY for SA, MYB for ABA, auxin response factor (ARF) for AUX, and APETALA2/ethylene-responsive element binding protein (AP2/EREPB) and AP2/ ethylene responsive factor (AP2/ERF) through 1-aminocyclopropane-1-carboxylate (ACC) oxidases for ET (Campos-Bermudez et al., 2013; Lanubile et al., 2014b; Wang et al., 2016).

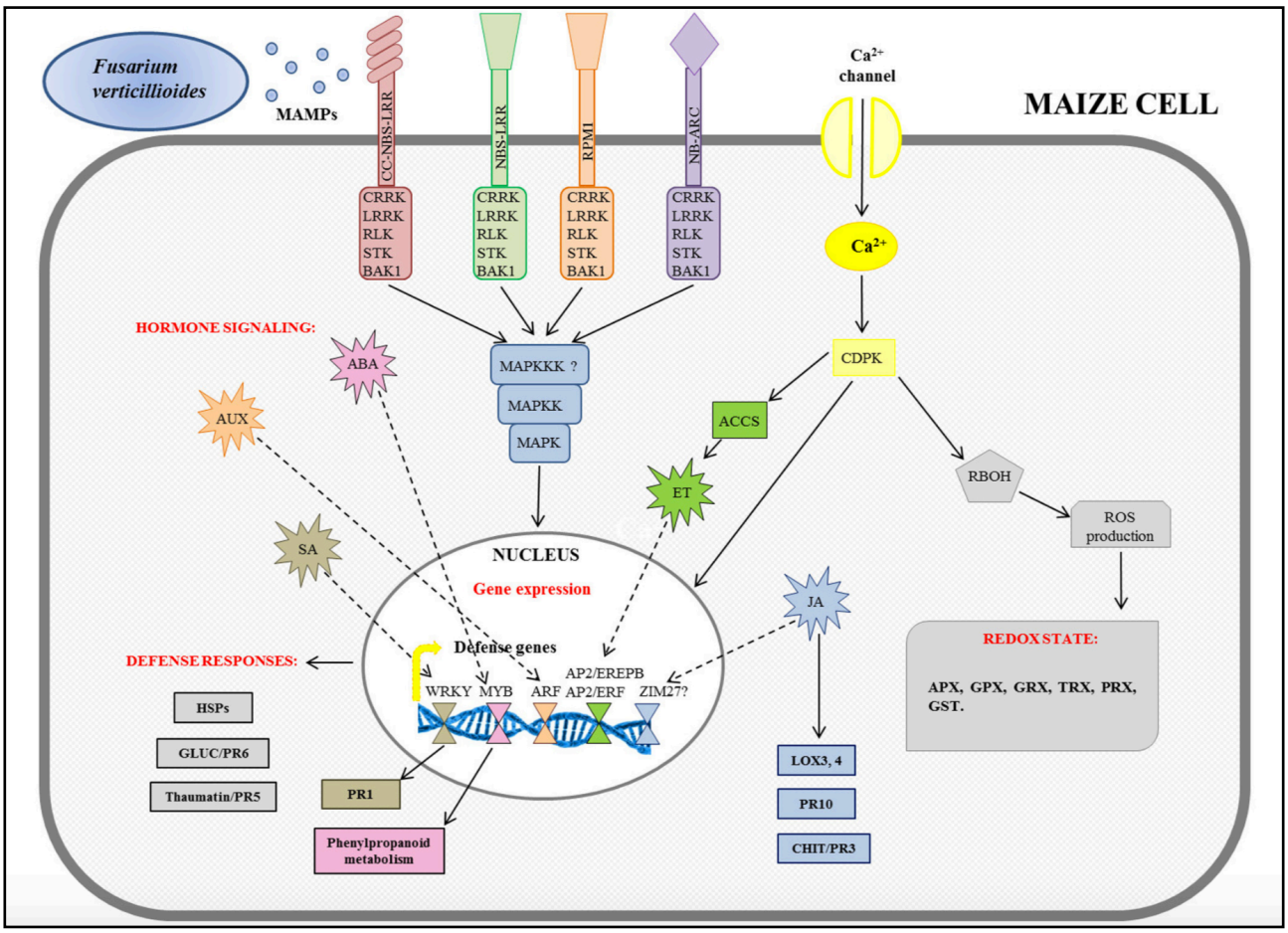

Figure 2. Schematic overview of maize defense gene activation in response to *Fusarium verticillioides* infection. The figure integrates the transcriptomic results previously reported in Campos-Bermudez et al. (2013), Lanubile et al. (2014b), and Wang et al. (2016). MAMPs (microbe-associated molecular patters); NBS-LRR (nucleotide binding site-leucine rich receptor); CC-NBS-LRR (coiled coil-NBS-LRR); NB-ARC (NB-adaptor shared by APAF-1, R proteins, and CED-4); BAK1 (BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1); CRRK (cysteine-rich receptor-like kinase); LRRK (leucine-rich receptor-like kinase); RLK (receptor-like kinase); STK (serine threonine kinase); CDPK (calcium-dependent protein kinase); MAPK (mitogen-activated protein kinase); MAPKK (MAPK kinase); MAPKKK (MAPKK kinase); RBOH (respiratory burst oxidase homolog protein); ROS (reactive oxygen species); APX (ascorbate peroxidase); GPX (glutathione peroxidase); GRX (glutaredoxin); TRX (thioredoxin); PRX

(peroxidase); GST (glutathione-S-transferase); ACCS (ACC synthase); ET (ethylene); AP2/EREPB (APETALA2/ethylene-responsive element binding protein); AP2/ERF (AP2/ethylene responsive factor); AUX (auxin); ARF (auxin response factor); ABA (abscisic acid); SA (salicylic acid); PR1 (pathogenesis-related 1); HSPs (heat shock proteins); CHIT (chitinase); GLUC (glucanase); JA (jasmonic acid); LOX (lipoxygenase).

WRKY are normally involved in the signal transduction pathway because they recognize the W-box of promoters of a large number of defense-related genes; in particular their association with the *PR1* gene has been described (Campos-Bermudez et al., 2013; Wang et al., 2016). Furthermore, it has been reported that Myb-like DNA binding proteins are involved in the signaling cascade for flavonol-specific gene activation in phenylpropanoid biosynthesis (Lanubile et al., 2014b). Other changes observed after *F. verticillioides* infection comprise the activation of genes encoding heat shock proteins (HSPs) as well as glucanases (GLUC or PR6) and thaumatin or PR5 proteins (Campos-Bermudez et al., 2013; Lanubile et al., 2014b; Wang et al., 2016). A JA signaling pathway has been found to promote the further down-stream activation of defense responsive genes for PR proteins, such as chitinases (CHIT or PR3) and PR10, and lipoxygenases (LOX3; LOX4). The role of JA in maize pathogen defense has recently been reviewed (Borrego and Kolomiets, 2016; Lim et al., 2017), and the relevance of genes for the lipoxygenase pathway in resistance to *F. verticillioides* is well established.

LOX genes have been found across animal, fungal, and plant kingdoms, and are presumed to be involved in plant susceptibility to fungal invasion and mycotoxin production (Kock et al., 2003; Christensen and Kolomiets, 2011; Maschietto et al., 2015). *LOX* genes are non-heme iron-containing dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) (Vick and Zimmerman, 1983), which are processed into an estimated 400 metabolites including the well-known hormone JA and green leaf volatiles (GLVs) (Mosblech et al., 2009). *LOX* genes are subdivided into two main functional groups, 9-LOXs and 13-LOXs, depending on which carbon on the fatty acid chain is oxygenated. A total of 13 different maize *LOXs* (*ZmLOXs*) with varying functions, localization, and regulation within the plant, have been reported (Yan et al., 2012). Of the 13 *ZmLOXs*, *ZmLOX4* and *ZmLOX5* located on chromosome 5 are the two most closely related paralogs, sharing only 40–67% of sequence identity with other *ZmLOXs* (Park et al., 2010). *ZmLOX4* and *ZmLOX5* are 9- LOXs and are segmentally duplicated genes. Other pairs of close paralogs include tandemly duplicated *ZmLOX1* and *ZmLOX2* and segmentally duplicated genes *ZmLOX7* and *ZmLOX8*, and *ZmLOX10* and *ZmLOX11* (Nemchenko et al., 2006; Christensen et al., 2013).

Maize mutants for a defective 9-LOX gene, *ZmLOX3*, resulted in reduced levels of several 9-LOX-derived fatty acid hydroperoxides. *F. verticillioides* conidiation and FB1 production, as well as other fungal diseases, were drastically reduced in kernels of *lox3* mutants (Gao et al., 2007, 2009). In addition, maize 9-LOX *ZmLOX12* suppressed contamination by *F. verticillioides* (Christensen et al., 2014). These observations suggest that a specific plant 9-LOX isoform is required for fungal pathogenesis, including disease development and production of spores and mycotoxins.

Localization and expression data supported the hypothesis that another *LOX* gene, *ZmLOX5* (expressed in the silks), affected resistance to other mycotoxigenic fungi, and a QTL affecting aflatoxin contamination was located where *ZmLOX5* also mapped (Warburton et al., 2010).

Key genes in the defense response are those of the phenylpropanoid pathway, encoding for phenylalanine ammonia lyase and chalcone synthase, leading to an accumulation of flavonoids, phenolic compounds, and phytoalexins. Phenolic compounds accumulate rapidly during host–pathogen interaction and may mediate disease suppression through the inactivation of fungal enzymes or the strengthening of plant structural components. High levels of phenylpropanoids in the kernel pericarp were associated with less severe FER and fumonisin accumulation (Assabgui et al., 1993; Sampietro et al., 2013). The most resistant genotypes exhibited high levels of phenylpropanoids (on average 23.7 mg/g of dry pericarp), related to low levels of disease severity and grain fumonisin concentration (5.6% of visibly diseased ear area and 56.7 ppm of fumonisin on average, respectively; Sampietro et al., 2013). In particular, total diferulates were the best explanatory parameter for the variability of disease severity, and grain fumonisin concentration was correlated to total diferulate, 8,5- diferulic acid benzofuran, and p-coumaric acid content. A potent inhibitory effect of α-tocopherol (0.1 mM) and ferulic acid (1 mM) on fumonisin biosynthesis was observed in *F. verticillioides* liquid cultures (Picot et al., 2013). These antioxidants were present in all stages of maize kernel development, indicating that the fumonisin-producing fungi were likely to face them during ear colonization.

Flavones in the silks contribute to FER resistance (Reid et al., 1992). Sekhon et al. (2006) investigated silk and kernel resistance to *F. verticillioides* and *F. proliferatum* in maize lines differing in 3-deoxyanthocyanidins and related 3-deoxyflavonoid (flavan-4-ols) content. Even though the degree of resistance was not strictly proportional to the amount of these secondary compounds in silks, the genes of the flavonoid pathway were active during the early stages of silk development. However, upon fungal inoculation, accumulation of 3-deoxyanthocyanidins was observed in resistant lines, suggesting a role of these compounds in resistance to *F. verticillioides*.

Higher susceptibility to FER was shown in ears of the *brown midrib* (*bm3*) mutant of maize, which cannot methylate either caffeic or hydroxyferulic acids to ferulic or sinapic acids due to a mutated *O*-methyltransferase (Vignols et al., 1995). Of the secondary metabolites, 6-methoxybenzoxazolin -2(3H)-one (MBOA) and benzoxazolin-2(3H)-one (BOA) have been found in corn and they are known for their antimicrobial properties (Glenn et al., 2002). Nevertheless, *F. verticillioides* is able to detoxify these compounds thanks to the presence of two specific loci, *Fdb1* and *Fdb2* (Glenn et al., 2002). Benzoxazinones are detoxified in 2-aminophenol (AP), which is converted to the less toxic *N*-(2-hydroxyphenyl) malonamic acid (HPMA) (Bacon et al., 2007). An endophytic bacterium, *Bacillus mojavensis*, is considered efficacious as a control of this *Fusarium* species, because it is able to produce a pigment identified as 2-amino-3H- phenoxazin-3-one (APO), which interacts with the fungus, thus preventing the usual transformation of AP into the non-toxic HPMA. The higher amounts of APO are toxic to *F. verticillioides* (Bacon et al., 2007).

The role of the biochemical composition of the endosperm has also been investigated. In particular, although Snijiders (1994) concluded that the biochemical composition of the endosperm had no intrinsic effect in proteins, sugars, and starches on resistance to the pathogen, Bluhm and Woloshuk (2005) found an influence on fumonisin B1 biosynthesis. Low amounts of amylopectin, required for fumonisin B1 biosynthesis, in early stages of kernel development and in some maize mutants, correlated with lower levels of mycotoxins (Bluhm and Woloshuk, 2005). The dynamic of water activity and humidity of maize kernels and their relevance for fumonisin accumulation in kernels was studied in medium to late season commercial hybrids by Battilani et al. (2011). The study revealed how “slow dry down” hybrids were more prone to fumonisin accumulation, irrespective of their maturity class.

More recently, the effect of fatty acid composition on fumonisin contamination and the occurrence of hidden fumonisins in maize (masking phenomenon consisting in the formation of covalent bonds between the tricarballylic groups of fumonisins and the hydroxyl groups of starch or the amino or sulfhydryl groups of the side chains of amino acids in proteins) has been investigated: higher fumonisin contamination was measured in hybrids showing a higher linoleic acid content and a higher masking action was observed in hybrids with higher oleic to linoleic ratio (Dall’Asta et al., 2012). Unsaturated fatty acids are often oxidized to produce oxylipins, whose role as signal molecules that regulate the response to biotic stress has been previously described (Wilson et al., 2001; Christensen and Kolomiets, 2011).

In general, it is worth mentioning that basal defense mechanisms against *F. verticillioides* were activated in maize- resistant kernels, as reported in several studies. Many proteins associated with

the defense response were found to be more abundant after infection, including PR10, chitinases, xylanase inhibitors, proteinase inhibitors, and PRXs. Kernels of the resistant line, even the non-inoculated ones, contained higher level of these defense-related proteins than the susceptible line, suggesting that these proteins may provide a basal defense against *Fusarium* infection in the resistant line (Mohammadi et al., 2011). These findings confirmed the conclusions of Lanubile et al. (2010, 2015a) and Maschietto et al. (2016) based on a transcriptomic analysis of the same resistant lines. Similar results were also obtained by Campos-Bermudez et al. (2013) using transcriptional and metabolite analysis in different resistant and susceptible inbreds. These results indicated that resistance was due to constitutive defense mechanisms preventing fungal infection. These mechanisms were poorly expressed in the susceptible line and, although the inoculation activated the defense response, this was not enough to prevent the disease's progress.

Genetic basis of the resistance to *Fusarium* infection

A deeper knowledge of the genetic basis underlying FER is necessary to speed up progress in breeding for resistance.

The most efficient way to improve FER resistance in hybrids is to evaluate and select among inbred lines, before using resources to produce hybrids (Hung and Holland, 2012). Lanubile et al. (2011) conducted screening trials for both FER and fumonisin concentration using public and private inbred lines, and identified several genotypes with good levels of resistance to both FER and fumonisin accumulation. In diallel mating of 18 inbred lines from different heterotic groups with different levels of resistance, hybrids had 27% less ear rot and 30% less fumonisin content than their inbred parents (Hung and Holland, 2012). General combing ability (GCA) and specific combining ability (SCA) were significant for disease resistance, and inbred performance *per se* and the corresponding GCA in hybrids were significantly correlated ($r \geq 0.78$).

FER resistance has proved to be a quantitative trait determined by polygenes (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Eller et al., 2008b). Pérez-Brito et al. (2001) tested two F_2 tropical maize populations of 238 and 206 F_2 individuals derived respectively from single crosses between resistant and susceptible inbred lines for FER resistance, and they measured relatively low heritability (h_2=0.26-0.42). Robertoson-Hoyt et al. (2006) tested two segregating populations of 213 $BC_1F_{1:2}$ families from the first backcross of GE440 to FR1064 (GEFR) and 143 recombinant inbred lines (RILs) from the cross of NC300 to B104 (NCB), respectively, both for fumonisin contamination and FER resistance traits. This experiment enhanced the breeding for resistance approach because family mean heritability for ear rot resistance increased by up to 0.47-0.80 and

for fumonisin contamination by up to 0.75-0.86. The increment of the heritability in comparison to Pérez-Brito's experiment can be explained by a reduction in the environmental influence obtained by doubling the number of evaluation environments and the number of artificial inoculations per plant. High positive correlations of FER resistance with fumonisin contamination and moderate-high heritabilities of both traits observed in the populations GEFR and NCB suggested that selecting for both traits at the same time was feasible (Robertson et al., 2006). Phenotypic correlation between the severity of FER and the amount of fumonisins has been reported to be moderate to low (Clements et al., 2003; Clements et al., 2004), probably because of symptomless endophytic infections (Oren et al., 2003). Moreover, genotypic correlation between the two traits was higher than the phenotypic correlation (0.87-0.96 versus 0.40-0.64) (Robertson et al., 2006). This demonstrated that genotypic effects on susceptibility to ear rot and fumonisin content were highly correlated (Robertson et al., 2006). The close correlation between FER and fumonisin accumulation suggests that toxin analysis is only rarely needed, if disease severity data are available. In breeding, selection against genotypes more susceptible to FER allows for simultaneous selection against genotypes accumulating high contents of fumonisins. Moreover, genetic mechanisms controlling both traits are the same or closely linked.

Maize quantitative trait loci (QTL) providing resistance to *Fusarium verticillioides*

The response to selection for resistance to FER can be increased by a wide variability in maize genotypes towards disease resistance and fumonisin contamination and by the moderate to high heritability of the traits. Nevertheless, phenotypic selection for the two traits is hampered by practical difficulties. Although many diseases could be evaluated during the plant's young stage or before flowering, FER and mycotoxin concentrations can only be analyzed on mature seeds and require artificial inoculations with calibrated fungal spore suspensions for consistent evaluation of the disease (Clements et al., 2003). Moreover, asymptomatic infections of this pathogen lead to time-consuming and expensive toxin assays for contamination assessment.

In addition, plant traits can affect pathogen access through the silk channel and the kernel. Hybrids with tight, adherent husks and less open apical parts of the ear were more resistant to FER (Butron et al., 2006; Warfield and Davies, 1996). Physiological traits such as earliness in flowering time have been shown to reduce susceptibility towards several pathogens, including *F. verticillioides*. FER was less common for inbred lines with green and actively growing silks at inoculation time rather than brown silks. Kernel properties and seed coat influenced pathogen success (Scott and King, 1984; Headrick and Pataky, 1991; Hoenish and Davis, 1994). A thicker pericarp made maize

more resistant to penetration. Disease severity was dependent on husk integrity, on drought stress that increased the amount of stalk rot, and agronomic practices, for instance irrigation at the silk stage (Munkvold, 2003b; Battilani et al., 2008).

Finally, FER is influenced by many environmental factors, and testing for multiple sites and years is required (Shelby et al., 1994; Munkvold, 2003a; Robertson et al., 2006; Zila et al., 2013; Maschietto et al., 2017).

Disease phenotyping

Selection for resistant hybrids must occur in areas with a known high incidence of FER. *F. verticillioides* can over-winter in the soil and may be spread by wind, rain splash, and insect larvae (Munkvold, 2003a), but to ensure equal distribution of the pathogen for all of the plants in the field, artificial inoculation is needed (Munkvold and Desjardins, 1997). Kernel infection through the seeds and infection through the silks are the best techniques for evaluating genetic resistance to FER (Munkvold and Desjardins, 1997; Robertson et al., 2006). These techniques refer to two types of inoculation method: with (type 1) and without (type 2) mechanical inoculation. Type 1 methods include toothpick inoculation methods and test kernel resistance (Reid et al., 1996), whereas in a typical type 2 method, a spore suspension is sprayed onto the maize silks with an atomizer, or injected into the silk channel near the cob tip. Type 1 inoculation methods usually screen for resistance to spreading on the host and simulate insect attack, as they bypass many of the plant's morphological barriers. Type 2 inoculation methods more closely resemble natural infection of a non-wounded host plant.

The best differentiation between resistant and susceptible genotypes has been obtained when inoculation occurred within a week after silking for type 2 inoculation (Reid et al., 1992; Lanubile et al., 2010; Campos-Bermudez et al., 2013); type 1 inoculation was effective 15 days after pollination (Lanubile et al., 2014b; Wang et al., 2016). Later inoculations resulted in significantly less severe disease symptoms, while the very early ones, i.e., 4–6 days, increased cases of disease outbreak.

As an alternative to field tests, *in vivo* bioassays including the rolled towel assay (RTA) or the Petri dish bioassay have been proposed for testing the ability of different pathogens to infect and colonize seedlings and kernels, respectively (Ellis et al., 2011; Lanubile et al., 2015b; Ju et al., 2017).

Fungal contamination of grains can be measured by various methods: the ergosterol level, representing a quantitative and qualitative measure of fungal contamination (Bakan et al., 2002), even though it is not strictly correlated with mycotoxin content; and the absolute quantification of

fungal housekeeping genes, such as β-*tubulin*, through quantitative PCR (Lanubile et al., 2010, 2012a, 2014b).

Accurate mycotoxin analysis can be conducted with high- performance liquid chromatography (HPLC), but its costs make this technique unsuitable for use in large-scale breeding programs. HPLC can be replaced by the ELISA assay (Eller et al., 2008a) and near-infrared spectroscopy (NIRS) (Siesler et al., 2002; Berardo et al., 2005). The NIRS methodology can potentially be used for large-scale selection of genotypes resistant to fungal and fumonisin contamination.

Mapping QTL for resistance and genome-wide association studies

Quantitative trait loci mapping and MAS, using PCR-based DNA markers associated to resistance genes, could be a successful strategy for selecting lines resistant to *F. verticillioides* (Beavis, 1998; Robertson et al., 2005). Localization of FER resistance QTL has often appeared to be contradictory in different studies (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Ding et al., 2008), probably because of a strong environmental influence on the spread of the disease. Figure 3 reports the localization of the main QTL and SNP markers for FER resistance on the maize chromosomes. Pérez-Brito et al. (2001) identified nine and seven QTL in two F_2 populations cross 3 × 18 and 5 × 18, respectively. The detected QTL explained between 30 and 44% of the phenotypic variation in the first population and 11–26% in the second. Three QTL on chromosomes 3 and 6 were co-located in both populations. Due to the number and limited effects of the QTL detected, Pérez-Brito et al. (2001) excluded MAS as a suitable strategy for this trait. Further studies contrasted with Pérez-Brito's conclusion (Robertson-Hoyt et al., 2006; Ding et al., 2008). Robertson-Hoyt et al. (2006) tested two segregating populations, a GEFR and an NCB population, derived, respectively, from FER resistant line GE440 crossed with FR1064 and the low fumonisin contamination line NC300 crossed with B104. In the GEFR population, seven QTL were identified, explaining 47% of the phenotypic variation for FER resistance, and nine were found for fumonisin content, explaining 67% of the variation. In the NCB population, five QTL explained 31% of the FER variation and six QTL explained 81% of the fumonisin variation. Three QTL for FER and two for fumonisin were mapped in similar positions in the two populations. In particular, two QTL, localized on chromosomes 4 and 5, appeared to be consistent for both traits in both populations. Ding et al. (2008) tested a RIL population of 187 genotypes for FER resistance. Of five identified QTL, two on chromosome 3 were stable across environments. The major QTL explained 13–22% of the phenotypic variation for FER, and it was flanked by SSR markers umc1025 and umc1742. More recently, a QTL on chromosome 4 (bin 4.05/06) was identified in the resistant inbred line BT-1

which explained 17.95% of the phenotypic variation for resistance to FER (Chen et al., 2012). Further verification of the QTL effect in near-isogenic lines (NILs) carrying the QTL region on chromosome 4 showed that if homozygous, this QTL can increase the resistance by 33.7–35.2%. The stable and significant resistance effect of the QTL on chromosomes 3 and 4 lays the foundation for further MAS and map-based cloning.

In conclusion, since QTL mapping used populations originated by crossing two homozygous lines, genetic variation in FER resistance was limited to the differences between the two parents.

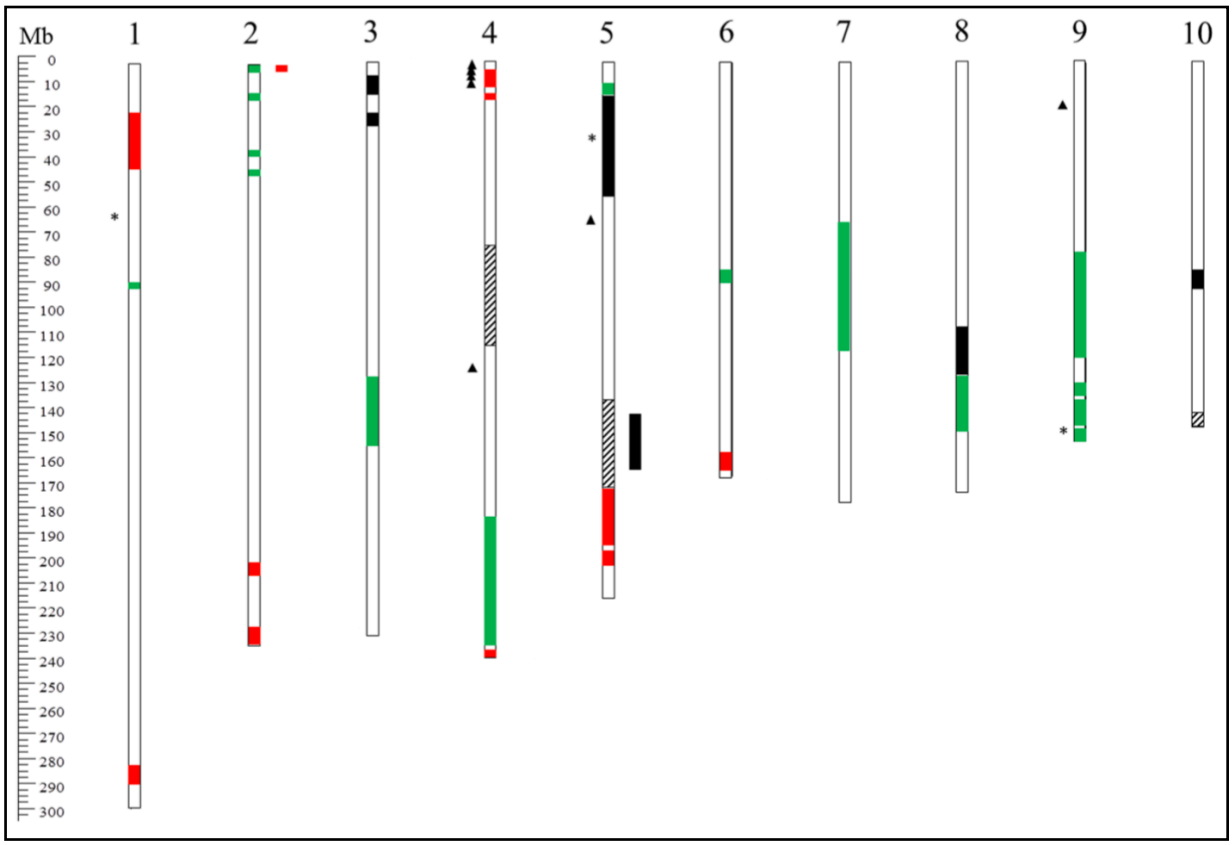

Figure 3. Overview of chromosomal locations on the B73 reference genome (version 2) of known QTL for Fusarium ear rot (FER) resistance. The bars inside the chromosomes indicate QTL intervals detected by Robertson-Hoyt et al. (2006) (red), Ding et al. (2008) (black), Chen et al. (2012) (dashed), and Maschietto et al. (2017) (green). The asterisks and the triangles on the left side of the chromosomes indicate FER-associated SNPs detected by Zila et al. (2013) and Zila et al. (2014), respectively.

Furthermore, the resolution power was often low and QTL positions spanned from a few to tens of centimorgans. These regions corresponded to several megabases which contained hundreds of genes. Such limitations, as well as the strong influence of environmental factors, hinder accurate QTL localization and the possibility of performing MAS efficiently (Robertson-Hoyt et al., 2006).

These issues may be partially overcome by increasing population size and the number of markers used, improving ear rot phenotyping protocols and integrating data from multiple environments (Robertson et al., 2005). In particular, initial QTL mapping studies on these traits were based on maps containing a few hundred restriction fragment length polymorphisms (RFLP; Pérez-Brito et al., 2001) and single sequence repeat (SSR) markers (Robertson-Hoyt et al., 2006; Ding et al., 2008; Chen et al., 2012).

In recent years, single-nucleotide polymorphisms (SNPs) have become the preferred genotyping system for genetic studies, being the cheapest and the most abundant markers in a genome (Rafalski, 2002), e.g., 1 SNP/100 bp in maize (Tenaillon et al., 2001).

With the advent of NGS technologies, SNP markers have shown their full potential with novel approaches combing SNP discovery and genotyping, such as Genotyping-by-Sequencing (GBS; Elshire et al., 2011).

Three GBS studies were performed on maize to detect allele variants associated with increased resistance to FER. In a maize core diversity panel of 267 inbred lines, three SNPs with significant effects on chromosomes 1, 5, and 9 were described (Zila et al., 2013). Seven SNPs in six genes associated with FER resistance were identified on chromosomes 4, 5, and 9 in a panel of 1,687 US maize inbred line collections (Zila et al., 2014). Maschietto et al. (2017) found eight QTL located on linkage groups (LGs) 1, 2, 3, 6, 7, and 9 that were common to FER response and FB1 contamination, making the selection of genotypes with both low disease severity and low fumonisin contamination possible. Five QTL were located close to previously reported QTL for resistance to other mycotoxigenic fungi. Moreover, combining previous transcriptomic data (Lanubile et al., 2014b) with QTL mapping, 24 candidate genes for resistance to *F. verticillioides* were positioned in the same chromosomal regions.

Furthermore, comparing studies addressed to detection of QTL for resistance against different diseases reveals that there is evidently an overlap of the genetic mechanisms involved. Several fumonisin contamination QTL (Robertson-Hoyt et al., 2006) were localized on chromosomes 1, 2, 3, 4, 5, and 9 close to QTL for aflatoxin contamination (Wisser et al., 2006). In addition, Robertson-Hoyt et al. (2007) discovered QTL affecting both fumonisin and aflatoxin contamination, and Fusarium and Aspergillus ear rots.

The role of fumonisins in the host-pathogen interaction

F. verticillioides produces fumonisins as secondary metabolites (Gelderblom et al., 1988), a family of mycotoxins that affect animal and human health (Munkvold and Desjardins, 1997). Among the most active fumonisins, *F. verticillioides* produces B series fumonisins, particularly FB1.

FB1 is synthesized via a polyketide biosynthetic pathway (Butchko et al., 2006). The fumonisin (*FUM*) gene cluster, including genes involved in FB1 biosynthesis, is known to contain 22 genes with a length of 42 kb (Proctor et al., 2003). Of the 22 genes, 15 genes are co-regulated, including the key gene *FUM1*, which encodes a polyketide synthase (PKS) (Proctor et al., 1999).

There are contrasting reports on the role of fumonisin production in the ability of *F. verticillioides* to cause maize ear rot. Fumonisin-nonproducing mutants were generated by disrupting *FUM1*, the gene encoding PKS, which is required for fumonisin biosynthesis (Proctor et al., 1999). *Fum1* mutants were 100% reduced in fumonisin production, but in field tests they were able to cause ear rot. The results provided evidence that production of fumonisins was not required for ear rot development and suggest that it is unlikely that fumonisin resistance would be an effective way to control this disease or fumonisin contamination in maize (Desjardins et al., 2002; Jardine and Leslie, 1999). Conversely, Lanubile et al. (2013) observed an enhanced reaction of incompatibility between resistant host and a *fum1* mutant of *F. verticillioides*, impaired in PKS activity, compared with the isogenic wild-type strain. In the early stages of infection, when the production of fumonisins was not detectable, the *fum1* mutant differed in its ability to colonize maize kernels compared to the wild type strain. In the resistant maize genotype, the *fum1* mutant provoked a delayed and weakened activation of defense-related genes, presumably as a consequence of reduced growth. The inability of the *fum1* mutant to infect maize ears may be related to PKS activity and its association with the LOX pathway. Plant and fungal *LOX* genes were up-regulated after *fum1* mutant inoculation, suggesting that *PKS* is a relevant gene, essential not only to the fumonisin biosynthetic pathway, but also to pathogen colonization.

Arias et al. (2012) focused on the role of fumonisins as possible pathogenicity factors in the maize-*F. verticillioides* interaction. The effect of fumonisin on the development of maize seedling disease was observed to be strongly influenced by toxin concentration. High levels of fumonisin triggered necrosis and wilting in maize seedlings, while on the other hand low doses activated detoxification processes, suggesting a strategy of recovery in the host plants. Death induced by FB1 usually presents features which resemble those of the hypersensitive response (HR), being fast and limited to the tissues that are exposed to the toxin (Asai et al., 2000; Stone et al., 2000), and determining

the induction of defense genes (pathogenesis-related, phenylalanine ammonia lyase), chromatin condensation and production of ROS, possibly in the apoplast through peroxydases. Different tissues and species have been used in the past for these toxicity studies, ranging from roots to leaves, from maize to *Arabidopsis* (Stone et al., 2000; Nadubinska and Ciamporova, 2001; Lin et al., 2008; Sánchez-Rangel et al., 2012).

FB1 acts through several pathways: salicylic acid, ethylene and jasmonates (Asai et al., 2000). It causes a depletion of extracellular ATP reservoirs and eventually involves the protease vacuolar-processing-enzyme (VPE) as regulator of programmed cell death (PCD) (Kuroyanagi et al., 2005). Finally, there is evidence that ubiquitination also plays an important role in FB1-induced PCD (Lin et al., 2008). Future knowledge of the toxicity mechanisms of this molecule might suggest new management strategies.

Future prospects

Several omics aspects of the *F. verticillioides*-maize interaction have been discussed in this review. Although down-stream processes of response to *F. verticillioides* infection have been well elucidated through transcriptomic studies, less information is available on the up-stream processes of recognition between maize and the fungus. To fill these gaps, recent advances in genomic technologies, such as GWAS, could resolve this complex trait down to the sequence level (Zhu et al., 2008). Moreover, GWAS applied to a large multi-parent population of RILs, termed MAGIC (Multi-parent Advanced Generation Inter-Cross; Cavanagh et al., 2008), will ensure the identification of multiple genes, determining resistance to both FER and fumonisin contamination.In addition, as resistance to *F. verticillioides* is quantitative and based on a diffused architecture of many minor genes, the best approach for future molecular breeding will shift from marker-assisted selection to genomic selection. Genomic-assisted breeding for quantitative resistance will necessitate whole genome marker profiles for the entire set of breeding lines, prediction models and selection methodology as implemented for classical complex traits such as yield (Poland and Rutkoski, 2017).A critical issue is that of the exploitation of candidate genes for resistance. RNASeq has been of great value in improving, validating and refining gene models, and can identify new genes not previously annotated. A new approach to identifying candidate genes and QTL for resistance is represented by plant metabolome investigation after pathogen infection. Growing efforts are being made in research into relating genomic to metabolic (phenotypic) information (Bueschl et al., 2014). Keurentjes et al. (2006) have shown the potential of untargeted metabolomics to reveal QTL in the model plant *Arabidopsis*. An increasing number of metabolites

are assigned to specific metabolic pathways and are the products of enzymatic reactions that depend on genome regulation. Moreover, the metabolic profile corresponds to the biochemical status of the organism that is a phenotypic expression. Metabolic profiling of resistant and susceptible cultivars can be used to detect biomarkers associated with the resistant trait.

In addition, genetic engineering permits the introduction or modification of gene coding for proteins with antifungal activities and enzymes that breach the plant cell wall, to increment pathogen resistance. In maize, several transgenic approaches can be exploited to reduce fumonisin content: reducing disease severity either by eliminating insect injury or by decreasing pathogen efficacy, by detoxifying or by blocking the synthesis of mycotoxins in seed (Duvick, 2001; Gao et al., 2007; Yuan et al., 2007). A limitation of this strategy is the possibility that other biosynthetic pathways might be altered, resulting in the biosynthesis of new plant secondary metabolites. Moreover, new identified dominant resistance genes (*R* genes) could be engineered in order to increase resistance in a specific response. More recently, efficient editing technologies for genome modification in multiple plant species have emerged. Of these, the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system has been used successfully in staple crops to modify single genes and change expression patterns. New gene variants conferred beneficial traits for plant breeding, including stress tolerance (Svitashev et al., 2015; Char et al., 2017; Shi et al., 2017). Finally, it is now generally accepted that efforts devoted to the improvement of resistance to FER will also determine increases in resistance to other ear rots and, in particular, to the rotting produced by *Aspergillus* spp. Several studies have dealt with the positive relationship between infection by *Fusarium* and *Aspergillus* spp. (Clements and White, 2004; Lanubile et al., 2011; Pechanova and Pechan , 2015). Such results suggest that these fungal species may require similar substances for growth and development, and that they interact in similar ways with the host plant.

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Molecular Basis of Resistance to Fusarium Ear Rot in Maize

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The impact of climate change has been identified as an emerging issue for food security and safety, and the increased incidence of mycotoxin contamination in maize over the last two decades is considered a potential emerging hazard. Disease control by chemical and agronomic approaches is often ineffective and increases the cost of production; for this reason the exploitation of genetic resistance is the most sustainable method for reducing contamination. The review focuses on the significant advances that have been made in the development of transcriptomic, genetic and genomic information for maize, *Fusarium verticillioides* molds, and their interactions, over recent years. Findings from transcriptomic studies have been used to outline a specific model for the intracellular signaling cascade occurring in maize cells against *F. verticillioides* infection. Several recognition receptors, such as receptor-like kinases and *R* genes, are involved in pathogen perception, and trigger down-stream signaling networks mediated by mitogen-associated protein kinases. These signals could be orchestrated primarily by hormones, including salicylic acid, auxin, abscisic acid, ethylene, and jasmonic acid, in association with calcium signaling, targeting multiple transcription factors that in turn promote the down-stream activation of defensive response genes, such as those related to detoxification processes, phenylpropanoid, and oxylipin metabolic pathways. At the genetic and genomic levels, several quantitative trait loci (QTL) and single-nucleotide polymorphism markers for resistance to Fusarium ear rot deriving from QTL mapping and genome-wide association studies are described, indicating the complexity of this polygenic trait. All these findings will contribute to identifying candidate genes for resistance and to applying genomic technologies for selecting resistant maize genotypes and speeding up a strategy of breeding to contrast disease, through plants resistant to mycotoxin-producing pathogens.

Keywords: ***Fusarium*, ear rot, fumonisins, genetic resistance, *Zea mays***

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INTRODUCTION

A large number of fungi can attack and invade developing maize ears and kernels, causing numerous diseases classified as ear rots. Many ear rot fungi produce mycotoxins that can affect the quality and marketability of grains. *Fusarium verticillioides* (Sacc.) Nirenberg (synonym *F. moniliforme* Sheldon, teleomorph *Gibberella moniliformis* Wineland) causes stalk rot and ear rot in maize, and is endemic in maize fields at harvest (Bottalico, 1998; Battilani et al., 2008).

F. verticillioides is the main causal agent of Fusarium ear rot (FER) (Logrieco et al., 2002; Folcher et al., 2009). Interest in *F. verticillioides* has been renewed by the discovery that the fungus can produce the secondary metabolite fumonisins (Gelderblom et al., 1988).

Breeding for resistance to FER and fumonisin contamination is considered the environmentally safest and most economical strategy (Munkvold, 2003a; Eller et al., 2008a), and many studies have focused on the search for resistance (Clements et al., 2004; Lanubile et al., 2011; Maschietto et al., 2017). These studies have demonstrated genetic variation for resistance to FER and fumonisin contamination, but no evidence of complete resistance to the pathogen has been observed. Quantitative trait loci (QTL) mapping studies in maize have indicated that resistance is a quantitative trait determined by polygenes having small effect (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Ding et al., 2008; Chen et al., 2012; Maschietto et al., 2017). Large genetic bases and the strong influence of the environment have slowed progress in accurate QTL localization, therefore reducing the efficiency of marker-assisted selection (MAS) (Robertson-Hoyt et al., 2006). Increasing population size and the number of markers used, improving ear rot phenotyping protocols and integrating data from multiple environments, will overcome such limitations (Robertson et al., 2005).

Transcriptomic and genome-wide association studies (GWAS) are useful tools for identifying candidate genes, especially when combined with QTL mapping in order to map and validate loci for quantitative traits (Korte and Farlow, 2013). The combination of these methods has overcome the limitations of either method performed alone (Brachi et al., 2010). Two recent GWASs were performed in maize to detect SNP associated with increased resistance to FER, resulting in 10 SNP markers with significant effects on several chromosomes (Zila et al., 2013, 2014).

As an alternative to plant breeding techniques, next-generation precision genome engineering relying on genome editing technologies can play a key role in accessing genetic resources and using them to increase plant disease resistance, by targeting suitable plant defense mechanisms. Such approaches, however, require efficient transformation protocols as well as extensive genomic resources and accurate knowledge, before they can be efficiently exploited in practical breeding programs.

In this review, we provide an extensive overview of recent developments related to basic research and breeding efforts aimed at improving resistance to FER and fumonisin contamination in one of the most important grain food crops, i.e., maize.

IMPORTANCE OF FUSARIUM EAR ROT DISEASE

Fusarium verticillioides, often in association with *F. subglutinans* and *F. proliferatum* (Logrieco et al., 2002), causes FER or pink ear rot, typically occurring on random groups of kernels or on physically injured kernels (White, 1999; Munkvold, 2003a; Lanubile et al., 2014a). FER prevails in drier and warmer climates, like those common in southern Europe and the United States (Logrieco et al., 2002; Eller et al., 2008a). FER strongly affects grain production, with yield reduction often estimated between 10 and 30% (Bottalico, 1998; Logrieco et al., 2002).

The interest in this fungus has arisen from mycotoxin accumulation in pre-harvest infected plants or in stored grains. *F. verticillioides* mycotoxins, including fumonisins, have been associated with chronic or acute mycotoxicoses in livestock. Feeds contaminated with FB1 caused leukoencephalomacia in horses and pulmonary edema and hepatic syndrome in swine (Ross et al., 1990). FB1 carcinogenic activity in rats (Gelderblom et al., 1996) and its relation with neural tube birth defects in humans (Missmer et al., 2006) have led to the classification of FB1 as carcinogenic for animals and humans. It has been estimated that 25% of world food crops are affected by mycotoxins, but for fumonisins the percentage could be even higher (Bottalico, 1998; Logrieco et al., 2002; Pietri et al., 2004; Eller et al., 2008a).

Regulations for permitted mycotoxin limits in food and feed have been set in most countries (Ferrigo et al., 2016). The European Commission has indicated maximum tolerable levels for fumonisins as 4000 ppb in unprocessed maize, 1000 ppb in maize intended for direct human consumption, and 800 ppb in maize-based breakfast cereals and snacks. Outside Europe, in the main maize producing countries, the US Food and Drug Administration (FDA) has recommended that fumonisin levels in dry milled corn products and cleaned maize used for popcorn should not exceed 2000 and 3000 ppb, respectively. The Health Surveillance Agency for Brazil (ANVISA) has established maximum tolerable limits of 1500 and 1000 ppb in maize meal and other maize-based products, respectively. Furthermore, the permissible levels of fumonisins in maize flour are not more than 200 ppb for the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor). The different regulations on mycotoxin levels are due to a global market, and since European regulations appear stringent, a common strategy would seem to be the best way forward to ensure food safety.

Fusarium verticillioides INFECTION IN MAIZE KERNELS

Fusarium verticillioides has been shown to behave as an endophytic fungus that tends to be symptomless in the kernels and can be systematic in the maize plant (Munkvold et al., 1997). Whitish pink fungal growth on kernels and/or silks is typical. Infected kernels may also exhibit a "starburst" symptom, i.e., white streaks radiating from the point of the silk attachment at the cap or from the base of the kernel (**Figure 1**). There are three main access pathways for the fungus into the ear: (i) fungal spores germinating on the silks and then fungal mycelia growing down the silks to infect the kernels and the cob (rachis); (ii) through wounds on the ear generated by insects, birds, or hail damage; (iii) systemic infection of the ear through infected stalks that generate infected seeds (Munkvold et al., 1997; Munkvold, 2003a). Kernel infection develops most efficiently from strains that are inoculated into the silks (Munkvold et al., 1997), but the

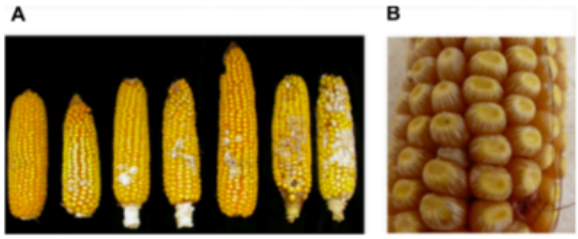

FIGURE 1 | Fusarium ear rot (FER) symptoms. **(A)** Different degrees of FER on ears of resistant (right) to highly susceptible maize lines (left). **(B)** Starburst showing white streaks radiating from the point of silk attachment at the cap of the kernel or from the base. Panel **(A)** is adapted from Maschietto et al. (2017).

prevalence of one or the other pathway depends on the insect pressure in the area.

Only recently the biology of maize kernel infection was investigated using a fluorescent-protein expressing transformant of *F. verticillioides* (Duncan and Howard, 2010). After the introduction of a conidial suspension through the silk channel, the fungus penetrated kernels via the stylar canal and spread within the pericarp, colonizing adjacent cells through pits. Starburst symptoms were observed only at the later times of inoculation, indicating the destruction of the pericarp cell wall (Duncan and Howard, 2010). Early reports focused on germinating seeds revealed that *F. verticillioides* penetrated directly by hyphae through the epidermal cells of the seedling and colonized the host tissue by inter- and intracellular modes of growth (Murillo et al., 1999; Oren et al., 2003). Scutellum colonization occurred earlier with branched hyphae growing into the parenchyma cells, and produced pronounced cell alterations and collapsed protoplasts. Pathogen ingress into the infected tissue induced defense-related ultrastructural modifications, such as appositions on the outer host cell wall surface, the occlusion of intercellular spaces, and the formation of papillae. Pathogenesis-related proteins from maize (PRms) represent the first barrier for fungal penetration and accumulated at very high levels in the aleurone layer and scutellar epithelial cells, as well as within the papillae. This suggests that signaling mechanisms that lead to their accumulation can operate at a distance from the infection point (Murillo et al., 1999).

MAIZE–*Fusarium verticillioides* MOLECULAR INTERACTION

Next-generation sequencing (NGS) and microarray approaches have been used to identify molecular mechanisms connected with *F. verticillioides* infection in resistant and susceptible maize genotypes (Lanubile et al., 2010, 2012a, 2014b; Campos-Bermudez et al., 2013; Wang et al., 2016). All these studies compared the response of resistant and susceptible lines to infection, considering early [12–48 h post-inoculation (hpi)] and late (from 72 to 120 hpi) stages of infection. Microarray hybridization studies were performed in the earliest published works (Lanubile et al., 2010, 2012a; Campos-Bermudez et al., 2013), whereas RNASeq technology has been employed in the more recent references (Lanubile et al., 2014b; Wang et al., 2016). Most of the information about differentially expressed genes has been obtained from infected maize kernels (Lanubile et al., 2010, 2012a, 2014b; Wang et al., 2016), whereas only two experiments have focused on infected silks (Lanubile et al., 2010; Campos-Bermudez et al., 2013). RNASeq has allowed for the identification of several thousands of differentially expressed genes and led to the possibility of detecting new expressed genes (Lanubile et al., 2014b; Wang et al., 2016).

A specific model for the intracellular signaling cascade against *F. verticillioides* infection occurring in maize cells is proposed by the integration of transcriptomic results deriving from Campos-Bermudez et al. (2013), Lanubile et al. (2014b), and Wang et al. (2016).

The first line of defense in plants is the recognition of conserved molecules characteristic of many microbes. These elicitors are also known as microbe-associated molecular patterns (MAMPs). Fungal enzymes breaching the plant cell wall produce oligogalacturonides that are typical MAMPs and elicit defense responses (Ridley et al., 2001; Sanabria et al., 2008; Boller and Felix, 2009). In maize the well-characterized β-1,3-glucanases and chitinases (Lanubile et al., 2012a) may be involved in the degradation of cell walls of *F. verticillioides*, releasing MAMPs-derived cell wall fragments.

Recognition of MAMPs by pattern recognition receptors (PRRs) that are plasma membrane localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs; Boutrot and Zipfel, 2017; Zhang et al., 2017) triggers MAMP-triggered immunity (MTI), thereby reinforcing the host defenses. Several PRRs, including cysteine-rich receptor-like kinase (CRRK), leucine-rich receptor-like kinase (LRRK), RLK, serine threonine kinase (STK), and BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) were identified in transcriptomic studies (Lanubile et al., 2014b; Wang et al., 2016).

A second line of the plants' defense is recognition of a given effector through a set of plant resistance (*R*) gene products resulting in effector-triggered immunity (ETI) (Jones and Dangl, 2006; Pel and Pieterse, 2013). *R* genes have been found in the interaction maize–*F. verticillioides* belonging to coiled coil-nucleotide binding site-leucine rich receptors (CC-NBS-LRR), NBS-LRR, and nucleotide-binding adaptors shared by APAF-1, R proteins, and CED-4 (NB-ARC) families (Lanubile et al., 2014b; Wang et al., 2016).

Both MTI and ETI triggered down-stream signaling networks in coordination with mitogen-associated protein kinase (MAPK) cascades, as reported in **Figure 2**.

In parallel, Ca^{2+} signaling through the cell membrane could be due to the induction of a specific calcium-dependent protein kinase (CDPK) gene expression after infection (Lanubile et al., 2014b). In turn, several CDPKs also activated respiratory burst oxidase homolog (RBOH) protein to induce early ROS production. The rapidly produced ROS affected the cellular oxidation state, inducing ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutaredoxin (GRX), thioredoxin (TRX), peroxidase (PRX), and glutathione-*S*-transferase (GST) gene expression, involved in plant cell wall reinforcement

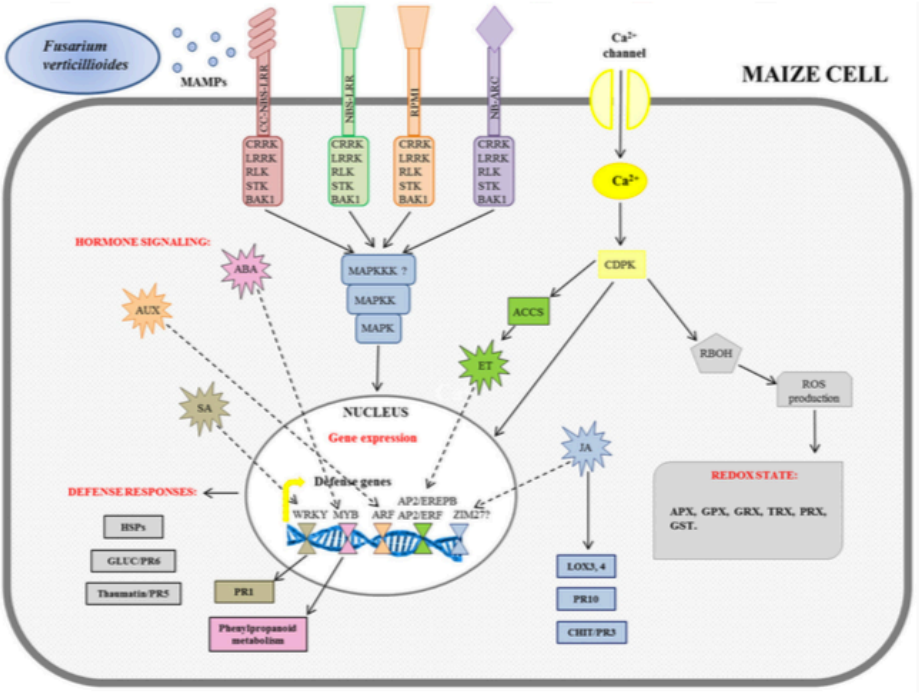

FIGURE 2 | Schematic overview of maize defense gene activation in response to *Fusarium verticillioides* infection. The figure integrates the transcriptomic results previously reported in Campos-Bermudez et al. (2013), Lanubile et al. (2014b), and Wang et al. (2016). MAMPs, microbe-associated molecular patters; NBS-LRR, nucleotide binding site-leucine rich receptor; CC-NBS-LRR, coiled coil-NBS-LRR; NB-ARC, NB-adaptor shared by APAF-1, R proteins, and CED-4; BAK1, BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1; CRRK, cysteine-rich receptor-like kinase; LRRK, leucine-rich receptor-like kinase; RLK, receptor-like kinase; STK, serine threonine kinase; CDPK, calcium-dependent protein kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; RBOH, respiratory burst oxidase homolog protein; ROS, reactive oxygen species; APX, ascorbate peroxidase; GPX, glutathione peroxidase; GRX, glutaredoxin; TRX, thioredoxin; PRX, peroxidase; GST, glutathione-*S*-transferase; ACCS, ACC synthase; ET, ethylene; AP2/EREPB, APETALA2/ethylene-responsive element binding protein; AP2/ERF, AP2/ethylene responsive factor; AUX, auxin; ARF, auxin response factor; ABA, abscisic acid; SA, salicylic acid; PR1, pathogenesis-related 1; HSPs, heat shock proteins; CHIT, chitinase; GLUC, glucanase; JA, jasmonic acid; LOX, lipoxygenase.

(Campo et al., 2004; Mohammadi et al., 2011). It has been shown that in resistant maize seedlings, before infection, APX and superoxide dismutase (SOD) enzymatic activities were higher than in the susceptible ones, while 5 days after inoculum, they remained unchanged. On the other hand, in the susceptible seedlings all enzymes assayed were activated only after *F. verticillioides* infection (Lanubile et al., 2012b).

These signals are primarily orchestrated by hormones until they reach the nucleus (Berens et al., 2017). The involvement of hormone-signaling genes, including salicylic acid (SA), auxin (AUX), abscisic acid (ABA), ethylene (ET), and jasmonic acid (JA), has been observed (**Figure 2**). The targets of the hormone-signaling transduction pathways have been found to be multiple transcriptional factor families, such as WRKY for SA, MYB for ABA, auxin response factor (ARF) for AUX, and APETALA2/ethylene-responsive element binding protein (AP2/EREPB) and AP2/ethylene responsive factor (AP2/ERF) through 1-aminocyclopropane-1-carboxylate (ACC) oxidases for ET (Campos-Bermudez et al., 2013; Lanubile et al., 2014b; Wang et al., 2016). WRKY are normally involved in the signal transduction pathway because they recognize the W-box of promoters of a large number of defense-related genes; in particular their association with the *PR1* gene has been described (Campos-Bermudez et al., 2013; Wang et al., 2016). Furthermore, it has been reported that Myb-like DNA binding proteins are involved in the signaling cascade for flavonol-specific gene activation in phenylpropanoid biosynthesis (Lanubile et al., 2014b). Other changes observed after *F. verticillioides* infection comprise the activation of genes encoding heat shock proteins (HSPs) as well as glucanases (GLUC or PR6) and thaumatin or PR5 proteins (Campos-Bermudez et al., 2013; Lanubile et al., 2014b; Wang et al., 2016). A JA signaling pathway has been found to promote the further down-stream activation of defense responsive genes for PR proteins, such as chitinases (CHIT or PR3) and PR10, and lipoxygenases (LOX3; LOX4). The role of JA in maize pathogen defense has recently been reviewed (Borrego and Kolomiets, 2016; Lim et al., 2017), and the relevance of genes for the lipoxygenase pathway in resistance to *F. verticillioides* is well established.

LOX genes have been found across animal, fungal, and plant kingdoms, and are presumed to be involved in plant susceptibility to fungal invasion and mycotoxin production (Kock et al., 2003; Christensen and Kolomiets, 2011; Maschietto et al., 2015). *LOX* genes are non-heme iron-containing dioxygenases

that catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) (Vick and Zimmerman, 1983), which are processed into an estimated 400 metabolites including the well-known hormone JA and green leaf volatiles (GLVs) (Mosblech et al., 2009). *LOX* genes are subdivided into two main functional groups, 9-LOXs and 13-LOXs, depending on which carbon on the fatty acid chain is oxygenated. A total of 13 different maize *LOXs* (*ZmLOXs*) with varying functions, localization, and regulation within the plant, have been reported (Yan et al., 2012). Of the 13 *ZmLOXs*, *ZmLOX4* and *ZmLOX5* located on chromosome 5 are the two most closely related paralogs, sharing only 40–67% of sequence identity with other *ZmLOXs* (Park et al., 2010). *ZmLOX4* and *ZmLOX5* are 9-LOXs and are segmentally duplicated genes. Other pairs of close paralogs include tandemly duplicated *ZmLOX1* and *ZmLOX2* and segmentally duplicated genes *ZmLOX7* and *ZmLOX8*, and *ZmLOX10* and *ZmLOX11* (Nemchenko et al., 2006; Christensen et al., 2013).

Maize mutants for a defective 9-LOX gene, *ZmLOX3*, resulted in reduced levels of several 9-LOX-derived fatty acid hydroperoxides. *F. verticillioides* conidiation and FB1 production, as well as other fungal diseases, were drastically reduced in kernels of *lox3* mutants (Gao et al., 2007, 2009). In addition, maize 9-LOX *ZmLOX12* suppressed contamination by *F. verticillioides* (Christensen et al., 2014). These observations suggest that a specific plant 9-LOX isoform is required for fungal pathogenesis, including disease development and production of spores and mycotoxins.

Localization and expression data supported the hypothesis that another *LOX* gene, *ZmLOX5* (expressed in the silks), affected resistance to other mycotoxigenic fungi, and a QTL affecting aflatoxin contamination was located where *ZmLOX5* also mapped (Warburton et al., 2010).

Key genes in the defense response are those of the phenylpropanoid pathway, encoding for phenylalanine ammonia lyase and chalcone synthase, leading to an accumulation of flavonoids, phenolic compounds, and phytoalexins. Phenolic compounds accumulate rapidly during host–pathogen interaction and may mediate disease suppression through the inactivation of fungal enzymes or the strengthening of plant structural components. High levels of phenylpropanoids in the kernel pericarp were associated with less severe FER and fumonisin accumulation (Assabgui et al., 1993; Sampietro et al., 2013). The most resistant genotypes exhibited high levels of phenylpropanoids (on average 23.7 mg/g of dry pericarp), related to low levels of disease severity and grain fumonisin concentration (5.6% of visibly diseased ear area and 56.7 ppm of fumonisin on average, respectively; Sampietro et al., 2013). In particular, total diferulates were the best explanatory parameter for the variability of disease severity, and grain fumonisin concentration was correlated to total diferulate, 8,5′-diferulic acid benzofuran, and p-coumaric acid content. A potent inhibitory effect of α-tocopherol (0.1 mM) and ferulic acid (1 mM) on fumonisin biosynthesis was observed in *F. verticillioides* liquid cultures (Picot et al., 2013). These antioxidants were present in all stages of maize kernel development, indicating that the fumonisin-producing fungi were likely to face them during ear colonization.

Flavones in the silks contribute to FER resistance (Reid et al., 1992). Sekhon et al. (2006) investigated silk and kernel resistance to *F. verticillioides* and *F. proliferatum* in maize lines differing in 3-deoxyanthocyanidins and related 3-deoxyflavonoid (flavan-4-ols) content. Even though the degree of resistance was not strictly proportional to the amount of these secondary compounds in silks, the genes of the flavonoid pathway were active during the early stages of silk development. However, upon fungal inoculation, accumulation of 3-deoxyanthocyanidins was observed in resistant lines, suggesting a role of these compounds in resistance to *F. verticillioides*.

Higher susceptibility to FER was shown in ears of the *brown midrib* (*bm3*) mutant of maize, which cannot methylate either caffeic or hydroxyferulic acids to ferulic or sinapic acids due to a mutated *O*-methyltransferase (Vignols et al., 1995). Of the secondary metabolites, 6-methoxybenzoxazolin-2(3H)-one (MBOA) and benzoxazolin-2(3H)-one (BOA) have been found in corn and they are known for their antimicrobial properties (Glenn et al., 2002). Nevertheless, *F. verticillioides* is able to detoxify these compounds thanks to the presence of two specific loci, *Fdb1* and *Fdb2* (Glenn et al., 2002). Benzoxazinones are detoxified in 2-aminophenol (AP), which is converted to the less toxic *N*-(2-hydroxyphenyl) malonamic acid (HPMA) (Bacon et al., 2007). An endophytic bacterium, *Bacillus mojavensis*, is considered efficacious as a control of this *Fusarium* species, because it is able to produce a pigment identified as 2-amino-3H-phenoxazin-3-one (APO), which interacts with the fungus, thus preventing the usual transformation of AP into the non-toxic HPMA. The higher amounts of APO are toxic to *F. verticillioides* (Bacon et al., 2007).

The role of the biochemical composition of the endosperm has also been investigated. In particular, although Snijiders (1994) concluded that the biochemical composition of the endosperm had no intrinsic effect in proteins, sugars, and starches on resistance to the pathogen, Bluhm and Woloshuk (2005) found an influence on fumonisin B1 biosynthesis. Low amounts of amylopectin, required for fumonisin B1 biosynthesis, in early stages of kernel development and in some maize mutants, correlated with lower levels of mycotoxins (Bluhm and Woloshuk, 2005). The dynamic of water activity and humidity of maize kernels and their relevance for fumonisin accumulation in kernels was studied in medium to late season commercial hybrids by Battilani et al. (2011). The study revealed how "slow dry down" hybrids were more prone to fumonisin accumulation, irrespective of their maturity class.

More recently, the effect of fatty acid composition on fumonisin contamination and the occurrence of hidden fumonisins in maize (masking phenomenon consisting in the formation of covalent bonds between the tricarballylic groups of fumonisins and the hydroxyl groups of starch or the amino or sulfhydryl groups of the side chains of amino acids in proteins) has been investigated: higher fumonisin contamination was measured in hybrids showing a higher linoleic acid content and a higher masking action was observed in hybrids with higher oleic to linoleic ratio (Dall'Asta et al., 2012). Unsaturated fatty

acids are often oxidized to produce oxylipins, whose role as signal molecules that regulate the response to biotic stress has been previously described (Wilson et al., 2001; Christensen and Kolomiets, 2011).

In general, it is worth mentioning that basal defense mechanisms against *F. verticillioides* were activated in maize-resistant kernels, as reported in several studies. Many proteins associated with the defense response were found to be more abundant after infection, including PR10, chitinases, xylanase inhibitors, proteinase inhibitors, and PRXs. Kernels of the resistant line, even the non-inoculated ones, contained higher level of these defense-related proteins than the susceptible line, suggesting that these proteins may provide a basal defense against *Fusarium* infection in the resistant line (Mohammadi et al., 2011). These findings confirmed the conclusions of Lanubile et al. (2010, 2015a) and Maschietto et al. (2016) based on a transcriptomic analysis of the same resistant lines. Similar results were also obtained by Campos-Bermudez et al. (2013) using transcriptional and metabolite analysis in different resistant and susceptible inbreds. These results indicated that resistance was due to constitutive defense mechanisms preventing fungal infection. These mechanisms were poorly expressed in the susceptible line and, although the inoculation activated the defense response, this was not enough to prevent the disease's progress.

GENETIC BASIS OF THE RESISTANCE TO *Fusarium* INFECTION

A deeper knowledge of the genetic basis underlying FER is necessary to speed up progress in breeding for resistance.

The most efficient way to improve FER resistance in hybrids is to evaluate and select among inbred lines, before using resources to produce hybrids (Hung and Holland, 2012). Lanubile et al. (2011) conducted screening trials for both FER and fumonisin concentration using public and private inbred lines, and identified several genotypes with good levels of resistance to both FER and fumonisin accumulation. In diallel mating of 18 inbred lines from different heterotic groups with different levels of resistance, hybrids had 27% less ear rot and 30% less fumonisin content than their inbred parents (Hung and Holland, 2012). General combing ability (GCA) and specific combining ability (SCA) were significant for disease resistance, and inbred performance *per se* and the corresponding GCA in hybrids were significantly correlated ($r \geq 0.78$).

Fusarium ear rot resistance has proved to be a quantitative trait determined by polygenes (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Eller et al., 2008b). Pérez-Brito et al. (2001) tested two F_2 tropical maize populations of 238 and 206 F_2 individuals derived, respectively, from single crosses between resistant and susceptible inbred lines for FER resistance, and they measured relatively low heritability ($h_2 = 0.26$–0.42). Robertson-Hoyt et al. (2006) tested two segregating populations of 213 $BC_1F_{1:2}$ families from the first backcross of GE440 to FR1064 (GEFR) and 143 recombinant inbred lines (RILs) from the cross of NC300 to B104 (NCB), respectively, both for fumonisin contamination and FER resistance traits. This experiment enhanced the breeding for resistance approach because family mean heritability for ear rot resistance increased by up to 0.47–0.80 and for fumonisin contamination by up to 0.75–0.86. The increment of the heritability in comparison to Pérez-Brito's experiment can be explained by a reduction in the environmental influence obtained by doubling the number of evaluation environments and the number of artificial inoculations per plant. High positive correlations of FER resistance with fumonisin contamination and moderate-high heritabilities of both traits observed in the populations GEFR and NCB suggested that selecting for both traits at the same time was feasible (Robertson et al., 2006).

Phenotypic correlation between the severity of FER and the amount of fumonisins has been reported to be moderate to low (Clements et al., 2003; Clements et al., 2004), probably because of symptomless endophytic infections (Oren et al., 2003). Moreover, genotypic correlation between the two traits was higher than the phenotypic correlation (0.87–0.96 versus 0.40–0.64) (Robertson et al., 2006). This demonstrated that genotypic effects on susceptibility to ear rot and fumonisin content were highly correlated (Robertson et al., 2006). The close correlation between FER and fumonisin accumulation suggests that toxin analysis is only rarely needed, if disease severity data are available. In breeding, selection against genotypes more susceptible to FER allows for simultaneous selection against genotypes accumulating high contents of fumonisins. Moreover, genetic mechanisms controlling both traits are the same or closely linked.

MAIZE QUANTITATIVE TRAIT LOCI (QTL) PROVIDING RESISTANCE TO *Fusarium verticillioides*

The response to selection for resistance to FER can be increased by a wide variability in maize genotypes toward disease resistance and fumonisin contamination and by the moderate to high heritability of the traits. Nevertheless, phenotypic selection for the two traits is hampered by practical difficulties. Although many diseases could be evaluated during the plant's young stage or before flowering, FER and mycotoxin concentrations can only be analyzed on mature seeds and require artificial inoculations with calibrated fungal spore suspensions for consistent evaluation of the disease (Clements et al., 2003). Moreover, asymptomatic infections of this pathogen lead to time-consuming and expensive toxin assays for contamination assessment.

In addition, plant traits can affect pathogen access through the silk channel and the kernel. Hybrids with tight, adherent husks, and less open apical parts of the ear were more resistant to FER (Warfield and Davies, 1996; Butron et al., 2006). Physiological traits such as earliness in flowering time have been shown to reduce susceptibility toward several pathogens, including *F. verticillioides*. FER was less common for inbred lines with green and actively growing silks at inoculation time rather than brown silks. Kernel properties and seed coat influenced pathogen success (Scott and King, 1984; Headrick and Pataky, 1991; Hoenish and Davis, 1994). A thicker pericarp made maize more resistant to

penetration. Disease severity was dependent on husk integrity, on drought stress that increased the amount of stalk rot, and agronomic practices, for instance irrigation at the silk stage (Munkvold, 2003b; Battilani et al., 2008).

Finally, FER is influenced by many environmental factors, and testing for multiple sites and years is required (Shelby et al., 1994; Munkvold, 2003a; Robertson et al., 2006; Zila et al., 2013; Maschietto et al., 2017).

Disease Phenotyping

Selection for resistant hybrids must occur in areas with a known high incidence of FER. *F. verticillioides* can over-winter in the soil and may be spread by wind, rain splash, and insect larvae (Munkvold, 2003a), but to ensure equal distribution of the pathogen for all of the plants in the field, artificial inoculation is needed (Munkvold and Desjardins, 1997). Kernel infection through the seeds and infection through the silks are the best techniques for evaluating genetic resistance to FER (Munkvold and Desjardins, 1997; Robertson et al., 2006). These techniques refer to two types of inoculation method: with (type 1) and without (type 2) mechanical inoculation. Type 1 methods include toothpick inoculation methods and test kernel resistance (Reid et al., 1996), whereas in a typical type 2 method, a spore suspension is sprayed onto the maize silks with an atomizer, or injected into the silk channel near the cob tip. Type 1 inoculation methods usually screen for resistance to spreading on the host and simulate insect attack, as they bypass many of the plant's morphological barriers. Type 2 inoculation methods more closely resemble natural infection of a non-wounded host plant.

The best differentiation between resistant and susceptible genotypes has been obtained when inoculation occurred within a week after silking for type 2 inoculation (Reid et al., 1992; Lanubile et al., 2010; Campos-Bermudez et al., 2013); type 1 inoculation was effective 15 days after pollination (Lanubile et al., 2014b; Wang et al., 2016). Later inoculations resulted in significantly less severe disease symptoms, while the very early ones, i.e., 4–6 days, increased cases of disease outbreak.

As an alternative to field tests, *in vivo* bioassays including the rolled towel assay (RTA) or the Petri dish bioassay have been proposed for testing the ability of different pathogens to infect and colonize seedlings and kernels, respectively (Ellis et al., 2011; Lanubile et al., 2015b; Ju et al., 2017).

Fungal contamination of grains can be measured by various methods: the ergosterol level, representing a quantitative and qualitative measure of fungal contamination (Bakan et al., 2002), even though it is not strictly correlated with mycotoxin content; and the absolute quantification of fungal housekeeping genes, such as β-*tubulin*, through quantitative PCR (Lanubile et al., 2010, 2012a, 2014b).

Accurate mycotoxin analysis can be conducted with high-performance liquid chromatography (HPLC), but its costs make this technique unsuitable for use in large-scale breeding programs. HPLC can be replaced by the ELISA assay (Eller et al., 2008a) and near-infrared spectroscopy (NIRS) (Siesler et al., 2002; Berardo et al., 2005). The NIRS methodology can potentially be used for large-scale selection of genotypes resistant to fungal and fumonisin contamination.

Mapping QTL for Resistance and Genome-Wide Association Studies

Quantitative trait loci mapping and MAS, using PCR-based DNA markers associated to resistance genes, could be a successful strategy for selecting lines resistant to *F. verticillioides* (Beavis, 1998; Robertson et al., 2005). Localization of FER resistance QTL has often appeared to be contradictory in different studies (Pérez-Brito et al., 2001; Robertson-Hoyt et al., 2006; Ding et al., 2008), probably because of a strong environmental influence on the spread of the disease. **Figure 3** reports the localization of the main QTL and SNP markers for FER resistance on the maize chromosomes. Pérez-Brito et al. (2001) identified nine and seven QTL in two F_2 populations cross 3 × 18 and 5 × 18, respectively. The detected QTL explained between 30 and 44% of the phenotypic variation in the first population and 11–26% in the second. Three QTL on chromosomes 3 and 6 were co-located in both populations. Due to the number and limited effects of the QTL detected, Pérez-Brito et al. (2001) excluded MAS as a suitable strategy for this trait. Further studies contrasted with Pérez-Brito's conclusion (Robertson-Hoyt et al., 2006; Ding et al., 2008). Robertson-Hoyt et al. (2006) tested two segregating populations, a GEFR and an NCB population, derived, respectively, from FER resistant line GE440 crossed with FR1064 and the low fumonisin contamination line NC300 crossed with B104. In the GEFR population, seven QTL were identified, explaining 47% of the phenotypic variation for FER resistance, and nine were found for fumonisin content, explaining 67% of the variation. In the NCB population, five QTL explained 31% of the FER variation and six QTL explained 81% of the fumonisin variation. Three QTL for FER and two for fumonisin were mapped in similar positions in the two populations. In particular, two QTL, localized on chromosomes 4 and 5, appeared to be consistent for both traits in both populations. Ding et al. (2008) tested a RIL population of 187 genotypes for FER resistance. Of five identified QTL, two on chromosome 3 were stable across environments. The major QTL explained 13–22% of the phenotypic variation for FER, and it was flanked by SSR markers umc1025 and umc1742. More recently, a QTL on chromosome 4 (bin 4.05/06) was identified in the resistant inbred line BT-1 which explained 17.95% of the phenotypic variation for resistance to FER (Chen et al., 2012). Further verification of the QTL effect in near-isogenic lines (NILs) carrying the QTL region on chromosome 4 showed that if homozygous, this QTL can increase the resistance by 33.7–35.2%. The stable and significant resistance effect of the QTL on chromosomes 3 and 4 lays the foundation for further MAS and map-based cloning.

In conclusion, since QTL mapping used populations originated by crossing two homozygous lines, genetic variation in FER resistance was limited to the differences between the two parents. Furthermore, the resolution power was often low and QTL positions spanned from a few to tens of centimorgans. These regions corresponded to several megabases which contained hundreds of genes.

Such limitations, as well as the strong influence of environmental factors, hinder accurate QTL localization and the

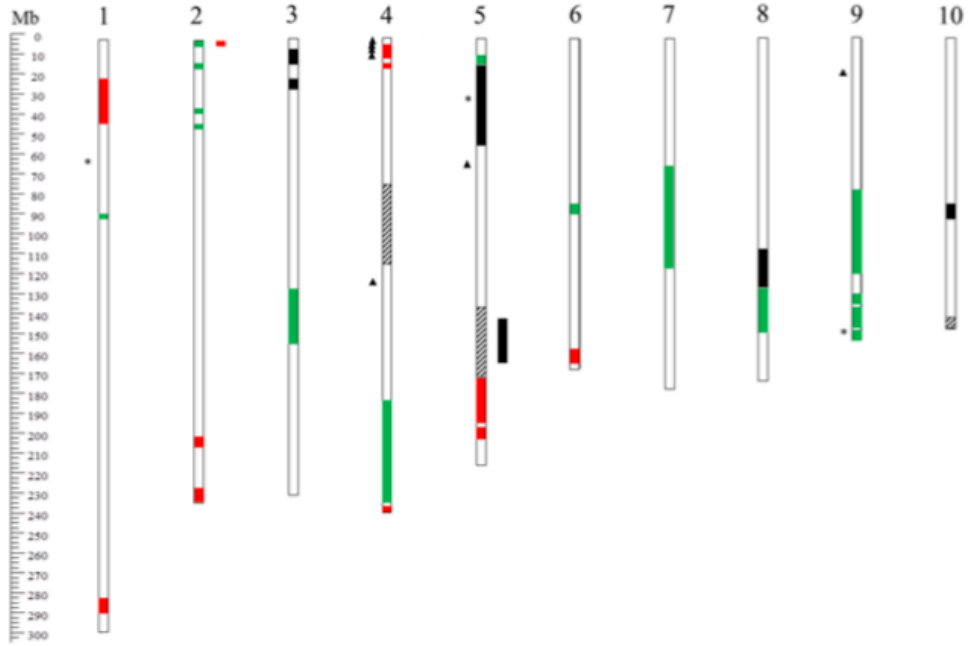

FIGURE 3 | Overview of chromosomal locations on the B73 reference genome (version 2) of known QTL for FER resistance. The bars inside the chromosomes indicate QTL intervals detected by Robertson-Hoyt et al. (2006) (red), Ding et al. (2008) (black), Chen et al. (2012) (dashed), and Maschietto et al. (2017) (green). The asterisks and the triangles on the left side of the chromosomes indicate FER-associated SNPs detected by Zila et al. (2013, 2014), respectively.

possibility of performing MAS efficiently (Robertson-Hoyt et al., 2006).

These issues may be partially overcome by increasing population size and the number of markers used, improving ear rot phenotyping protocols and integrating data from multiple environments (Robertson et al., 2005). In particular, initial QTL mapping studies on these traits were based on maps containing a few hundred restriction fragment length polymorphisms (RFLP; Pérez-Brito et al., 2001) and single sequence repeat (SSR) markers (Robertson-Hoyt et al., 2006; Ding et al., 2008; Chen et al., 2012). In recent years, single-nucleotide polymorphisms (SNPs) have become the preferred genotyping system for genetic studies, being the cheapest and the most abundant markers in a genome (Rafalski, 2002), e.g., 1 SNP/100 bp in maize (Tenaillon et al., 2001).

With the advent of NGS technologies, SNP markers have shown their full potential with novel approaches combing SNP discovery and genotyping, such as Genotyping-by-Sequencing (GBS; Elshire et al., 2011).

Three GBS studies were performed on maize to detect allele variants associated with increased resistance to FER. In a maize core diversity panel of 267 inbred lines, three SNPs with significant effects on chromosomes 1, 5, and 9 were described (Zila et al., 2013). Seven SNPs in six genes associated with FER resistance were identified on chromosomes 4, 5, and 9 in a panel of 1,687 US maize inbred line collections (Zila et al., 2014). Maschietto et al. (2017) found eight QTL located on linkage groups (LGs) 1, 2, 3, 6, 7, and 9 that were common to FER response and FB1 contamination, making the selection of genotypes with both low disease severity and low fumonisin contamination possible. Five QTL were located close to previously reported QTL for resistance to other mycotoxigenic fungi. Moreover, combining previous transcriptomic data (Lanubile et al., 2014b) with QTL mapping, 24 candidate genes for resistance to *F. verticillioides* were positioned in the same chromosomal regions.

Furthermore, comparing studies addressed to detection of QTL for resistance against different diseases reveals that there is evidently an overlap of the genetic mechanisms involved. Several fumonisin contamination QTL (Robertson-Hoyt et al., 2006) were localized on chromosomes 1, 2, 3, 4, 5, and 9 close to QTL for aflatoxin contamination (Wisser et al., 2006). In addition, Robertson-Hoyt et al. (2007) discovered QTL affecting both fumonisin and aflatoxin contamination, and Fusarium and Aspergillus ear rots.

THE ROLE OF FUMONISINS IN THE HOST–PATHOGEN INTERACTION

Fusarium verticillioides produces fumonisins as secondary metabolites (Gelderblom et al., 1988), a family of mycotoxins that affects animal and human health (Munkvold and Desjardins, 1997). Among the most active fumonisins, *F. verticillioides* produces B series fumonisins, particularly FB1.

FB1 is synthesized via a polyketide biosynthetic pathway (Butchko et al., 2006). The fumonisin (*FUM*) gene cluster, including genes involved in FB1 biosynthesis, is known to contain 22 genes with a length of 42 kb (Proctor et al., 2003). Of the 22 genes, 15 genes are co-regulated, including the key gene

FUM1, which encodes a polyketide synthase (PKS) (Proctor et al., 1999).

There are contrasting reports on the role of fumonisin production in the ability of *F. verticillioides* to cause maize ear rot. Fumonisin-nonproducing mutants were generated by disrupting *FUM1*, the gene encoding PKS, which is required for fumonisin biosynthesis (Proctor et al., 1999). *Fum1* mutants were 100% reduced in fumonisin production, but in field tests they were able to cause ear rot. The results provided evidence that production of fumonisins was not required for ear rot development and suggest that it is unlikely that fumonisin resistance would be an effective way to control this disease or fumonisin contamination in maize (Jardine and Leslie, 1999; Desjardins et al., 2002). Conversely, Lanubile et al. (2013) observed an enhanced reaction of incompatibility between resistant host and a *fum1* mutant of *F. verticillioides*, impaired in PKS activity, compared with the isogenic wild-type strain. In the early stages of infection, when the production of fumonisins was not detectable, the *fum1* mutant differed in its ability to colonize maize kernels compared to the wild-type strain. In the resistant maize genotype, the *fum1* mutant provoked a delayed and weakened activation of defense-related genes, presumably as a consequence of reduced growth. The inability of the *fum1* mutant to infect maize ears may be related to PKS activity and its association with the LOX pathway. Plant and fungal *LOX* genes were up-regulated after *fum1* mutant inoculation, suggesting that *PKS* is a relevant gene, essential not only to the fumonisin biosynthetic pathway, but also to pathogen colonization.

Arias et al. (2012) focused on the role of fumonisins as possible pathogenicity factors in the maize–*F. verticillioides* interaction. The effect of fumonisin on the development of maize seedling disease was observed to be strongly influenced by toxin concentration. High levels of fumonisin triggered necrosis and wilting in maize seedlings, while on the other hand low doses activated detoxification processes, suggesting a strategy of recovery in the host plants.

Death induced by FB1 usually presents features which resemble those of the hypersensitive response (HR), being fast and limited to the tissues that are exposed to the toxin (Asai et al., 2000; Stone et al., 2000), and determining the induction of defense genes (pathogenesis-related, phenylalanine ammonia lyase), chromatin condensation, and production of ROS, possibly in the apoplast through peroxydases. Different tissues and species have been used in the past for these toxicity studies, ranging from roots to leaves, from maize to *Arabidopsis* (Stone et al., 2000; Nadubinska and Ciamporova, 2001; Lin et al., 2008; Sánchez-Rangel et al., 2012).

FB1 acts through several pathways: SA, ET, and jasmonates (Asai et al., 2000). It causes a depletion of extracellular ATP reservoirs and eventually involves the protease vacuolar-processing-enzyme (VPE) as regulator of programmed cell death (PCD) (Kuroyanagi et al., 2005). Finally, there is evidence that ubiquitination also plays an important role in FB1-induced PCD (Lin et al., 2008). Future knowledge of the toxicity mechanisms of this molecule might suggest new management strategies.

FUTURE PROSPECTS

Several omics aspects of the *F. verticillioides*–maize interaction have been discussed in this review. Although down-stream processes of response to *F. verticillioides* infection have been well elucidated through transcriptomic studies, less information is available on the up-stream processes of recognition between maize and the fungus. To fill these gaps, recent advances in genomic technologies, such as GWAS, could resolve this complex trait down to the sequence level (Zhu et al., 2008). Moreover, GWAS applied to a large multi-parent population of RILs, termed multi-parent advanced generation inter-cross (MAGIC; Cavanagh et al., 2008), will ensure the identification of multiple genes, determining resistance to both FER and fumonisin contamination. In addition, as resistance to *F. verticillioides* is quantitative and based on a diffused architecture of many minor genes, the best approach for future molecular breeding will shift from MAS to genomic selection. Genomic-assisted breeding for quantitative resistance will necessitate whole-genome marker profiles for the entire set of breeding lines, prediction models and selection methodology as implemented for classical complex traits such as yield (Poland and Rutkoski, 2017).

A critical issue is that of the exploitation of candidate genes for resistance. RNASeq has been of great value in improving, validating, and refining gene models, and can identify new genes not previously annotated. A new approach to identifying candidate genes and QTL for resistance is represented by plant metabolome investigation after pathogen infection. Growing efforts are being made in research into relating genomic to metabolic (phenotypic) information (Bueschl et al., 2014). Keurentjes et al. (2006) have shown the potential of untargeted metabolomics to reveal QTL in the model plant *Arabidopsis*. An increasing number of metabolites are assigned to specific metabolic pathways and are the products of enzymatic reactions that depend on genome regulation. Moreover, the metabolic profile corresponds to the biochemical status of the organism that is a phenotypic expression. Metabolic profiling of resistant and susceptible cultivars can be used to detect biomarkers associated with the resistant trait.

In addition, genetic engineering permits the introduction or modification of gene coding for proteins with antifungal activities and enzymes that breach the plant cell wall, to increment pathogen resistance. In maize, several transgenic approaches can be exploited to reduce fumonisin content: reducing disease severity either by eliminating insect injury or by decreasing pathogen efficacy, by detoxifying or by blocking the synthesis of mycotoxins in seed (Duvick, 2001; Gao et al., 2007; Yuan et al., 2007). A limitation of this strategy is the possibility that other biosynthetic pathways might be altered, resulting in the biosynthesis of new plant secondary metabolites. Moreover, new identified dominant resistance genes (*R* genes) could be engineered in order to increase resistance in a specific response.

More recently, efficient editing technologies for genome modification in multiple plant species have emerged. Of these,

the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system has been used successfully in staple crops to modify single genes and change expression patterns. New gene variants conferred beneficial traits for plant breeding, including stress tolerance (Svitashev et al., 2015; Char et al., 2017; Shi et al., 2017).

Finally, it is now generally accepted that efforts devoted to the improvement of resistance to FER will also determine increases in resistance to other ear rots and, in particular, to the rotting produced by *Aspergillus* spp. Several studies have dealt with the positive relationship between infection by *Fusarium* and *Aspergillus* spp. (Clements and White, 2004; Lanubile et al., 2011; Pechanova and Pechan, 2015). Such results suggest that these fungal species may require similar substances for growth and development, and that they interact in similar ways with the host plant.

AUTHOR CONTRIBUTIONS

AL contributed for writing and editing the major part of the review and was involved in approving the final version of the review. VM, VB, and LS organized and prepared some of the parts of this review. AFL critically revised the manuscript. AM contributed to the design of this work outlay and was responsible for drafting the manuscript and final approval from all others.

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Enhancement of plant disease resistance by CRISPR/Cas9 technology

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Article types

Review

Abstract

Genome editing technologies have progressed rapidly and become one of the most important genetic tools in the implementation of pathogen resistance in plants. Recent years have witnessed the emergence of site directed modification methods using meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). Recently, CRISPR/Cas9 has largely overtaken the other genome editing technologies due to the fact that it is easier to design and implement, has a higher success rate, and is more versatile and less expensive. This review focuses on the recent advances in plant protection using CRISPR/Cas9 technology in model plants and crops in response to viral, fungal and bacterial diseases. As regards the achievement of viral disease resistance, the main strategies employed in model species such as *Arabidopsis* and *Nicotiana benthamiana*, which include the integration of CRISPR-encoding

sequences that target and interfere with the viral genome and the induction of a CRISPR-mediated targeted mutation in the host plant genome, will be discussed. Furthermore, as regards fungal and bacterial disease resistance, the strategies based on CRISPR/Cas9 targeted modification of susceptibility genes in crop species such as rice, tomato, wheat, and citrus will be reviewed. After spending years deciphering and reading genomes, researchers are now editing and rewriting them to develop crop plants resistant to specific pests and pathogens.

Introduction

Plant breeding has been the most successful approach for developing new crop varieties since domestication occurred, making possible major advances in feeding the world and societal development. Crops are susceptible to a large set of pathogens including fungi, bacteria, and viruses, which cause important economic losses (FAO, 2017); the enhancement of plant resistance plays an important role in adjusting crop production to meet global population increases. Approaches to disease control that depend on resistant varieties and agrochemicals are usually highly effective whenever they are deployed. However, due to the high evolutionary potential of many plant pathogens, novel genotypes no longer sensitive to the resistance gene or the phytosanitary product can rapidly emerge via mutation or recombination. When this happens, particular disease control approaches can rapidly be rendered ineffective as the novel genotypes increase in frequency through natural selection and quickly spread to other locations, causing failure of control over large geographic areas.

An understanding of interactions between plants and communities of bacteria, fungi, and other microorganisms has been a major area of investigation for many years. The advent of high-throughput molecular technologies has made a more complete inventory of the pathogens associated with particular crops possible, and provided insight into how these communities may be affected by environmental factors and the crop genotype. Disease involves a complex inter-play between a host plant and a pathogen, and the resistance/susceptibility response can involve several components. Natural and induced mutations may change the interaction and inhibit certain steps in the mechanism of infection (Boyd and O'Toole, 2012; Dracatos et al., 2018).

During pre-genomic years, traditional breeding programs were based on the identification of natural and induced mutant alleles for resistance, and their incorporation into elite genotypes through breeding techniques. These approaches were uncertain and imprecise, leading for instance to the transfer of large genome regions instead of just single gene insertions. Nevertheless, mutation breeding methods have been quite successful in improving disease resistance, and traditional plant breeding has been used to generate new crop varieties for decades. Numerous mutants have been developed through mutation induction, showing enhanced resistance to various diseases. Among the

most widely known mutants are those induced at the mildew resistance locus (*MLO*) in barley for resistance to powdery mildew (Miklis et al., 2007), and mutations conferring resistance to several lettuce diseases (Christopoulou et al., 2015). The *mlo* mutant is interesting, as the allele has not broken down and has provided unprecedented resistance to mildew for two decades (Panstruga and Schulze - Lefert, 2002). This longevity is due to a gene knockout. In other cases where resistance to specific pathotypes is conferred by a specific host gene allele, mutagenesis needs to be deployed to provide more precise single nucleotide mutations in the target gene sequence. The revolution driven by the availability of genome and transcriptome sequences offers a new start for plant breeding programs. Association genetics based on single nucleotide polymorphisms (SNPs) and other molecular markers are spreading in plant breeding, creating high throughput data fundamental for the identification of quantitative trait loci (QTL). Major QTL are employed in crops to provide quantitative resistance to pathogens, together with the use of major resistance (*R*) genes introduced into varieties with superior agronomic characteristics.

New breeding techniques (NBTs) are attracting attention in plant research and concern many different areas, such as developmental biology, abiotic stress tolerance or plant- pathogen resistance (Nelson et al., 2018). NBT include the most recent and powerful molecular approaches for precise genetic modifications of single or multiple gene targets. They employ site-directed nucleases to introduce double stranded breaks at predetermined sites in DNA. These breaks are repaired by different host cell repair mechanisms, resulting either in small insertions or deletions via near homologous end-joining (NHEJ) or micro-homology-mediated end-joining (MMEJ), or in a modified gene carrying predetermined nucleotide changes copied from a repair matrix via homologous recombination (HR). Meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) correspond to the four types of nucleases used in genome editing. The exponential increase in publications reporting the use of CRISPR/Cas9 illustrates the fact that this technology requires less know-how and financial means and has a higher success rate in gene modification compared to the other available nucleases. The application of CRISPR/Cas9 editing has become a powerful tool for future enhancement of agronomic traits in crops (Mohanta et al., 2017).

The objective of this review is to recall the main features of the CRISPR/Cas9 genome editing technique and discuss its application for the enhancement of pathogen resistance in model plants and important crops, with a focus on rice, wheat, and maize.

CRISPR/Cas9: advances, limitations, and new combinations

CRISPR/Cas9 from *Streptococcus pyogenes* (SpCas9) has rapidly assumed an important role in different application areas of plant research and many other fields (Ding et al., 2018; Liu and Moschou, 2018). In the CRISPR/Cas9 system a single- guide RNA (sgRNA) can bind to Cas9 and target it to specific DNA sequences (Figure 1). The requirement of a protospacer adjacent motif (PAM) limits the possible target sequences in a gene of interest. This limitation is of minor importance if the aim is simply to inactivate a gene by targeted mutagenesis at any position. It has much more importance for genome editing aiming at the precise change of specific nucleotides in a gene.

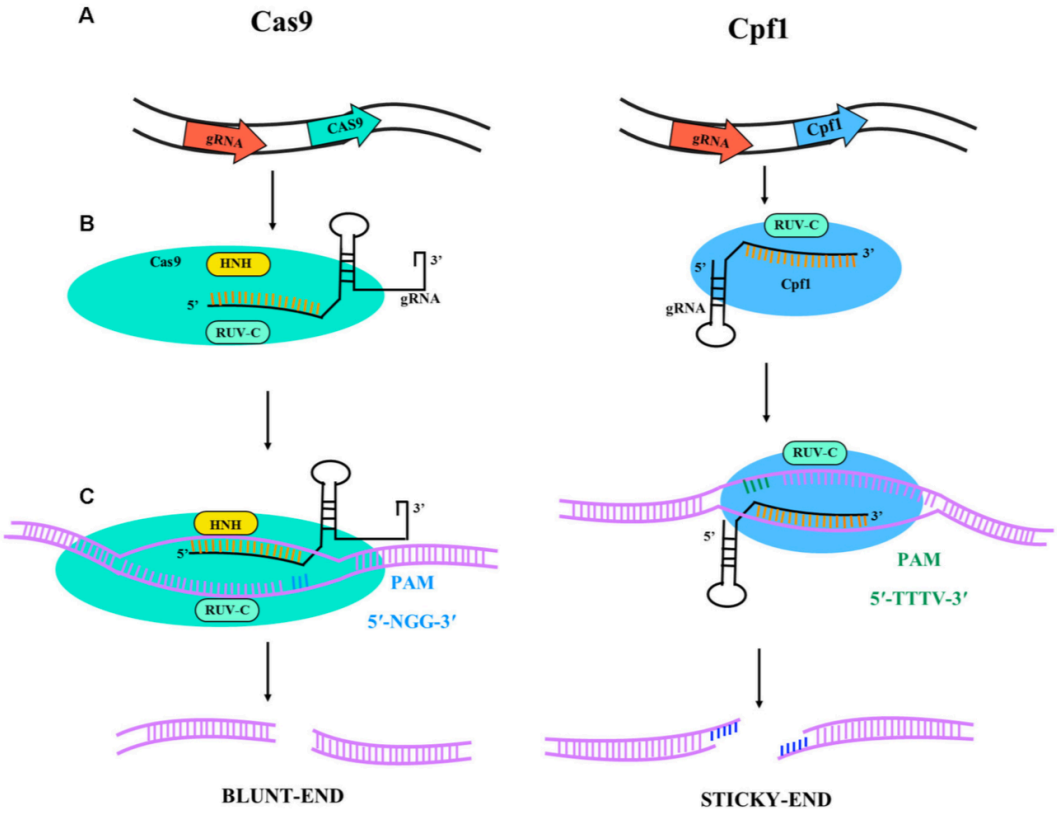

Figure1. Illustrative diagram of Cas9 and Cpf1 activities. The target specificity is given by the 17-20 nt located at the 5'end of the sgRNA sequence. **(A)** Primary transcript and gRNA-nuclease (Cas9 or Cpf1) complex formation. The catalytic domains are RUV-C (light blue) and HNH (yellow) for Cas9 and RUV-C for Cpf1. The Cas9 is colored in light blue and the Cpf1 in dark blue; in black is represented the gRNA for gene targeting. **(B)** Gene target activity. Cas9 has 5'-NGG-3'PAM sequence (blue bars) and Cpf1 has 5'-TTTV-3'PAM sequence (green bars). **(C)** DNA ends after nuclease activity. Cas9 lead to blunt-end and Cpf1 to sticky-ends.

Consequently, major efforts are under way to find Cas9-like proteins with different PAM sequences or to engineer the original Cas9 from *S. pyogenes* to recognize other PAM sequences. For example, xCas9, an evolved version of SpCas9, has been shown to recognize a broad range of PAM sequences including NG, GAA, and GAT in mammalian cells (Hu et al., 2018). In plants, the most widely explored alternative to SpCas9 is Cpf1 from *Prevotella* and *Francisella* with the PAM sequence TTTV, where "V" is A, C, or G (Endo et al., 2016), and an illustrative diagram is shown in Figure 1. Cpf1 is also considerably smaller than Cas9, is capable of RNAse activity to process its guide RNA, and introduces a staggered double break, which can be useful for enhancing homology-directed recombination and generating efficient gene insertion.

Multiplex genome editing: when does it become useful?

The ease of multiplexing, i.e., the simultaneous targeting of several genes with a single molecular construct, is one of the major advantages of CRISPR/Cas9 technology with respect to MN, ZFN, or TALEN. For example, the simultaneous mutation of 14 different genes by a single construct has been demonstrated in *Arabidopsis* (Peterson et al., 2016). In crops, several multiplex genome editing (MGE) strategies were reported early on (Ma et al., 2014; Xing et al., 2014; Zhou et al., 2014; Xu et al., 2016), which were all based on a common strategy, i.e., the assembly of multiple gRNAs under the control of a U3 or U6 promoter into a single construct. In maize, the ISU Maize CRISPR platform (Char et al., 2017) permits the cloning of up to four gRNAs for multiplex gene targeting.

More recent multiplex systems exploit self-cleavage capacity of RNA molecules containing tRNA sequences. Constructs alternating sgRNA and tRNA sequences under the control of a single U3 or U6 promoter permit reduction of the size of the construct and limit the risk of silencing due to direct repetitions of promoter sequences. The use of such a strategy employing polycistronic tRNA-gRNA (PTG) to generate hereditable mutation in *TaLpx-1* and *TaMLO* genes has been reported in hexaploid wheat (Wang et al., 2018); the PTG system is described in Figure 2. Starting from a previous study on gene silencing of *TaLpx-1*, which encodes the wheat 9-lipoxygenase resistance gene to *Fusarium graminearum* (Nalam et al., 2015), the editing of homologs in wheat was tested. The PTG system containing gRNA activity was validated in wheat confirming gene editing efficacy and providing an effective tool for rapid trait pyramiding in breeding programs.

Recently, an alternative approach for MGEs based on PTG has been reported in rice, where crRNA transcription was obtained from introns inserted into Cpf1 and Cas9 sequences (Ding et al., 2018). Multiplex PTG/Cas9 systems can help with multigene family analysis, as reported for the closely related mitogen-activated protein kinase *MPK1* and *MPK6* in rice (Minkenberg et al., 2017). 67% of all lines were double mutants for *MPK* genes with a high frequency of biallelic mutations on

multiple target sites. The possibility of programming the PTG/Cas9 to delete chromosomal fragments could be adopted to remove genes and regulatory elements in order to produce transgene free plants.

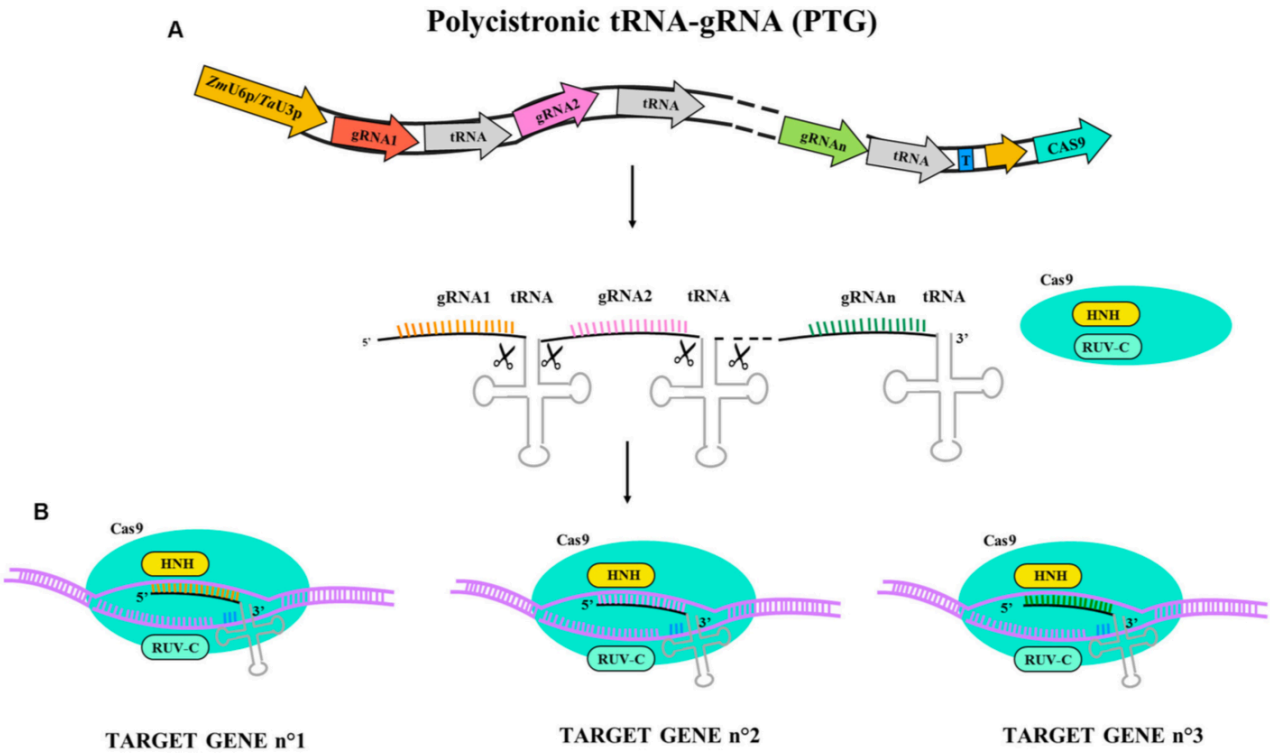

Figure 2. Illustrative diagram of polycistronic tRNA-gRNA (PTG) gene construct and targeting activity for Cas9. PTG is composed of t-RNA-gRNA repeats and is upregulated by ZmU6 promoter or TaU3 promoter according the experimental design as different terminator region (T) are adopted. (A) PTG primary transcript. Endogenous endonuclease cuts the tRNA ends and let each tRNA-gRNA targeting the corresponding gene sequence. (B) In PTG system more sequence targets are available (n°gene targets) and the different gRNA are represented in different colors (orange, pink, and green).

Off- target mutations: frequency and limitations

High specificity is frequently put forward as a major argument in favor of CRISPR/Cas9 technology, for example in comparison to chemically or irradiation-induced mutagenesis. This raises the question of to what extent a gRNA targets only fully complementary genomic DNA sequences, and to what extent other genomic regions (off-target regions) can also be recognized and cleaved by the CRISPR/Cas9 tool, provoking potentially unwanted damage. Two types of off-target effects are evoked by scientists and regulatory agencies: (i) expected off-target in genome regions with high sequence similarity to the target and (ii) unexpected off-target in unrelated genome regions. The former is generally addressed by PCR amplification and sequencing of regions known to be similar to the target, the latter by whole genome sequencing (Feng et al., 2016).

Genome sequence information is necessary for the prediction of expected off-target effects. The search focuses on the 20 bp target sequence involved in base pairing with the gRNA but excludes the PAM 5' -NGG-3'. The PAM functions as a recognition site outside of the targeted element and does not give specificity for nuclease cleavage (Shah et al., 2013). Moreover, the CRISPR/Cas9 system accepts at least three mismatches in the 20 bp DNA target sequence. Most CRISPR/Cas9 design tools take this into account and propose only specific gRNA designs that do not bind theoretical off-target sites with more than 17 bp identity anywhere else in the genome. Such state-of- the art design is effortless if the gene is unique in the genome, but it becomes rather challenging if the gene has one or more paralogs. This also means that the design is generally easier for diploid genomes without recent duplications than for recently duplicated or polyploid genomes. *In silico* genome analysis of potential target sequences in dicots and monocots has confirmed that, as expected, larger genomes contain more PAMs and more potential targets (Bortesi et al., 2016). High specificity of between 87.3% and 94.3% was observed in relatively simple genomes of *Arabidopsis*, rice, tomato, and soybean, whereas maize, a recent allotetraploid with high levels of repetitive DNA, revealed only 29.5% specific targeting (Bortesi et al., 2016).

Analysis of expected off-target sites, with only one to several mismatches with the primary target, has revealed that the position of the mismatches in the sequence is significant. Mismatches in the seed sequence ("seed" is defined as the 12 bp close to the PAM) are generally not supported or poorly supported by the sgRNA/Cas9 complex (Tsai et al., 2015), causing mutation less frequently at off-target sites, although in some cases mutations have been observed, as in barley (Lawrenson et al., 2015), soybean (Jacobs et al., 2015), and rice (Xie and Yang, 2013). Unwanted off-target mutations become more frequent when mismatches are located far from the seed region (Zhang et al., 2014).

To clarify the off-targeting issue in crops, recent investigations have screened progenies of CRISPR/Cas9 knockout in polyploid species. A study of CRISPR mutation frequency and mutation heritability of *TaGW2*, *TaLpx-1*, and *TaMLO* genes in the allohexaploid wheat was conducted (Wang et al., 2018). The results were different for the three genes: highly conserved for *TaGW2* (target sequence was specific for all three genomes), moderate for *TaLpx-1* (target sequence specific in two genomes), and low for *TaMLO*. The study showed the flexibility of CRISPR/Cas9 technology for implementing complex gene editing where the majority of genes have more than three homologous copies. Also, the gene editing process was investigated across generations: new mutant variants were recovered across multiple gene targets suggesting the transgenerational activity of CRISPR/Cas9 (Wang et al., 2018).

Another study on target accuracy and efficiency was performed in rice on paralogs *OsBEIIb* and *OSBEIIa* (Baysal et al., 2016). The study reveals the discrepancy in gRNA prediction and mutagenesis efficiency, confirming that gRNA with low predicted efficiency can achieve high mutation frequency even though the prediction suggested different targets with high mutagenesis scores. Empirical testing seems necessary in order to avoid putative gRNA inefficiency. Moreover, the authors also investigated off-target mutagenesis, reporting no mutation in the *OSBEIIa* paralog when only *OsBEIIb* was targeted, confirming the high accuracy of the strategy. CRISPR accuracy has been shown also in tomato (Cermák et al., 2015; Pan et al., 2016; Nekrasov et al., 2017).

To conclude, the CRISPR/Cas9 complex can bind with lower efficiency sequences with one to three mismatches. Therefore, expected off-target mutations do occur but can be avoided by rigorous design of the CRISPR/Cas9 tool. Unexpected off-target mutations do not occur at a frequency above the spontaneous mutation rate of plants.

Plant transformations: conventional and alternative techniques

The bottleneck in the application of CRISPR/Cas9 technology to a wide range of crops is clearly the regeneration of fertile plants from the cells into which the CRISPR/Cas9 tool has been introduced (Altpeter et al., 2016). Consequently, the efficiency of the entire process remains very species- and genotype-dependent, meaning that in many crop species only a few lab varieties are accessible to CRISPR/Cas9 technology. Other important parameters are the quality of the design of the CRISPR/Cas9 tool and the method chosen for its introduction into the plant cell. As in conventional transgenesis, the introduction of the CRISPR/Cas9 tool can be achieved by the *Agrobacterium*- mediated and biolistic transformation of explants, or by direct transformation of protoplasts. The latter two systems have the advantage that not only can the DNA coding for Cas9 and the sgRNA be transferred, but this also applies for ribonucleoproteins (RNPs), i.e., an *in vitro* assembled complex of Cas9 protein with an sgRNA (Malnoy et al., 2016; Svitashev et al., 2016; Liang et al., 2017), or intermediate versions such as a DNA or RNA coding for Cas9 and an RNA representing the sgRNA (Svitashev et al., 2015; Zhang et al., 2016). In addition, both biolistics and direct DNA transfer permit an increase in the ratio of repair matrix DNA over DNA encoding Cas9 and sgRNA readily, thereby favoring HR over NHEJ/MMEJ.

In maize, ISU Maize CRISPR is a high efficiency public platform using *Agrobacterium*-mediated transformation (Char et al., 2017). The main genotypes used for immature embryo transformation are A188, A634, H99, W117 (Ishida et al., 2007), B104 and the hybrid Hi-II (Char et al., 2017). Private companies seem to prefer biolistic transformation to *Agrobacterium*- mediated transformation in the case of gene editing with donor template (Shi et al., 2017), particularly where multiple copies of donor template DNA molecules can be delivered (Svitashev et al., 2015). Even

though both transformation processes have decent efficiencies nowadays, they remain limited to the above genotypes with poor agronomic traits. This limitation has recently been overcome by the overexpression of *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes, which stimulated callus growth and increased the overall transformation frequency in maize, including in recalcitrant genotypes. Proof of concept has also been provided for enhanced transformation in sorghum (Lowe et al., 2016).

In rice, most genotypes can easily be transformed both via *Agrobacterium*-mediated transformation and by biolistic methods. In order to achieve CRISPR-mediated HR the DNA template is normally introduced via the biolistic method to increase its copy number in the host (Baysal et al., 2016). As for maize, but involving a higher number of studies, protoplast transient assay is becoming an efficient tool for testing CRISPR- target before starting the transformation of embryos or scutellum derived calli by *Agrobacterium* or particle bombardment (Gao et al., 2013; Jiang et al., 2013; Xie and Yang, 2013; Zhou et al., 2014; Lowder et al., 2015; Li et al., 2016; Luo et al., 2016; Wang et al., 2016). Regeneration of rice protoplasts is still very challenging, but important optimization efforts may render it feasible in the near future. In wheat, although very high *Agrobacterium*- mediated transformation efficiencies of up to 90% have been reported for specific wheat genetic backgrounds (Ishida et al., 2015 a ,b), particle bombardment has been more widely accepted as the standard method in wheat genetic transformation (Hakam et al., 2015; Wang et al., 2018). Remarkable success has been achieved by particle bombardment of both immature embryos and callus cells to obtain transient expression of the CRISPR/Cas9 DNA, and transgene-free homozygous mutant T0 plants have been generated in the absence of any selection (Zhang et al., 2016). Three studies have reported CRISPR mutagenesis in barley by using *Agrobacterium*-mediated transformation of immature embryos (Lawrenson et al., 2015; Holme et al., 2017; Kapusi et al., 2017), while in Kapusi et al. (2017) a comparison with particle bombardment was carried out. Higher numbers of mutants were reported with the *Agrobacterium*-mediated compared to the biolistic transformation approach.

In conclusion, although preferences for certain delivery methods exist for certain species, efficiency is not only linked to the technique itself, but also to the know-how of a given lab as regards a given technique. Polyethylene glycol (PEG) or electroporation-mediated DNA transient expression in protoplasts have proven very useful for the evaluation of the efficiency of CRISPR/Cas9 designs (Malnoy et al., 2016). The importance of preliminary screens will certainly increase with the foreseeable shift from targeted mutagenesis to repair matrix based genome editing, which will increase the number of events to analyze due to lower efficiency. RNP technology has been

established in plants and may help toward exemption from regulatory oversight, but its efficiency needs to be improved to make it a routine tool.

CRISPR/Cas-based strategies conferring biotic resistance

Biotic stresses including viral, fungal, and bacterial diseases are responsible for losses ranging from 20% to 40% of global agricultural productivity (Savary et al., 2012). Conferring host plant resistance to pathogens can reduce the impact of disease on crop development and yield, thereby addressing the challenge of feeding the world's growing population.

Advances in genome editing tools have opened new ways to achieve the improvement of resistance in crops. In recent years, the CRISPR/Cas system has been employed to respond to several agricultural challenges, including the achievement of improved biotic stress resistance (Arora and Narula, 2017). The application of CRISPR/Cas tools has mainly been explored against virus infection, followed by efforts to improve fungal and bacterial disease resistance. Recent studies demonstrating the power of the CRISPR/Cas technology in establishing resistance to these pathogen categories will be further discussed below.

Virus Resistance via CRISPR/Cas

Plant viruses are a serious threat to many economically important staple and specialty crops. Based on their genome nature they are classified into six major groups: double-stranded DNA (dsDNA) viruses with no plant viruses in this group, single-stranded DNA (ssDNA), reverse-transcribing viruses, double-stranded RNA (dsRNA), negative sense single-stranded RNA (ssRNA-), and positive sense single-stranded RNA (ssRNA+) viruses (Roossinck et al., 2015). Most studies involving CRISPR-edited plants for virus resistance targeted ssDNA geminivirus genomes (Ali et al., 2015; 2016; Baltes et al., 2015; Ji et al., 2015; Table 1).

Geminiviridae is a large family of plant viruses causing worldwide crop losses of several important families such as Cucurbitaceae, Euphorbiaceae, Solanaceae, Malvaceae, and Fabaceae (Zaidi et al., 2016). Their genome is replicated through a rolling-circle amplification mechanism via a dsDNA replicative form, or by recombination-mediated replication (Hanley-Bowdoin et al., 2013). The economically most important genus of geminiviruses is Begomovirus. Begomoviruses infect dicotyledonous plants via the sweet potato/tobacco/silverleaf whitefly (*Bemisia tabaci*) and are found mostly associated to the phloem of infected plants (Gilbertson et al., 2015). Their genome is organized in one (A, monopartite) or two (A and B, bipartite) components containing a common region of ~ 220 bp (Fondong, 2013).

The first two studies focusing on resistance to geminiviruses, beet severe curly top virus (BSCTV) and bean yellow dwarf virus (BeYDV), in model plants N. benthamiana and Arabidopsis were

reported by Ji et al. (2015) and Baltes et al. (2015) (Table 1). Ji and co-workers (2015) screened 43 candidate sgRNA/Cas9 target sites in coding and non-coding regions of the BSCTV genome. All the sgRNA/Cas9 constructs reduced virus accumulation in inoculated leaves at varying levels, but a greater resistance to virus infection was observed in Nicotiana and Arabidospis plants showing the highest levels of expression of Cas9 and sgRNAs. Similar findings were described by Baltes et al. (2015) who employed 11 sgRNAs targeting Rep motifs, Rep-binding sites, hairpin, and the nonanucleotide sequence of BeYDV, and reported up to 87% reduction in targeted viral load in *N. benthamiana.*

Two recent works also employed a CRISPR/Cas9 approach for achieving resistance to begomoviruses (Ali et al., 2015; 2016; Table 1). Both studies were based on the strategy to express the CRISPR/Cas9 system in the host cell nucleus to target and cleave the virus genome during replication. Ali et al. (2015) developed sgRNA molecules delivered via a tobacco rattle virus (TRV) vector into *Nicotiana benthamiana* plants stably overexpressing the Cas9 endonuclease. SgRNAs were specific for different tomato yellow leaf curl virus (TYLCV) coding and non-coding sequences, targeting the viral capsid protein (CP), the RCRII motif of the replication protein (Rep) and the intergenic region (IR). All sgRNAs were able to interfere with TYLCV genome sequences, but targeting the stem-loop invariant sequence contained in the IR caused a more significant reduction of viral replication and accumulation. The same CRISPR/Cas9 system was tested for targeting simultaneously the monopartite beet curly top virus (BCTV) and the bipartite Merremia mosaic virus (MeMV), geminiviruses that share the same stem-loop sequence in the IR. The results showed attenuated symptoms for both viruses demonstrating that mixed infection immunity can be developed via a single sgRNA specific for conserved sequences of multiple viral strains.

Furthermore, Ali et al. (2016) analyzed not only the targeting efficiencies of the CRISPR/Cas9 tool but also the emergence of mutated viruses capable of replication and systemic movement. The CRISPR/Cas9 tool was designed to interfere with different coding and non-coding sequences of cotton leaf curl Kokhran virus (CLCuKoV), MeMV and different severe and mild strains of TYLCV. The work revealed that when the sgRNA/Cas9 complex edited sites in the coding regions of all viruses, virus variants were generated capable to replicate and move escaping the CRISPR/ Cas9 machinery. Conversely, no novel variants were detected in *N. benthamiana* plants carrying sgRNAs addressing the IR sequences. Even though the non-homologous end-joining repair (NHEJ) machinery repaired the double strand breaks caused by the Cas9 protein, the IR-repaired variants

generated virus genomes unable to replicate, thus providing a better overall interference with the viral life cycle.

Protection against RNA viruses seemed more difficult to achieve, since the classical SpCas9 from *Streptococcus pyogenes* only cuts double stranded DNA. However, the search for and characterization of related nucleases led to the discovery of enzymes that can bind to and cut RNA, such as FnCas9 from *Francisella novicida* or LwaCas13a from *Leptotrichia wadei*. A first report demonstrating resistance to RNA viruses (Zhang et al., 2018; Table 1) expressed FnCas9 and RNA-targeting sgRNAs specific for cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) in *N. benthamiana* and Arabidopsis plants. Transgenic plants showed reduced CMV and TMV accumulation by 40-80% compared with control plants. Furthermore, the resistance obtained by expressing the sgRNA-FnCas9 system was quite stable and still active in the T6 generation. Importantly, Zhang et al. (2018) observed that the endonuclease activity of FnCas9 was not required for the interference of CMV genome, whereas its RNA-binding activity was essential, meaning that this particular application of FnCas9 can be considered as a CRISPR interference (CRISPRi) tool, similar to catalytically inactive SpCas9 proteins programmed to mitigate gene expression (Larson et al., 2013). The use of a catalytically inactive variant of FnCas9 has the advantage to limit the onset of mutated viral variants capable of escaping CRISPR/Cas9. Moreover, in contrast to the previously described interference with geminivirus replication in the nucleus, no nuclear localization signal is necessary for FnCas9 which interferes with the RNA viruses in the cytoplasm.

Similar work was carried out with Cas13a. Aman et al. (2018) exploited this RNA-guided ribonuclease to manipulate the turnip mosaic virus (TuMV) RNA genome (Table 1). Four different viral genomic regions were targeted: two targets in the green fluorescent protein (GFP) region, one in the helper component proteinase silencing suppressor (HC-Pro), and one in the coat protein (CP). The most efficient virus interference was observed with CRISPR RNA editing HC-Pro and GFP2 genes and resulted in a reduced replication and spread of TuMV in tobacco leaves. Furthermore, due to the innate ability of Cas13 to process pre-CRISPR RNA into functional CRISPR RNA, the multiplex targeting of several viral mRNA could be markedly improved through this alternative system (Aman et al., 2018).

All of the systems aiming at protection against viruses described so far require the maintenance of a transgene for Cas9 and sgRNA in the genome of the crop plants, submitting them to GMO regulation. A second strategy for the achievement of viral disease resistance consists in modifying plant genes that will generate virus resistance traits, to segregate the CRISPR/Cas9 tool and to

release non-transgenic mutants in the field (Chandrasekaran et al., 2016; Pyott et al., 2016; Table 1). Plant host factors are required by RNA viruses to maintain their life cycle, including the eukaryotic translation initiation factors eIF4E and eIF(iso)4E (Sanfacon, 2015). Chandrasekaran et al. (2016) developed cucumber resistant plants to potyviruses by mutating independently two different sites of the host susceptibility gene eIF4E by CRISPR/Cas9. Non-transgenic Cucumis *eif4e* mutant plants were obtained by segregation of the CRISPR/Cas9 tool by three generations of backcrossing, making these plants safe for human consumption and for release into the environment according to the authors. When challenged with viruses from the Potyviridae family, cucumber vein yellowing virus (CVYV), zucchini yellow mosaic virus (ZYMV) and papaya ring spot mosaic virus-W (PRSV-W), homozygous *eif4e* mutants showed immunity. Conversely, heterozygous knockout plants and non-mutant plants did not give rise to any resistance to these viruses.

A similar editing approach was utilized by Pyott et al. (2016) in order to introduce site-specific mutations at the closely related *eIF(iso)4E* locus in Arabidopsis plants. Both 1 bp insertions and 1 bp deletions in *eIF(iso)4E* conferred complete resistance to the single-stranded RNA potyvirus (+ssRNA) TuMV and no off-target modification was detected in this study. Furthermore, homozygous T3 *eIF(iso)4E* mutants did not significantly differ in growth and development compared to wild-type plants.

The advantage of knocking-out host susceptibility genes is that it is a relatively simple method that allows to follow the mutation easily. The loss of a host factor required for the viral life cycle is a form of recessive resistance that should be a more durable than that of dominant R genes, because viruses undergo a lower selective pressure preventing their evolution to hinder host defense mechanisms. A possible disadvantage of the knockout strategy is that it may negatively influence plant vigor supporting the selection of virus variants breaking the resistance, as already observed in nature (Abdul - Razzak et al., 2009). Albeit Pyott et al. (2016) did not observe any significant growth defect between *eIF(iso)4E* mutants and normal plants, further investigations remain to be carried out in order to test the durability of this edited recessive resistance.

Table 1. CRISPR/Cas9 applications for virus resistance. BeYDV, bean yellow dwarf virus; BSCTV, beet severe curly top virus; TYLCV, tomato yellow leaf curl virus; BCTV, beet curly top virus; MeMV, Merremia mosaic virus; TRV, tobacco rattle virus; CLCuKoV, cotton leaf curl Kokhran virus; TuMV, turnip mosaic virus; CMV, cucumber mosaic virus; TMV, tobacco mosaic virus; CVYV, cucumber vein yellowing virus; ZYMV, zucchini yellow mosaic virus; PRSV-W, papaya ring spot mosaic virus-W; PVX, potato virus X; TCV, turnip crinkle virus; CMV, cucumber mosaic virus; RTSV, rice tungro spherical virus; CP, coat protein; Rep, replication association protein; IR, intergenic region; RCA, rolling-circle amplification; LIR, long intergenic region; GFP1, green fluorescent protein 1; GFP2, green fluorescent protein 2; HC-Pro, helper component proteinase silencing suppressor; ORF, open reading frame; UTR, untranslated terminal repeat; eIF4E, eukaryotic translation initiation factor 4E; eIF4G, eukaryotic translation initiation factor 4G.

Plant species	Virus	Target gene	Gene function	Strategy	Reference
Nicotiana benthamiana and *Arabidopsis thaliana*	BeYDV	CP, Rep and IR	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with Cas9/gRNA expression plasmid vectors	Ji et al., 2015
Nicotiana benthamiana	BSCTV	LIR and Rep/ RepA	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with Cas9/gRNA expression plasmid vectors	Baltes et al., 2015
Nicotiana benthamiana	TYLCV BCTV MeMV	CP, Rep and IR	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas9 overexpressing plants	Ali et al., 2015
Nicotiana benthamiana	CLCuKoV MeMV TYLCV	CP, Rep and IR	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas9 overexpressing plants	Ali et al., 2016
Nicotiana benthamiana	TuMV	GFP1, GFP2, HC-Pro, CP	Replication mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas13a overexpressing plants	Aman et al., 2018
Nicotiana benthamiana and *Arabidopsis thaliana*	CMV TMV	ORF1, 2, 3, CP and 3'UTR	Replication mechanism	*Agrobacterium*-mediated transformation of leaves with FnCas9/gRNA expression binary vectors Floral dipping for *Arabidospsis*	Zhang et al., 2018
Cucumis sativus	CVYV ZYMV PRSV-W	eIF4E	Host factor for RNA viruses Translation	*Agrobacterium*-mediated transformation of cut cotyledons (without embryo) with Cas9/ gRNA binary vectors	Chandrasekara et al., 2016
Arabidopsis thaliana	TuMV	eIF(iso)4E	Host factor for RNA viruses Translation	*Agrobacterium*-mediated transformation with Cas9/gRNA recombinant plasmid binary vectors (floral dipping)	Pyott et al., 2016

Resistance to Fungi Through CRISPR/Cas

Fungal pathogens are responsible for numerous diseases such as mildew, smut, rust, rot and many more. These diseases not only cause dramatic yield losses annually throughout the world but also compromise the quality of the harvested products. Moreover, mycotoxigenic fungi represent a serious concern due to the production of secondary metabolites known as mycotoxins, which cause severe health problems in humans and animals after exposure to contaminated food and feed. Several strategies have been evolved to enhance fungal resistance in plant species based on the current knowledge of molecular mechanisms implicated in plant-pathogen interaction. Potential candidate genes and gene products involved in plant resistance against fungi have been described, and nowadays these are prime targets for editing through the CRISPR/Cas9 approach.

As previously partially discussed, *MLO* loci have been targeted by RNA-guided Cas9 endonuclease in three different plant species: bread wheat, tomato, and grapevine (Wang et al., 2014; Malnoy et al., 2016; Nekrasov et al., 2017) (Table 2). *MLO* encodes a protein with seven transmembrane domains localized in the plasma membrane and is ubiquitously present in monocots and dicots (Acevedo-Garcia et al., 2014). It had previously been reported that *MLO* were susceptibility (*S*) genes and that homozygous loss-of-function mutants had significantly increased resistance to powdery mildew in barley, *Arabidopsis* and tomato (Piffanelli et al., 2004; Consonni et al., 2006; Bai et al., 2008). Bread wheat plants mutated by CRISPR/Cas9 in one (*TaMLO-A1*) of the three *MLO* homeoalleles showed improved resistance to *Blumeria graminis* f. sp. tritici infection, a finding that once again demonstrated the important role of *TaMLO* genes in powdery mildew disease (Wang et al., 2014). In tomato, *SlMlo1*, previously identified as the most important of 16 *SlMlo* genes, was targeted at two sites and a deletion of 48 bp was obtained. The edited plants were self-pollinated in order to generate CRISPR/Cas cassette-free individuals. This new non-transgenic variety, " Tomelo " was fully resistant to *Oidium neolycopersici*. Furthermore, off-target analysis did not reveal any effect on the genomic regions outside the *SlMlo1* locus (Nekrasov et al., 2017). In grapevine, the molecular feasibility of *VvMLO7* knockout has been demonstrated through CRISPR/Cas9 RNP in protoplasts, but no plants have been regenerated (Malnoy et al., 2016). Parallel experiments with RNAi plants showed that the loss of *VvMLO7* reduced susceptibility to *Erysiphe necator* in grapevine (Pessina et al., 2016).

The RNP approach has also been used for editing *DIPM-1*, *DIPM-2*, and *DIPM-4* genes in apple protoplasts in order to confer resistance to fire blight disease (Malnoy et al., 2016). Again, only the molecular analysis attesting mutations has been carried out, not disease assay on regenerated plants. In perennial crops such as grapevine and apple, which take several years to flower, the transient introduction of genome editing tools in protoplasts is particularly interesting, since the segregation

of stably integrated CRISPR/Cas9 cassettes by backcrosses would take a lot longer than in annual crops with generation times of only a few months. Secondly, the delivery of Cas9/sgRNA complex as RNP is a rapid approach, making possible the achievement of transformed protoplasts and the evaluation of sgRNA efficiency within 1 or 2 days. Thirdly, no foreign DNA is integrated into the genome and the Cas9/sgRNA complexes can be degraded rapidly during the cell culture regeneration process. Furthermore, even in transient approaches, the employment of plasmids can sometimes cause their undesired integration into the host genome, and the prolonged presence of CRISPR/Cas9 tools in the genome increases the risk of off-target mutations, while the CRISPR/Cas9 RNP shows improved on-target specificity. The drawback of this approach is the need to optimize plant regeneration protocols in order to apply this technology.

An example of the successful protection of grapevine by the CRISPR/Cas9 system is the *VvWRKY52* transcription factor, which was targeted by four gRNAs (Wang et al., 2018) (Table 2). About 21% of the transgenic plants showed biallelic mutations and were more resistant to *Botrytis cinerea* compared to the monoallelic mutants. No marked difference was observed in phenotype between wild-type and biallelic mutant plants, confirming the efficiency of the CRISPR/Cas9 strategy in woody plants with long reproductive cycles.

A further strategy to expedite genome editing application in slow generation tree crops is the employment of transient leaf transformation coupled to disease assays as demonstrated in *Theobroma cacao* (Fister et al., 2018) (Table 2). The authors reported for the first time the transient introduction of CRISPR/Cas9 components into cacao leaves targeting the *Non-Expressor of Pathogenesis-Related 3* (*NPR3*) gene, a suppressor of the immune system, and obtained leaves with increased resistance to *Phytophthora tropicalis*. This new system of *in vivo* mutagenesis in adult cacao trees is a fast and useful technique for validating sgRNA design and observing CRISPR mutagenized phenotypes. It encouraged the authors to regenerate genome-edited somatic embryos to validate the observed results at whole-plant level.

Plants resistant to rice blast disease were generated through CRISPR/Cas9 by disrupting *OsERF922* and *OsSEC3A* genes in rice (Wang et al., 2016; Ma et al., 2018) (Table 2). *Ossec3a* mutant plants disrupted in a putative subunit of a complex involved in exocytosis, revealed a pleiotropic phenotype including improved resistance against *Magnaporthe oryzae*, higher levels of salicylic acid (SA) content and up-regulation of pathogenesis- and SA- related genes, but also dwarf stature (Ma et al., 2018). In contrast, no alteration of a number of agronomic traits was observed in T1 and T2 transgene free plants mutated in the ethylene responsive factor (ERF)922, a transcription factor implicated in multiple stress responses. The mutant plants had a reduced number of blast lesions at both seedling and tillering stages (Wang et al., 2016). Overall, these results demonstrate the

powerful and advantageous application of the CRISPR/Cas9 system for crop improvement as regards fungal disease resistance.

Table 2. CRISPR/Cas9 applications for fungal resistance. MLO, MILDEW RESISTANT *LOCUS; NPR3, non-expressor of pathogenesis-related 3; ERF922, ethylene responsive factor.*

Plant species	**Fungus**	**Target gene**	**Gene function**	**Strategy**	**Reference**
Triticum aestivum	Powdery mildew (*Blumeria graminis* f. sp. *tritici*)	MLO-A1	Susceptibility (S) gene involved in powdery mildew disease	Particle bombardment of immature wheat embryos with Cas9/gRNA expression plasmid vectors	Wang et al., 2014
Solanum lycopersicum	Powdery mildew (*Oidium neolycopersici*)	MLO1	Major responsible for powdery mildew vulnerability	*Agrobacterium*-mediated transformation of cotyledons with Cas9/gRNA expression plasmid vectors	Nekrasov et al., 2017
Vitis vinifera *Malus domestica*	Powdery mildew (*Erysiphe necator*) Fire blight (*Erwinia amylovora*)	MLO-7 DIPM-1 DIPM-2 DIPM-4	Susceptibility (S) gene involved in powdery mildew disease.Susceptibility factor involved in fire blight disease	PEG-mediated protoplast transformation with CRISPR ribonucleoproteins	Malnoy et al., 2016
Vitis vinifera	Grey mold (*Botrytis cinerea*)	WRKY52	Transcription factor involved in response to biotic stress	*Agrobacterium*-mediated transformation of proembryonal masses with Cas9/gRNA expression binary vectors	Wang et al., 2018
Theobroma cacao	Black pod disease (*Phytophthora tropicalis*)	NPR3	Regulator of the immune system	*Agrobacterium*-mediated transient transformation of stage C leaves with Cas9/gRNA expression binary vectors	Fister et al., 2018
Oryza sativa L. *japonica*	Rice blast disease (*Magnaporthe oryzae*)	SEC3A	Subunit of the exocyst complex	Protoplast transformation with Cas9/gRNA expression binary vectors	Ma et al., 2018
Oryza sativa L. *japonica*	Rice blast disease (*Magnaporthe oryzae*)	ERF922	Transcription factor implicated in multiple stress responses	*Agrobacterium*-mediated transformation of embryogenic calli with Cas9/gRNA expression binary vectors	Wang et al., 2016

Resistance to Bacteria Through CRISPR/Cas

Among the bacterial species living on earth, just a few hundred are involved in crop damage, which often reveals multiple symptoms of disease (Schloss and Handelsman, 2004). Phytopathogenic bacteria are difficult to control, mainly because of undetected asymptomatic infections and the lack of suitable agrochemicals. Generally speaking, bacteriological plant control is based on prevention and exclusion of the pathogen by using genetic resistance, agronomic practices, and biocontrol agents (Kerr, 2016).

Phytopathogenic bacteria can be classified as crop specific, such as *Clavibacter michiganensis*, which is the causal agent of tomato bacterial ring rot; polyphagous specific, such as *Ralstonia solanacearum*, which causes disease in multiple monocot and dicot species; and "kingdom crosser," such as *Dickeya dadantii*, an entomo-phytopathogen, which can affect plants and animals.

Relatively few studies (Table 3) have been published on the application of the CRISPR/Cas system to counteract crop bacterial diseases. CRISPR/Cas9 mutagenesis of *OsSWEET13* has been performed in rice to achieve resistance to bacterial blight disease caused by γ-proteobacterium *Xanthomonas oryzae* pv. *oryzae* (Zhou et al., 2015). *OsSWEET13* is a susceptibility (*S*) gene encoding a sucrose transporter involved in plant-pathogen interaction. *X. oryzae* produces an effector protein, PthXo2, which induces *OsSWEET13* expression in the host and the consequent condition of susceptibility. In a previous work concerning *OsSWEET14* promoter mutagenesis adopting a TALEN approach, the disruption of this gene rendered the *X. oryzae* effector unable to bind *OsSWEET14* and ultimately resulted in disease resistance (Li et al., 2012). Similarly, Zhou et al. (2015) obtained a null mutation in *OsSWEET13* in order to better explore PthXo2-dependent disease susceptibility, and resultant mutants were resistant to bacterial blight. Further genome editing strategies for multiplexed recessive resistance using a combination of the major effectors and other resistance (*R*) genes will be the next step toward achieving bacterial blight resistance.

Two recent works have reported the employment of CRISPR/Cas9 with the aim of producing citrus plants resistant to citrus bacterial canker (CBC). CBC is caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) and is the most widespread disease among commercial cultivars. In the first work, Jia et al. (2016) generated canker resistant mutants by editing the PthA4 effector binding elements in the promoter of the *Lateral Organ Boundaries 1* (*CsLOB1*) gene in Duncan grapefruit. Mutated lines showed a decrease in typical canker symptoms 4 days post inoculation with *Xcc*, and no further phenotypic alterations were detectable. Furthermore, no potential off- target mutations in other *LOB* family genes were found by PCR-sequencing. The second work, by Peng et al. (2017), confirmed the link between *CsLOB1* promoter activity and CBC disease susceptibility in Wanjincheng orange

(*Citrus sinensis* Osbeck). The complete deletion of the EBE_{PthA4} sequence from both *CsLOB1* alleles induced resistance enhancement to CBC. Moreover, no alteration in plant development was observed after *CsLOB1* promoter modification. Additional efforts will be required to generate non-transgenic canker-resistant citrus varieties for facilitating their agronomic application in CBC prevention.

Table 3. CRISPR/Cas9 applications for bacterial resistance. Lateral Organ Boundaries 1 (CsLOB1), DspE - interacting proteins of Malus (DIPM).

Plant species	Fungus	Target gene	Gene function	Strategy	Reference
Oryza sativa	Bacterial blight (*Xanthomonas oryzae* pv. *oryzae*)	SWEET13	Sucrose transporter gene	*Agrobacterium*-mediated transformation of embryogenic callus with Cas9/gRNA expression plasmid vectors and TALEN	Zhou et al., 2015; Li et al., 2012
Citrus paradisi	Citrus canker (*Xanthomonas citri* subspecies *citric*)	LOB1	Susceptibility (S) gene promoting pathogen growth and pustule formation	*Agrobacterium*-mediated transformation of epicotyl with Cas9/gRNA expression plasmid vectors	Jia et al., 2016
Citrus sinensis Osbeck	Citrus canker (*Xanthomonas citri* subspecies *citric*)	LOB1	Susceptibility (S) gene promoting pathogen growth and pustule formation	*Agrobacterium*-mediated transformation of epicotyl with Cas9/gRNA expression plasmid vectors	Peng et al., 2017
Malus domestica	Fire blight (*Erwinia amylovora*)	DIPM-1 DIPM-2 DIPM-4	Susceptibility factor involved in fire blight disease	PEG-mediated protoplast transformation with CRISPR ribonucleoproteins	Malnoy et al., 2016

Future prospects

In an era marked by political and societal pressure to reduce the use of pesticides, crop protection by genetic improvement provides a promising alternative with no obvious impact on human health or the environment. Genome editing is one of the genetic levers that can be adopted, and disease resistance is frequently cited as the most promising application of CRISPR/Cas9 technology in agriculture. There are three main reasons for this: firstly, scientific knowledge of the molecular mechanisms underlying numerous pathosystems is sufficiently advanced to enable the proposal of genes to be edited in order to achieve resistance. Secondly, disease resistance can frequently be achieved by the modification of a single gene, which is technically less challenging. This is similar to the modification of metabolic pathways, where the editing of a single gene can also have an all-

or-nothing effect, but different from abiotic stress tolerance, where generally numerous genes have to be modified in a coordinated fashion to achieve incremental improvements. Thirdly, targeted mutagenesis, the only use of CRISPR/Cas9 technology at present mastered with respect to crops, is readily applicable to disease resistance, since the inactivation of susceptibility genes leads to protection. For other agriculturally interesting traits the achievement of positive effects by the loss-of-function of genes is a more delicate matter. However, acting as the spearhead of genome editing in crops also puts a certain responsibility on plant pathologists.

The first challenge is to demonstrate that the promises made by proofs of concept in confined environments can be maintained under field conditions. It is one thing to show that the population of a pathogen or the size of disease lesions is reduced in a greenhouse and another to protect a crop year after year under varying environmental conditions. Field tests are also necessary for correct evaluation of the agronomic fitness of the edited crops. Most of the genes inactivated by CRISPR/Cas9 technology in order to obtain disease resistance are likely to have roles in the physiology of the plant other than that linked to the life cycle of the pathogen. For example, triple knockouts of wheat *TaMLO* were not only resistant to powdery mildew but also showed leaf chlorosis (Wang et al., 2014), whereas EMS-induced triple mutants with non-conservative point mutations in *TaMLO* did not show obvious pleiotropic phenotypes (Acevedo-Garcia et al., 2017). Therefore, encouraging greenhouse observations of plant development or measurements of key parameters such as height, leaf area or grain weight absolutely must be confirmed under field conditions by multi-environmental yield trials in order to measure the relative importance of negative side effects. A final limitation of many published proofs of concept is that they involve lab varieties, which can easily be regenerated after the introduction of Cas9 and sgRNA, but which often have only a limited agronomic value. It remains to be shown that the phenotypic effects are maintained in elite lines under field conditions.

The second challenge is the durability of the disease resistances, and their agronomic management. This challenge needs to be dealt with seriously, in order to convince a public often hostile to this technology. Durability is not a specific aspect of resistance genes obtained by genome editing, and the answers are the same as for introgressed resistance genes discovered in the genetic variability of the species: (i) the stacking of several resistance genes, preferably with different modes of action, (ii) a focus on systems other than NBS-LRR receptor kinases known to break down rapidly, and (iii) good agronomic practices, including, in particular, crop rotation and the concomitant use of biocontrol agents. An example of two independent CRISPR/Cas9-derived resistances against the same disease are the knockouts of *TaMLO* (Wang et al., 2014) and *TaEDR1* (Zhang et al., 2017), both conferring resistance to powdery mildew in wheat. Beyond the creation of novel alleles

conferring protection, CRISPR/Cas9 technology can also be helpful in the stacking process itself. In contrast with the introgression of conventional resistance genes, the technology not only avoids genetic drag on neighboring regions with potentially negative impacts on agronomic performance, but also permits the simultaneous creation of multiple resistances in a single generation by multiplexing, i.e., the parallel use of several sgRNAs targeting different genes. Admittedly, multiplexing becomes more challenging with increasing ploidy levels, and in the above example in hexaploid wheat (A, B, and D genome), three *TaMLO* genes and three *TaEDR1* genes would need to be modified in parallel.

The third challenge is to overcome the present technical limitation regarding targeted mutagenesis and to implement true genome editing in crop plants. Targeted mutagenesis introduces random mutations (generally short insertions or deletions) at a predetermined site of a given gene, leading generally to loss-of- function, whereas true genome editing introduces predetermined base changes at one or several specific positions in a gene. For example, the elongation initiation factor 4E (eIF4E) is necessary for the translation of RNA into protein for both the host cell and single-stranded RNA viruses of the Potyviridae family. As described above, loss-of-function of eIF4E by targeted mutagenesis has been achieved in several model and crop species, consistently conferring resistance to potyviruses but also impacting the host plants to varying degrees. The specific modification of amino acids known to be important for the translation of viral but not host proteins would permit driving resistance to potyviruses without affecting plant physiology (Bastet et al., 2017). The expression of a transgene carrying a synthetic allele with six mutations in an *Arabidopsis eif4e* mutant validated the concept (Bastet et al., 2018), demonstrating indirectly the potential benefit of genome editing over targeted mutagenesis. However, at present true genome editing by HR is still hampered by very low efficiencies in plants, although it has recently become routine in many animal species. Continued efforts to improve its efficiency, for example by the use of *lig4* (Endo et al., 2016) or *polQ* mutations (Saito et al., 2017), or a copy number increase of the repair matrix by virus vectors (Čermák et al., 2015), are crucial to increasing the range of tools available to plant pathologists. Base editing, to date permitting C to T and A to G transitions in plants, is more limited in scope but has recently emerged as a readily available alternative for certain editing projects (Zhang et al., 2017; Hua et al., 2018).

The long term success of CRISPR/Cas9 technology in plant protection is dependent on new scientific knowledge. CRISPR/Cas9 technology can only be used if one knows which gene(s) to modify and which modification(s) to carry out in these genes in order to render plants resistant to disease. When pathogen resistance is achieved by the knock-out of one or several genes, inactivating mutations can easily be provoked by CRISPR-mediated specific DNA break and

activation of the cell's error prone DNA repair, based on NHEJ. In this case, CRISPR can be used to target and inactivate a single gene or large gene families, both through single gRNA which matches several targets, or by multiplexing the system by introducing several gRNAs simultaneously. On the contrary, when specific allelic variants are involved in resistance, CRISPR-DNA break can be coupled with the less frequent cell repair mechanism based on HDR. The DNA template for HDR should be introduced into the cell together with the effector nuclease. This permits the introduction of a custom-designed sequence into the genome. The use of HDR, compared to NHEJ, can indefinitely expand the possibility of the type of mutations inserted by CRISPR, as any sequence can be inserted into a site of choice. Nevertheless, HDR is still technically challenging due to its low efficiency, the difficulty of having a selective marker and the lack of multiplexing protocols. These are aspects that will need to be improved if CRISPR applications are to expand in plant breeding. Despite the recent judgment of the Court of Justice of the European Union issued that organisms created using genome editing techniques are to be regulated as GMOs (Callaway, 2018), anyhow continuous efforts in plant pathology are necessary, in order to identify and characterize the genes involved in plant pathogen interactions. For example, the past decade was marked by the discovery of hundreds of effector molecules that are synthesized by different classes of pathogens and transferred into the host cell. A major challenge is to identify the host proteins targeted by these effectors and to characterize the underlying genes, which are one of many possible targets for future genome editing approaches. New knowledge does not necessarily have to stem from the crop species of interest. For example, the targeted mutagenesis of wheat *TaMLO* was based on knowledge of another crop, barley, where *Hvmlo* mutant varieties have provided good protection against powdery mildew that has not yet broken down, and the modification of *TaERF1* exploited knowledge from the model species *Arabidopsis*. These examples perfectly illustrate the added value of genome editing, which permits the enlargement of the gene pool of a crop species beyond all the available natural variability, by means of the transfer of knowledge acquired in other crops or model species.

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REVIEW
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The Enhancement of Plant Disease Resistance Using CRISPR/Cas9 Technology

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Genome editing technologies have progressed rapidly and become one of the most important genetic tools in the implementation of pathogen resistance in plants. Recent years have witnessed the emergence of site directed modification methods using meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). Recently, CRISPR/Cas9 has largely overtaken the other genome editing technologies due to the fact that it is easier to design and implement, has a higher success rate, and is more versatile and less expensive. This review focuses on the recent advances in plant protection using CRISPR/Cas9 technology in model plants and crops in response to viral, fungal and bacterial diseases. As regards the achievement of viral disease resistance, the main strategies employed in model species such as *Arabidopsis* and *Nicotiana benthamiana*, which include the integration of CRISPR-encoding sequences that target and interfere with the viral genome and the induction of a CRISPR-mediated targeted mutation in the host plant genome, will be discussed. Furthermore, as regards fungal and bacterial disease resistance, the strategies based on CRISPR/Cas9 targeted modification of susceptibility genes in crop species such as rice, tomato, wheat, and citrus will be reviewed. After spending years deciphering and reading genomes, researchers are now editing and rewriting them to develop crop plants resistant to specific pests and pathogens.

Keywords: CRISPR/Cas9, crop improvement, genome editing, disease resistance, virus, fungus, bacteria

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INTRODUCTION

Plant breeding has been the most successful approach for developing new crop varieties since domestication occurred, making possible major advances in feeding the world and societal development. Crops are susceptible to a large set of pathogens including fungi, bacteria, and viruses, which cause important economic losses (FAO, 2017); the enhancement of plant resistance plays an important role in adjusting crop production to meet global population increases. Approaches to disease control that depend on resistant varieties and agrochemicals are usually

highly effective whenever they are deployed. However, due to the high evolutionary potential of many plant pathogens, novel genotypes no longer sensitive to the resistance gene or the phytosanitary product can rapidly emerge via mutation or recombination. When this happens, particular disease control approaches can rapidly be rendered ineffective as the novel genotypes increase in frequency through natural selection and quickly spread to other locations, causing failure of control over large geographic areas.

An understanding of interactions between plants and communities of bacteria, fungi, and other microorganisms has been a major area of investigation for many years. The advent of high-throughput molecular technologies has made a more complete inventory of the pathogens associated with particular crops possible, and provided insight into how these communities may be affected by environmental factors and the crop genotype. Disease involves a complex inter-play between a host plant and a pathogen, and the resistance/susceptibility response can involve several components. Natural and induced mutations may change the interaction and inhibit certain steps in the mechanism of infection (Boyd and O'Toole, 2012; Dracatos et al., 2018).

During pre-genomic years, traditional breeding programs were based on the identification of natural and induced mutant alleles for resistance, and their incorporation into elite genotypes through breeding techniques. These approaches were uncertain and imprecise, leading for instance to the transfer of large genome regions instead of just single gene insertions. Nevertheless, mutation breeding methods have been quite successful in improving disease resistance, and traditional plant breeding has been used to generate new crop varieties for decades. Numerous mutants have been developed through mutation induction, showing enhanced resistance to various diseases. Among the most widely known mutants are those induced at the mildew resistance locus (*MLO*) in barley for resistance to powdery mildew (Miklis et al., 2007), and mutations conferring resistance to several lettuce diseases (Christopoulou et al., 2015). The *mlo* mutant is interesting, as the allele has not broken down and has provided unprecedented resistance to mildew for two decades (Panstruga and Schulze-Lefert, 2002). This longevity is due to a gene knockout. In other cases where resistance to specific pathotypes is conferred by a specific host gene allele, mutagenesis needs to be deployed to provide more precise single nucleotide mutations in the target gene sequence.

The revolution driven by the availability of genome and transcriptome sequences offers a new start for plant breeding programs. Association genetics based on single nucleotide polymorphisms (SNPs) and other molecular markers are spreading in plant breeding, creating high throughput data fundamental for the identification of quantitative trait loci (QTL). Major QTL are employed in crops to provide quantitative resistance to pathogens, together with the use of major resistance (*R*) genes introduced into varieties with superior agronomic characteristics.

New breeding techniques (NBTs) are attracting attention in plant research and concern many different areas, such as developmental biology, abiotic stress tolerance or plant-pathogen resistance (Nelson et al., 2018). NBT include the most recent and powerful molecular approaches for precise genetic modifications of single or multiple gene targets. They employ site-directed nucleases to introduce double stranded breaks at predetermined sites in DNA. These breaks are repaired by different host cell repair mechanisms, resulting either in small insertions or deletions via near homologous end-joining (NHEJ) or micro-homology-mediated end-joining (MMEJ), or in a modified gene carrying predetermined nucleotide changes copied from a repair matrix via homologous recombination (HR). Meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) correspond to the four types of nucleases used in genome editing. The exponential increase in publications reporting the use of CRISPR/Cas9 illustrates the fact that this technology requires less know-how and financial means and has a higher success rate in gene modification compared to the other available nucleases. The application of CRISPR/Cas9 editing has become a powerful tool for future enhancement of agronomic traits in crops (Mohanta et al., 2017).

The objective of this review is to recall the main features of the CRISPR/Cas9 genome editing technique and discuss its application for the enhancement of pathogen resistance in model plants and important crops, with a focus on rice, wheat, and maize.

CRISPR/Cas9: ADVANCES, LIMITATIONS, AND NEW COMBINATIONS

CRISPR/Cas9 from *Streptococcus pyogenes* (SpCas9) has rapidly assumed an important role in different application areas of plant research and many other fields (Ding et al., 2018; Liu and Moschou, 2018). In the CRISPR/Cas9 system a single-guide RNA (sgRNA) can bind to Cas9 and target it to specific DNA sequences (**Figure 1**). The requirement of a protospacer adjacent motif (PAM) limits the possible target sequences in a gene of interest. This limitation is of minor importance if the aim is simply to inactivate a gene by targeted mutagenesis at any position. It has much more importance for genome editing aiming at the precise change of specific nucleotides in a gene. Consequently, major efforts are under way to find Cas9-like proteins with different PAM sequences or to engineer the original Cas9 from *S. pyogenes* to recognize other PAM sequences. For example, xCas9, an evolved version of SpCas9, has been shown to recognize a broad range of PAM sequences including NG, GAA, and GAT in mammalian cells (Hu et al., 2018). In plants, the most widely explored alternative to SpCas9 is Cpf1 from *Prevotella* and *Francisella* with the PAM sequence TTTV, where "V" is A, C, or G (Endo et al., 2016), and an illustrative diagram is shown in **Figure 1**. Cpf1 is also considerably smaller than Cas9, is capable of RNAse activity to process its guide RNA, and introduces

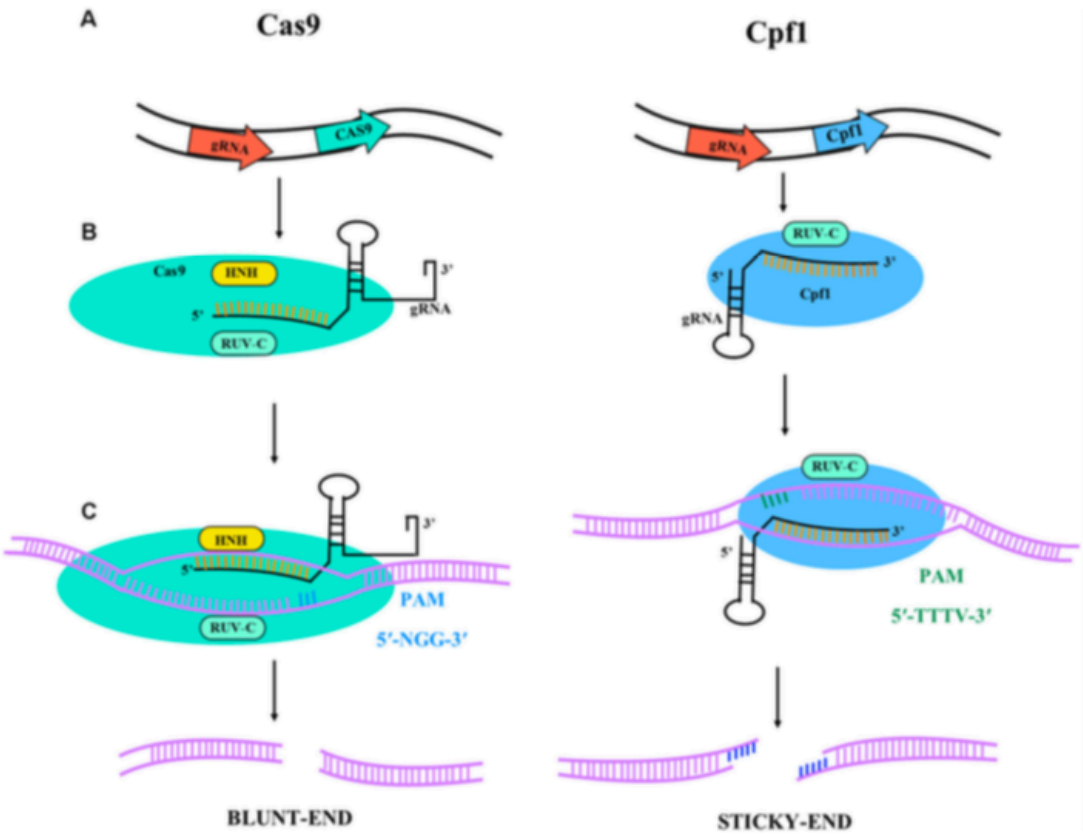

FIGURE 1 | Illustrative diagram of Cas9 and Cpf1 activities. The target specificity is given by the 17-20 nt located at the 5′ end of the sgRNA sequence. **(A)** Primary transcript and gRNA-nuclease (Cas9 or Cpf1) complex formation. The catalytic domains are RUV-C (light blue) and HNH (yellow) for Cas9 and RUV-C for Cpf1. The Cas9 is colored in light blue and the Cpf1 in dark blue; in black is represented the gRNA for gene targeting. **(B)** Gene target activity. Cas9 has 5′-NGG-3′ PAM sequence (blue bars) and Cpf1 has 5′-TTTV-3′ PAM sequence (green bars). **(C)** DNA ends after nuclease activity. Cas9 lead to blunt-end and Cpf1 to sticky-ends.

a staggered double break, which can be useful for enhancing homology-directed recombination and generating efficient gene insertion.

MULTIPLEX GENOME EDITING: WHEN DOES IT BECOME USEFUL?

The ease of multiplexing, i.e., the simultaneous targeting of several genes with a single molecular construct, is one of the major advantages of CRISPR/Cas9 technology with respect to MN, ZFN, or TALEN. For example, the simultaneous mutation of 14 different genes by a single construct has been demonstrated in *Arabidopsis* (Peterson et al., 2016). In crops, several multiplex genome editing (MGE) strategies were reported early on (Ma et al., 2014; Xing et al., 2014; Zhou et al., 2014; Xu et al., 2016), which were all based on a common strategy, i.e., the assembly of multiple gRNAs under the control of a U3 or U6 promoter into a single construct. In maize, the ISU Maize CRISPR platform (Char et al., 2017) permits the cloning of up to four gRNAs for multiplex gene targeting.

More recent multiplex systems exploit self-cleavage capacity of RNA molecules containing tRNA sequences. Constructs alternating sgRNA and tRNA sequences under the control of a single U3 or U6 promoter permit reduction of the size of the construct and limit the risk of silencing due to direct repetitions of promoter sequences. The use of such a strategy employing polycistronic tRNA-gRNA (PTG) to generate hereditable mutation in *TaLpx-1* and *TaMLO* genes has been reported in hexaploid wheat (Wang et al., 2018); the PTG system is described in **Figure 2**. Starting from a previous study on gene silencing of *TaLpx-1*, which encodes the wheat 9-lipoxygenase resistance gene to *Fusarium graminearum* (Nalam et al., 2015), the editing of homologs in wheat was tested. The PTG system containing gRNA activity was validated in wheat confirming gene editing efficacy and providing an effective tool for rapid trait pyramiding in breeding programs.

Recently, an alternative approach for MGEs based on PTG has been reported in rice, where crRNA transcription was obtained from introns inserted into Cpf1 and Cas9 sequences (Ding et al., 2018). Multiplex PTG/Cas9 systems can help with multigene family analysis, as reported for the closely related mitogen-activated protein kinase *MPK1* and *MPK6* in rice

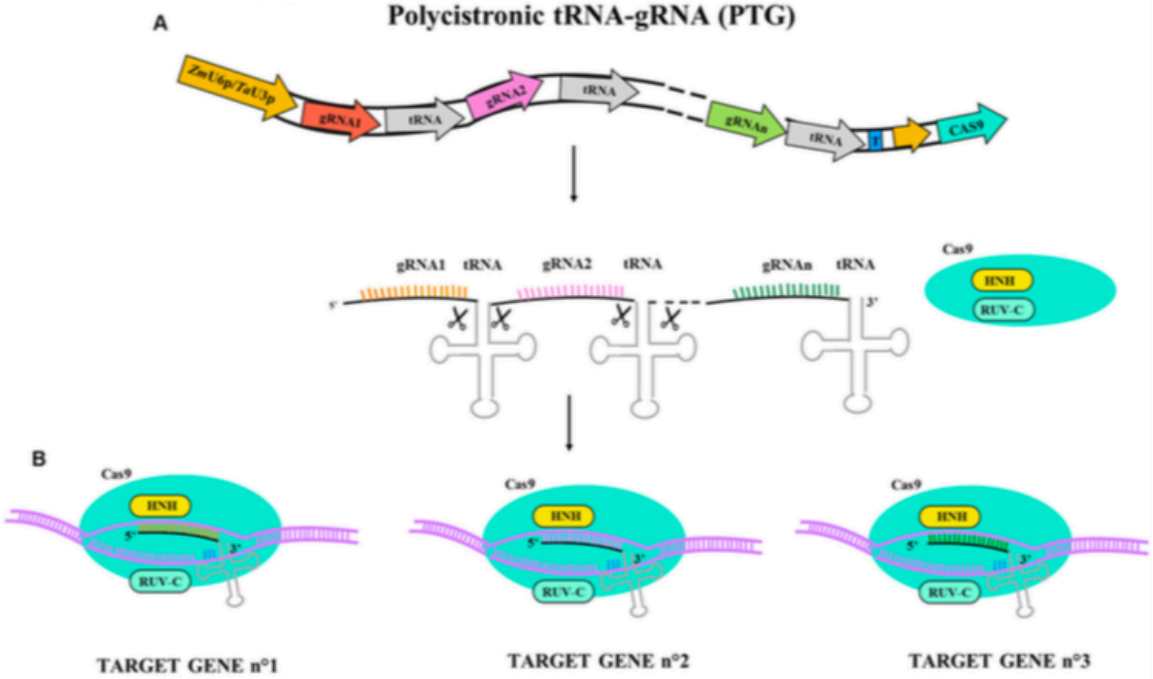

FIGURE 2 | Illustrative diagram of polycistronic tRNA-gRNA (PTG) gene construct and targeting activity for Cas9. PTG is composed of t-RNA-gRNA repeats and is upregulated by ZmU6 promoter or TaU3 promoter according the experimental design as different terminator region (T) are adopted. **(A)** PTG primary transcript. Endogenous endonuclease cuts the tRNA ends and let each tRNA-gRNA targeting the corresponding gene sequence. **(B)** In PTG system more sequence targets are available (n° gene targets) and the different gRNA are represented in different colors (orange, pink, and green).

(Minkenberg et al., 2017). 67% of all lines were double mutants for *MPK* genes with a high frequency of biallelic mutations on multiple target sites. The possibility of programming the PTG/Cas9 to delete chromosomal fragments could be adopted to remove genes and regulatory elements in order to produce transgene free plants.

OFF-TARGET MUTATIONS: FREQUENCY AND LIMITATIONS

High specificity is frequently put forward as a major argument in favor of CRISPR/Cas9 technology, for example in comparison to chemically or irradiation-induced mutagenesis. This raises the question of to what extent a gRNA targets only fully complementary genomic DNA sequences, and to what extent other genomic regions (off-target regions) can also be recognized and cleaved by the CRISPR/Cas9 tool, provoking potentially unwanted damage. Two types of off-target effects are evoked by scientists and regulatory agencies: (i) expected off-target in genome regions with high sequence similarity to the target and (ii) unexpected off-target in unrelated genome regions. The former is generally addressed by PCR amplification and sequencing of regions known to be similar to the target, the latter by whole genome sequencing (Feng et al., 2016).

Genome sequence information is necessary for the prediction of expected off-target effects. The search focuses on the 20 bp target sequence involved in base pairing with the gRNA but excludes the PAM 5′-NGG-3′. The PAM functions as a recognition site outside of the targeted element and does not give specificity for nuclease cleavage (Shah et al., 2013). Moreover, the CRISPR/Cas9 system accepts at least three mismatches in the 20 bp DNA target sequence. Most CRISPR/Cas9 design tools take this into account and propose only specific gRNA designs that do not bind theoretical off-target sites with more than 17 bp identity anywhere else in the genome. Such state-of-the art design is effortless if the gene is unique in the genome, but it becomes rather challenging if the gene has one or more paralogs. This also means that the design is generally easier for diploid genomes without recent duplications than for recently duplicated or polyploid genomes. *In silico* genome analysis of potential target sequences in dicots and monocots has confirmed that, as expected, larger genomes contain more PAMs and more potential targets (Bortesi et al., 2016). High specificity of between 87.3% and 94.3% was observed in relatively simple genomes of *Arabidopsis*, rice, tomato, and soybean, whereas maize, a recent allotetraploid with high levels of repetitive DNA, revealed only 29.5% specific targeting (Bortesi et al., 2016).

Analysis of expected off-target sites, with only one to several mismatches with the primary target, has revealed that the position of the mismatches in the sequence is significant. Mismatches in the seed sequence ("seed" is defined as the 12 bp close to the PAM) are generally not supported or poorly supported by the sgRNA/Cas9 complex (Tsai et al., 2015), causing

mutation less frequently at off-target sites, although in some cases mutations have been observed, as in barley (Lawrenson et al., 2015), soybean (Jacobs et al., 2015), and rice (Xie and Yang, 2013). Unwanted off-target mutations become more frequent when mismatches are located far from the seed region (Zhang et al., 2014).

To clarify the off-targeting issue in crops, recent investigations have screened progenies of CRISPR/Cas9 knockout in polyploid species. A study of CRISPR mutation frequency and mutation heritability of *TaGW2*, *TaLpx-1*, and *TaMLO* genes in the allohexaploid wheat was conducted (Wang et al., 2018). The results were different for the three genes: highly conserved for *TaGW2* (target sequence was specific for all three genomes), moderate for *TaLpx-1* (target sequence specific in two genomes), and low for *TaMLO*. The study showed the flexibility of CRISPR/Cas9 technology for implementing complex gene editing where the majority of genes have more than three homologous copies. Also, the gene editing process was investigated across generations: new mutant variants were recovered across multiple gene targets suggesting the transgenerational activity of CRISPR/Cas9 (Wang et al., 2018).

Another study on target accuracy and efficiency was performed in rice on paralogs *OsBEIIb* and *OSBEIIa* (Baysal et al., 2016). The study reveals the discrepancy in gRNA prediction and mutagenesis efficiency, confirming that gRNA with low predicted efficiency can achieve high mutation frequency even though the prediction suggested different targets with high mutagenesis scores. Empirical testing seems necessary in order to avoid putative gRNA inefficiency. Moreover, the authors also investigated off-target mutagenesis, reporting no mutation in the *OSBEIIa* paralog when only *OsBEIIb* was targeted, confirming the high accuracy of the strategy. CRISPR accuracy has been shown also in tomato (Čermák et al., 2015; Pan et al., 2016; Nekrasov et al., 2017).

To conclude, the CRISPR/Cas9 complex can bind with lower efficiency sequences with one to three mismatches. Therefore, expected off-target mutations do occur but can be avoided by rigorous design of the CRISPR/Cas9 tool. Unexpected off-target mutations do not occur at a frequency above the spontaneous mutation rate of plants.

PLANT TRANSFORMATIONS: CONVENTIONAL AND ALTERNATIVE TECHNIQUES

The bottleneck in the application of CRISPR/Cas9 technology to a wide range of crops is clearly the regeneration of fertile plants from the cells into which the CRISPR/Cas9 tool has been introduced (Altpeter et al., 2016). Consequently, the efficiency of the entire process remains very species- and genotype-dependent, meaning that in many crop species only a few lab varieties are accessible to CRISPR/Cas9 technology. Other important parameters are the quality of the design of the CRISPR/Cas9 tool and the method chosen for its introduction into the plant cell. As in conventional transgenesis, the introduction of the CRISPR/Cas9 tool can be achieved by the *Agrobacterium*-mediated and biolistic transformation of explants, or by direct transformation of protoplasts. The latter two systems have the advantage that not only can the DNA coding for Cas9 and the sgRNA be transferred, but this also applies for ribonucleoproteins (RNPs), i.e., an *in vitro* assembled complex of Cas9 protein with an sgRNA (Malnoy et al., 2016; Svitashev et al., 2016; Liang et al., 2017), or intermediate versions such as a DNA or RNA coding for Cas9 and an RNA representing the sgRNA (Svitashev et al., 2015; Zhang et al., 2016). In addition, both biolistics and direct DNA transfer permit an increase in the ratio of repair matrix DNA over DNA encoding Cas9 and sgRNA readily, thereby favoring HR over NHEJ/MMEJ.

In maize, ISU Maize CRISPR is a high efficiency public platform using *Agrobacterium*-mediated transformation (Char et al., 2017). The main genotypes used for immature embryo transformation are A188, A634, H99, W117 (Ishida et al., 2007), B104 and the hybrid Hi-II (Char et al., 2017). Private companies seem to prefer biolistic transformation to *Agrobacterium*-mediated transformation in the case of gene editing with donor template (Shi et al., 2017), particularly where multiple copies of donor template DNA molecules can be delivered (Svitashev et al., 2015). Even though both transformation processes have decent efficiencies nowadays, they remain limited to the above genotypes with poor agronomic traits. This limitation has recently been overcome by the overexpression of *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes, which stimulated callus growth and increased the overall transformation frequency in maize, including in recalcitrant genotypes. Proof of concept has also been provided for enhanced transformation in sorghum (Lowe et al., 2016).

In rice, most genotypes can easily be transformed both via *Agrobacterium*-mediated transformation and by biolistic methods. In order to achieve CRISPR-mediated HR the DNA template is normally introduced via the biolistic method to increase its copy number in the host (Baysal et al., 2016). As for maize, but involving a higher number of studies, protoplast transient assay is becoming an efficient tool for testing CRISPR-target before starting the transformation of embryos or scutellum derived calli by *Agrobacterium* or particle bombardment (Gao et al., 2013; Jiang et al., 2013; Xie and Yang, 2013; Zhou et al., 2014; Lowder et al., 2015; Li et al., 2016; Luo et al., 2016; Wang et al., 2016). Regeneration of rice protoplasts is still very challenging, but important optimization efforts may render it feasible in the near future. In wheat, although very high *Agrobacterium*-mediated transformation efficiencies of up to 90% have been reported for specific wheat genetic backgrounds (Ishida et al., 2015a,b), particle bombardment has been more widely accepted as the standard method in wheat genetic transformation (Hakam et al., 2015; Wang et al., 2018). Remarkable success has been achieved by particle bombardment of both immature embryos and callus cells to obtain transient expression of the CRISPR/Cas9 DNA, and transgene-free homozygous mutant T0 plants have been generated in the absence of any selection (Zhang et al., 2016). Three studies have reported CRISPR mutagenesis in barley by using *Agrobacterium*-mediated transformation of immature embryos (Lawrenson et al., 2015; Holme et al., 2017;

Kapusi et al., 2017), while in Kapusi et al. (2017) a comparison with particle bombardment was carried out. Higher numbers of mutants were reported with the *Agrobacterium*-mediated compared to the biolistic transformation approach.

In conclusion, although preferences for certain delivery methods exist for certain species, efficiency is not only linked to the technique itself, but also to the know-how of a given lab as regards a given technique. Polyethylene glycol (PEG) or electroporation-mediated DNA transient expression in protoplasts have proven very useful for the evaluation of the efficiency of CRISPR/Cas9 designs (Malnoy et al., 2016). The importance of preliminary screens will certainly increase with the foreseeable shift from targeted mutagenesis to repair matrix based genome editing, which will increase the number of events to analyze due to lower efficiency. RNP technology has been established in plants and may help toward exemption from regulatory oversight, but its efficiency needs to be improved to make it a routine tool.

CRISPR/Cas-BASED STRATEGIES CONFERRING BIOTIC RESISTANCE

Biotic stresses including viral, fungal, and bacterial diseases are responsible for losses ranging from 20% to 40% of global agricultural productivity (Savary et al., 2012). Conferring host plant resistance to pathogens can reduce the impact of disease on crop development and yield, thereby addressing the challenge of feeding the world's growing population.

Advances in genome editing tools have opened new ways to achieve the improvement of resistance in crops. In recent years, the CRISPR/Cas system has been employed to respond to several agricultural challenges, including the achievement of improved biotic stress resistance (Arora and Narula, 2017). The application of CRISPR/Cas tools has mainly been explored against virus infection, followed by efforts to improve fungal and bacterial disease resistance. Recent studies demonstrating the power of the CRISPR/Cas technology in establishing resistance to these pathogen categories will be further discussed below.

Virus Resistance via CRISPR/Cas

Plant viruses are a serious threat to many economically important staple and specialty crops. Based on their genome nature, they are classified into six major groups: double-stranded DNA (dsDNA) viruses with no plant viruses in this group, single-stranded DNA (ssDNA), reverse-transcribing viruses, double-stranded RNA (dsRNA), negative sense single-stranded RNA (ssRNA−), and positive sense single-stranded RNA (ssRNA+) viruses (Roossinck et al., 2015). Most studies involving CRISPR-edited plants for virus resistance have targeted ssDNA geminivirus genomes (Ali et al., 2015, 2016; Baltes et al., 2015; Ji et al., 2015) (**Table 1**).

Geminiviridae is a large family of plant viruses causing worldwide crop losses among several important families, such as Cucurbitaceae, Euphorbiaceae, Solanaceae, Malvaceae, and Fabaceae (Zaidi et al., 2016). The virus genome is replicated through a rolling-circle amplification mechanism via a dsDNA replicative form, or by recombination-mediated replication (Hanley-Bowdoin et al., 2013). The most important genus of geminiviruses in economic terms is Begomovirus. Begomoviruses infect dicotyledonous plants via the sweet potato/tobacco/silverleaf whitefly (*Bemisia tabaci*) and are mainly found associated to the phloem of infected plants (Gilbertson et al., 2015). Their genome is organized in one (A, monopartite) or two (A and B, bipartite) components containing a common region of ∼220 bp (Fondong, 2013).

The first two studies focusing on resistance to geminiviruses, beet severe curly top virus (BSCTV) and bean yellow dwarf virus (BeYDV) in model plants *N. benthamiana* and *Arabidopsis* were reported by Baltes et al. (2015) and Ji et al. (2015) (**Table 1**). Ji et al. (2015) screened 43 candidate sgRNA/Cas9 target sites in coding and non-coding regions of the BSCTV genome. All the sgRNA/Cas9 constructs reduced virus accumulation in inoculated leaves at varying levels, but a greater resistance to virus infection was observed in *Nicotiana* and *Arabidopsis* plants showing the highest levels of expression of Cas9 and sgRNAs. Similar findings were described by Baltes et al. (2015), who employed 11 sgRNAs targeting Rep motifs, Rep-binding sites, hairpin, and the nonanucleotide sequence of BeYDV, and reported up to 87% reduction in the targeted viral load in *N. benthamiana*.

Two recent works have also employed a CRISPR/Cas9 approach for achieving resistance to begomoviruses (Ali et al., 2015, 2016) (**Table 1**). Both studies were based on the strategy of expressing the CRISPR/Cas9 system in the host cell nucleus to target and cleave the virus genome during replication. Ali et al. (2015) developed sgRNA molecules delivered via a tobacco rattle virus (TRV) vector into *N. benthamiana* plants stably overexpressing the Cas9 endonuclease. SgRNAs were specific for different tomato yellow leaf curl virus (TYLCV) coding and non-coding sequences, targeting the viral capsid protein (CP), the RCRII motif of the replication protein (Rep) and the intergenic region (IR). All sgRNAs were able to interfere with TYLCV genome sequences, but targeting the stem-loop invariant sequence contained in the IR caused a more significant reduction of viral replication and accumulation. The same CRISPR/Cas9 system was tested for targeting simultaneously the monopartite beet curly top virus (BCTV) and the bipartite Merremia mosaic virus (MeMV), geminiviruses that share the same stem-loop sequence in the IR. The results showed attenuated symptoms for both viruses, demonstrating that mixed infection immunity can be developed via a single sgRNA specific for conserved sequences of multiple viral strains.

Furthermore, Ali et al. (2016) analyzed not only the targeting efficiencies of the CRISPR/Cas9 tool but also the emergence of mutated viruses capable of replication and systemic movement. The CRISPR/Cas9 tool was designed to interfere with different coding and non-coding sequences of cotton leaf curl Kokhran virus (CLCuKoV), MeMV, and different severe and mild strains of TYLCV. The work revealed that when the sgRNA/Cas9 complex edited sites in the coding regions of all viruses, virus variants were generated capable of replicating and moving to escape the CRISPR/Cas9 machinery. Conversely, no novel variants were detected in *N. benthamiana* plants carrying sgRNAs addressing the IR sequences. Even though the NHEJ machinery

TABLE 1 | CRISPR/Cas9 applications for virus resistance.

Plant species	Virus	Target gene	Gene function	Strategy	Reference
Nicotiana benthamiana and *Arabidopsis thaliana*	BeYDV	CP, Rep, and IR	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with Cas9/gRNA expression plasmid vectors	Ji et al., 2015
Nicotiana benthamiana	BSCTV	LIR and Rep/RepA	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with Cas9/gRNA expression plasmid vectors	Baltes et al., 2015
Nicotiana benthamiana	TYLCV BCTV MeMV	CP, Rep and IR	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas9 overexpressing plants	Ali et al., 2015
Nicotiana benthamiana	CLCuKoV MeMV TYLCV	CP, Rep, and IR	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas9 overexpressing plants	Ali et al., 2016
Nicotiana benthamiana	TuMV	GFP1, GFP2, HC-Pro, CP	Replication mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas13a overexpressing plants	Aman et al., 2018
Nicotiana benthamiana and *Arabidopsis thaliana*	CMV TMV	ORF1, 2, 3, CP and 3′UTR	Replication mechanism	*Agrobacterium*-mediated transformation of leaves with FnCas9/gRNA expression binary vectors Floral dipping for *Arabidopsis*	Zhang et al., 2018
Cucumis sativus	CVYV ZYMV PRSV-W	eIF4E	Host factor for RNA viruses translation	*Agrobacterium*-mediated transformation of cut cotyledons (without embryo) with Cas9/gRNA binary vectors	Chandrasekaran et al., 2016
Arabidopsis thaliana	TuMV	eIF(iso)4E	Host factor for RNA viruses translation	*Agrobacterium*-mediated transformation with Cas9/gRNA recombinant plasmid binary vectors (floral dipping)	Pyott et al., 2016
Oryza sativa L. *japonica*	RTSV	eIF4G	Host factor for RNA viruses translation	*Agrobacterium*-mediated transformation of immature embryos with Cas9/gRNA expression plasmid vectors	Macovei et al., 2018

BeYDV, bean yellow dwarf virus; BSCTV, beet severe curly top virus; TYLCV, tomato yellow leaf curl virus; BCTV, beet curly top virus; MeMV, Merremia mosaic virus; TRV, tobacco rattle virus; CLCuKoV, cotton leaf curl Kokhran virus; TuMV, turnip mosaic virus; CMV, cucumber mosaic virus; TMV, tobacco mosaic virus; CVYV, cucumber vein yellowing virus; ZYMV, zucchini yellow mosaic virus; PRSV-W, papaya ring spot mosaic virus-W; PVX, potato virus X; TCV, turnip crinkle virus; CMV, cucumber mosaic virus; RTSV, rice tungro spherical virus; CP, coat protein; Rep, replication association protein; IR, intergenic region; RCA, rolling-circle amplification; LIR, long intergenic region; GFP1, green fluorescent protein 1; GFP2, green fluorescent protein 2; HC-Pro, helper component proteinase silencing suppressor; ORF, open reading frame; UTR, untranslated terminal repeat; eIF4E, eukaryotic translation initiation factor 4E; eIF4G, eukaryotic translation initiation factor 4G.

repaired the double strand breaks caused by the Cas9 protein, the IR-repaired variants generated virus genomes unable to replicate, thus providing a better overall interference with the viral life cycle.

Protection against RNA viruses has seemed more difficult to achieve, since the classical SpCas9 from *Streptococcus pyogenes* only recognizes dsDNA. However, the search for and characterization of related nucleases has led to the discovery of enzymes that can bind to and cut RNA, such as FnCas9 from *Francisella novicida* or LwaCas13a from *Leptotrichia wadei*. A first report demonstrating resistance to RNA viruses (Zhang et al., 2018) (**Table 1**) expressed FnCas9 and RNA-targeting sgRNAs specific for cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) in *N. benthamiana* and *Arabidopsis* plants. Transgenic plants showed CMV and TMV accumulation reduced by 40–80% compared with control plants. Furthermore, the resistance obtained by expressing the sgRNA-FnCas9 system was quite stable and still active in the T6 generation. Importantly, Zhang et al. (2018) observed that the endonuclease activity of FnCas9 was not required for interference with the CMV genome, whereas its RNA-binding activity was essential, meaning that this particular application of FnCas9 can be considered as a CRISPR interference (CRISPRi) tool, similar to catalytically inactive SpCas9 proteins programmed to mitigate gene expression (Larson et al., 2013). The use of a catalytically inactive variant of FnCas9 has the advantage of limiting the onset of mutated viral variants capable of escaping CRISPR/Cas9. Moreover, in contrast with the previously described interference with geminivirus replication in the nucleus, no nuclear localization signal is necessary for FnCas9, which interferes with the RNA viruses in the cytoplasm.

Similar work has been carried out with Cas13a. Aman et al. (2018) exploited this RNA-guided ribonuclease to manipulate the turnip mosaic virus (TuMV) RNA genome (**Table 1**). Four different viral genomic regions were targeted: two targets in the green fluorescent protein (GFP) region, one in the helper component proteinase silencing suppressor (HC-Pro), and one in the coat protein (CP). The most efficient virus interference was observed with CRISPR RNA editing HC-Pro and GFP2 genes and resulted in a reduced replication and spread of TuMV in tobacco leaves. Furthermore, due to the innate ability of Cas13 to process pre-CRISPR RNA into functional CRISPR RNA, the multiplex targeting of several viral mRNA could be markedly improved through this alternative system (Aman et al., 2018).

All the systems aiming at protection against viruses described so far require the maintenance of a transgene for Cas9 and sgRNA in the genome of the crop plants, rendering them subject to genetically modified organism (GMO) regulation. A second strategy for the achievement of viral disease resistance consists in modifying plant genes that will generate virus resistance traits, to segregate the CRISPR/Cas9 tool and to release non-transgenic mutants in the field (Chandrasekaran et al., 2016; Pyott et al., 2016; Macovei et al., 2018) (**Table 1**). Plant host factors are required by RNA viruses to maintain their life cycle, including the eukaryotic translation initiation factors eIF4E, eIF(iso)4E and eIF4G (Sanfacon, 2015). Chandrasekaran et al. (2016) developed cucumber plants resistant to potyviruses by mutating independently two different sites of the host susceptibility gene *eIF4E* by CRISPR/Cas9. Non-transgenic Cucumis *eif4e* mutant plants were obtained by segregation of the CRISPR/Cas9 tool by three generations of backcrossing, making these plants safe for human consumption and for release into the environment, according to the authors. When challenged with viruses from the Potyviridae family, cucumber vein yellowing virus (CVYV), zucchini yellow mosaic virus (ZYMV), and papaya ring spot mosaic virus-W (PRSV-W), homozygous *eif4e* mutants showed immunity. Conversely, heterozygous knockout plants and non-mutant plants did not reveal any resistance to these viruses.

A similar editing approach was utilized by Pyott et al. (2016) in order to introduce site-specific mutations at the closely related *eIF(iso)4E* locus in *Arabidopsis* plants. Both 1 bp insertions and 1 bp deletions in *eIF(iso)4E* conferred complete resistance to the single-stranded RNA potyvirus (+ssRNA) TuMV and no off-target modification was detected in this study. Furthermore, homozygous T3 *eIF(iso)4E* mutants did not significantly differ in growth and development compared to wild-type plants.

Recently, Macovei et al. (2018) have developed new sources of resistance to rice tungro spherical virus (RTSV) through mutagenesis of *eIF4G* alleles in rice plants. The RTSV-resistant T_2 plants obtained did not show any detectable mutation in the off-target sites and were negative when tested for the presence of Cas9. Furthermore, after inoculation with RTSV, agronomic parameters such as plant height and grain yield were enhanced in the edited rice plants compared to their wild-type counterparts under glasshouse conditions.

The advantage of knocking out host susceptibility genes is that this is a relatively simple method that renders following the mutation easy. The loss of a host factor required for the viral life cycle is a form of recessive resistance that should be more durable than that of dominant *R* genes because viruses undergo a lower selective pressure, preventing their evolution to hinder host defense mechanisms. A possible disadvantage of the knockout strategy is that it may negatively influence plant vigor, supporting the selection of virus variants breaking the resistance, as already observed in nature (Abdul-Razzak et al., 2009). Pyott et al. (2016) and Macovei et al. (2018) did not observe any significant difference in growth defects between mutants and normal plants, although further investigations should be carried out in order to test the durability of this edited recessive resistance.

Resistance to Fungi Through CRISPR/Cas

Fungal pathogens are responsible for numerous diseases such as mildew, smut, rust, rot and many more. These diseases not only cause dramatic yield losses annually throughout the world but also compromise the quality of the harvested products. Moreover, mycotoxigenic fungi represent a serious concern due to the production of secondary metabolites known as mycotoxins, which cause severe health problems in humans and animals after exposure to contaminated food and feed. Several strategies have been evolved to enhance fungal resistance in plant species based on the current knowledge of molecular mechanisms implicated in plant-pathogen interaction. Potential candidate genes and gene

products involved in plant resistance against fungi have been described, and nowadays these are prime targets for editing through the CRISPR/Cas9 approach.

As previously partially discussed, *MLO* loci have been targeted by RNA-guided Cas9 endonuclease in three different plant species: bread wheat, tomato, and grapevine (Wang et al., 2014; Malnoy et al., 2016; Nekrasov et al., 2017) (**Table 2**). *MLO* encodes a protein with seven transmembrane domains localized in the plasma membrane and is ubiquitously present in monocots and dicots (Acevedo-Garcia et al., 2014). It had previously been reported that *MLO* were susceptibility (*S*) genes and that homozygous loss-of-function mutants had significantly increased resistance to powdery mildew in barley, *Arabidopsis* and tomato (Piffanelli et al., 2004; Consonni et al., 2006; Bai et al., 2008). Bread wheat plants mutated by CRISPR/Cas9 in one (*TaMLO-A1*) of the three *MLO* homeoalleles showed improved resistance to *Blumeria graminis* f. sp. tritici infection, a finding that once again demonstrated the important role of *TaMLO* genes in powdery mildew disease (Wang et al., 2014). In tomato, *SlMlo1*, previously identified as the most important of 16 *SlMlo* genes, was targeted at two sites and a deletion of 48 bp was obtained. The edited plants were self-pollinated in order to generate CRISPR/Cas cassette-free individuals. This new non-transgenic variety, "Tomelo," was fully resistant to *Oidium neolycopersici*. Furthermore, off-target analysis did not reveal any effect on the genomic regions outside the *SlMlo1* locus (Nekrasov et al., 2017). In grapevine, the molecular feasibility of *VvMLO7* knockout has been demonstrated through CRISPR/Cas9 RNP in protoplasts, but no plants have been regenerated (Malnoy et al., 2016). Parallel experiments with RNAi plants showed that the loss of *VvMLO7* reduced susceptibility to *Erysiphe necator* in grapevine (Pessina et al., 2016).

The RNP approach has also been used for editing *DIPM-1*, *DIPM-2*, and *DIPM-4* genes in apple protoplasts in order to confer resistance to fire blight disease (Malnoy et al., 2016). Again, only the molecular analysis attesting mutations has been carried out, not disease assay on regenerated plants. In perennial crops such as grapevine and apple, which take several years to flower, the transient introduction of genome editing tools in protoplasts is particularly interesting, since the segregation of stably integrated CRISPR/Cas9 cassettes by backcrosses would take a lot longer than in annual crops with generation times of only a few months. Secondly, the delivery of Cas9/sgRNA complex as RNP is a rapid approach, making possible the achievement of transformed protoplasts and the evaluation of sgRNA efficiency within 1 or 2 days. Thirdly, no foreign DNA is integrated into the genome and the Cas9/sgRNA complexes can be degraded rapidly during the cell culture regeneration process. Furthermore, even in transient approaches, the employment of plasmids can sometimes cause their undesired integration into the host genome, and the prolonged presence of CRISPR/Cas9 tools in the genome increases the risk of off-target mutations, while the CRISPR/Cas9 RNP shows improved on-target specificity. The drawback of this approach is the need to optimize plant regeneration protocols in order to apply this technology.

TABLE 2 | CRISPR/Cas9 applications for fungal resistance.

Plant species	Fungus	Target gene	Gene function	Strategy	Reference
Triticum aestivum	Powdery mildew (*Blumeria graminis* f. sp. *tritici*)	MLO-A1	Susceptibility (S) gene involved in powdery mildew disease	Particle bombardment of immature wheat embryos with Cas9/gRNA expression plasmid vectors	Wang et al., 2014
Solanum lycopersicum	Powdery mildew (*Oidium neolycopersici*)	MLO1	Major responsible for powdery mildew vulnerability	*Agrobacterium*-mediated transformation of cotyledons with Cas9/gRNA expression plasmid vectors	Nekrasov et al., 2017
Vitis vinifera	Powdery mildew (*Erysiphe necator*)	MLO-7	Susceptibility (S) gene involved in powdery mildew disease	PEG-mediated protoplast transformation with CRISPR ribonucleoproteins	Malnoy et al., 2016
Vitis vinifera	Gray mold (*Botrytis cinerea*)	WRKY52	Transcription factor involved in response to biotic stress	*Agrobacterium*-mediated transformation of proembryonal masses with Cas9/gRNA expression binary vectors	Wang et al., 2018
Theobroma cacao	Black pod disease (*Phytophthora tropicalis*)	NPR3	Regulator of the immune system	*Agrobacterium*-mediated transient transformation of stage C leaves with Cas9/gRNA expression binary vectors	Fister et al., 2018
Oryza sativa L. *japonica*	Rice blast disease (*Magnaporthe oryzae*)	SEC3A	Subunit of the exocyst complex	Protoplast transformation with Cas9/gRNA expression binary vectors	Ma et al., 2018
Oryza sativa L. *japonica*	Rice blast disease (*Magnaporthe oryzae*)	ERF922	Transcription factor implicated in multiple stress responses	*Agrobacterium*-mediated transformation of embryogenic calli with Cas9/gRNA expression binary vectors	Wang et al., 2016

MLO, MILDEW RESISTANT LOCUS; NPR3, non-expressor of pathogenesis-related 3; ERF922, ethylene responsive factor.

An example of the successful protection of grapevine by the CRISPR/Cas9 system is the *VvWRKY52* transcription factor, which was targeted by four gRNAs (Wang et al., 2018) (**Table 2**). About 21% of the transgenic plants showed biallelic mutations and were more resistant to *Botrytis cinerea* compared to the monoallelic mutants. No marked difference was observed in phenotype between wild-type and biallelic mutant plants, confirming the efficiency of the CRISPR/Cas9 strategy in woody plants with long reproductive cycles.

A further strategy to expedite genome editing application in slow generation tree crops is the employment of transient leaf transformation coupled to disease assays as demonstrated in *Theobroma cacao* (Fister et al., 2018) (**Table 2**). The authors reported for the first time the transient introduction of CRISPR/Cas9 components into cacao leaves targeting the *Non-Expressor of Pathogenesis-Related 3* (*NPR3*) gene, a suppressor of the immune system, and obtained leaves with increased resistance to *Phytophthora tropicalis*. This new system of *in vivo* mutagenesis in adult cacao trees is a fast and useful technique for validating sgRNA design and observing CRISPR mutagenized phenotypes. It encouraged the authors to regenerate genome-edited somatic embryos to validate the observed results at whole-plant level.

Plants resistant to rice blast disease were generated through CRISPR/Cas9 by disrupting *OsERF922* and *OsSEC3A* genes in rice (Wang et al., 2016; Ma et al., 2018) (**Table 2**). *Ossec3a* mutant plants disrupted in a putative subunit of a complex involved in exocytosis, revealed a pleiotropic phenotype including improved resistance against *Magnaporthe oryzae*, higher levels of salicylic acid (SA) content and up-regulation of pathogenesis- and SA-related genes, but also dwarf stature (Ma et al., 2018). In contrast, no alteration of a number of agronomic traits was observed in T1 and T2 transgene free plants mutated in the ethylene responsive factor (ERF)922, a transcription factor implicated in multiple stress responses. The mutant plants had a reduced number of blast lesions at both seedling and tillering stages (Wang et al., 2016). Overall, these results demonstrate the powerful and advantageous application of the CRISPR/Cas9 system for crop improvement as regards fungal disease resistance.

Resistance to Bacteria Through CRISPR/Cas

Among the bacterial species living on earth, just a few hundred are involved in crop damage, which often reveals multiple symptoms of disease (Schloss and Handelsman, 2004). Phytopathogenic bacteria are difficult to control, mainly because of undetected asymptomatic infections and the lack of suitable agrochemicals. Generally speaking, bacteriological plant control is based on prevention and exclusion of the pathogen by using genetic resistance, agronomic practices, and biocontrol agents (Kerr, 2016).

Phytopathogenic bacteria can be classified as crop specific, such as *Clavibacter michiganensis*, which is the causal agent of tomato bacterial ring rot; polyphagous specific, such as *Ralstonia solanacearum*, which causes disease in multiple monocot and dicot species; and "kingdom crosser," such as *Dickeya dadantii*, an entomo-phytopathogen, which can affect plants and animals.

Relatively few studies (**Table 3**) have been published on the application of the CRISPR/Cas system to counteract crop bacterial diseases. CRISPR/Cas9 mutagenesis of *OsSWEET13* has been performed in rice to achieve resistance to bacterial blight disease caused by γ-proteobacterium *Xanthomonas oryzae* pv. *oryzae* (Zhou et al., 2015). *OsSWEET13* is a susceptibility (*S*) gene encoding a sucrose transporter involved in plant-pathogen interaction. *X. oryzae* produces an effector protein, PthXo2, which induces *OsSWEET13* expression in the host and the consequent condition of susceptibility. In a previous work concerning *OsSWEET14* promoter mutagenesis adopting a TALEN approach, the disruption of this gene rendered the *X. oryzae* effector unable to bind *OsSWEET14* and ultimately resulted in disease resistance (Li et al., 2012). Similarly, Zhou et al. (2015) obtained a null mutation in *OsSWEET13* in order to better explore PthXo2-dependent disease susceptibility, and resultant mutants were resistant to bacterial blight. Further genome editing strategies for multiplexed recessive resistance using a combination of the major effectors and other resistance (*R*) genes will be the next step toward achieving bacterial blight resistance.

Two recent works have reported the employment of CRISPR/Cas9 with the aim of producing citrus plants resistant to citrus bacterial canker (CBC). CBC is caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) and is the most widespread disease among commercial cultivars. In the first work, Jia et al. (2016) generated canker resistant mutants by editing the PthA4 effector binding elements in the promoter of the *Lateral Organ Boundaries 1* (*CsLOB1*) gene in Duncan grapefruit. Mutated lines showed a decrease in typical canker symptoms 4 days post inoculation with *Xcc*, and no further phenotypic alterations were detectable. Furthermore, no potential off-target mutations in other *LOB* family genes were found by PCR-sequencing. The second work, by Peng et al. (2017), confirmed the link between *CsLOB1* promoter activity and CBC disease susceptibility in Wanjincheng orange (*Citrus sinensis* Osbeck). The complete deletion of the EBE_{PthA4} sequence from both *CsLOB1* alleles induced resistance enhancement to CBC. Moreover, no alteration in plant development was observed after *CsLOB1* promoter modification. Additional efforts will be required to generate non-transgenic canker-resistant citrus varieties for facilitating their agronomic application in CBC prevention.

FUTURE PROSPECTS

In an era marked by political and societal pressure to reduce the use of pesticides, crop protection by genetic improvement provides a promising alternative with no obvious impact on human health or the environment. Genome editing is one of the genetic levers that can be adopted, and disease resistance is frequently cited as the most promising application of CRISPR/Cas9 technology in agriculture. There are three main

TABLE 3 | CRISPR/Cas9 applications for bacterial resistance.

Plant species	Fungus	Target gene	Gene function	Strategy	Reference
Oryza sativa	Bacterial blight (*Xanthomonas oryzae* pv. *oryzae*)	SWEET13	Sucrose transporter gene	*Agrobacterium*-mediated transformation of embryogenic callus with Cas9/gRNA expression plasmid vectors and TALEN	Li et al., 2012; Zhou et al., 2015
Citrus paradisi	Citrus canker (*Xanthomonas citri* subspecies *citric*)	LOB1	Susceptibility (S) gene promoting pathogen growth and pustule formation	*Agrobacterium*-mediated transformation of epicotyl with Cas9/gRNA expression plasmid vectors	Jia et al., 2016
Citrus sinensis Osbeck	Citrus canker (*Xanthomonas citri* subspecies *citric*)	LOB1	Susceptibility (S) gene promoting pathogen growth and pustule formation	*Agrobacterium*-mediated transformation of epicotyl with Cas9/gRNA expression plasmid vectors	Peng et al., 2017
Malus domestica	Fire blight (*Erwinia amylovora*)	DIPM-1 DIPM-2 DIPM-4	Susceptibility factor involved in fire blight disease	PEG-mediated protoplast transformation with CRISPR ribonucleoproteins	Malnoy et al., 2016

CsLOB1, Lateral Organ Boundaries 1; DIPM, DspE-interacting proteins of Malus.

reasons for this: firstly, scientific knowledge of the molecular mechanisms underlying numerous pathosystems is sufficiently advanced to enable the proposal of genes to be edited in order to achieve resistance. Secondly, disease resistance can frequently be achieved by the modification of a single gene, which is technically less challenging. This is similar to the modification of metabolic pathways, where the editing of a single gene can also have an all-or-nothing effect, but different from abiotic stress tolerance, where generally numerous genes have to be modified in a coordinated fashion to achieve incremental improvements. Thirdly, targeted mutagenesis, the only use of CRISPR/Cas9 technology at present mastered with respect to crops, is readily applicable to disease resistance, since the inactivation of susceptibility genes leads to protection. For other agriculturally interesting traits the achievement of positive effects by the loss-of-function of genes is a more delicate matter. However, acting as the spearhead of genome editing in crops also puts a certain responsibility on plant pathologists.

The first challenge is to demonstrate that the promises made by proofs of concept in confined environments can be maintained under field conditions. It is one thing to show that the population of a pathogen or the size of disease lesions is reduced in a greenhouse and another to protect a crop year after year under varying environmental conditions. Field tests are also necessary for correct evaluation of the agronomic fitness of the edited crops. Most of the genes inactivated by CRISPR/Cas9 technology in order to obtain disease resistance are likely to have roles in the physiology of the plant other than that linked to the life cycle of the pathogen. For example, triple knockouts of wheat *TaMLO* were not only resistant to powdery mildew but also showed leaf chlorosis (Wang et al., 2014), whereas EMS-induced triple mutants with non-conservative point mutations in *TaMLO* did not show obvious pleiotropic phenotypes (Acevedo-Garcia et al., 2017). Therefore, encouraging greenhouse observations of plant development or measurements of key parameters such as height, leaf area or grain weight absolutely must be confirmed under field conditions by multi-environmental yield trials in order to measure the relative importance of negative side effects. A final limitation of many published proofs of concept is that they involve lab varieties, which can easily be regenerated after the introduction of Cas9 and sgRNA, but which often have only a limited agronomic value. It remains to be shown that the phenotypic effects are maintained in elite lines under field conditions.

The second challenge is the durability of the disease resistances, and their agronomic management. This challenge needs to be dealt with seriously, in order to convince a public often hostile to this technology. Durability is not a specific aspect of resistance genes obtained by genome editing, and the answers are the same as for introgressed resistance genes discovered in the genetic variability of the species: (i) the stacking of several resistance genes, preferably with different modes of action, (ii) a focus on systems other than NBS-LRR receptor kinases known to break down rapidly, and (iii) good agronomic practices,

including, in particular, crop rotation and the concomitant use of biocontrol agents. An example of two independent CRISPR/Cas9-derived resistances against the same disease are the knockouts of *TaMLO* (Wang et al., 2014) and *TaEDR1* (Zhang et al., 2017), both conferring resistance to powdery mildew in wheat. Beyond the creation of novel alleles conferring protection, CRISPR/Cas9 technology can also be helpful in the stacking process itself. In contrast with the introgression of conventional resistance genes, the technology not only avoids genetic drag on neighboring regions with potentially negative impacts on agronomic performance, but also permits the simultaneous creation of multiple resistances in a single generation by multiplexing, i.e., the parallel use of several sgRNAs targeting different genes. Admittedly, multiplexing becomes more challenging with increasing ploidy levels, and in the above example in hexaploid wheat (A, B, and D genome), three *TaMLO* genes and three *TaEDR1* genes would need to be modified in parallel.

The third challenge is to overcome the present technical limitation regarding targeted mutagenesis and to implement true genome editing in crop plants. Targeted mutagenesis introduces random mutations (generally short insertions or deletions) at a predetermined site of a given gene, leading generally to loss-of-function, whereas true genome editing introduces predetermined base changes at one or several specific positions in a gene. For example, the elongation initiation factor 4E (eIF4E) is necessary for the translation of RNA into protein for both the host cell and single-stranded RNA viruses of the Potyviridae family. As described above, loss-of-function of eIF4E by targeted mutagenesis has been achieved in several model and crop species, consistently conferring resistance to potyviruses but also impacting the host plants to varying degrees. The specific modification of amino acids known to be important for the translation of viral but not host proteins would permit driving resistance to potyviruses without affecting plant physiology (Bastet et al., 2017). The expression of a transgene carrying a synthetic allele with six mutations in an *Arabidopsis eif4e* mutant validated the concept (Bastet et al., 2018), demonstrating indirectly the potential benefit of genome editing over targeted mutagenesis. However, at present true genome editing by HR is still hampered by very low efficiencies in plants, although it has recently become routine in many animal species. Continued efforts to improve its efficiency, for example by the use of *lig4* (Endo et al., 2016) or *polQ* mutations (Saito et al., 2017), or a copy number increase of the repair matrix by virus vectors (Čermák et al., 2015), are crucial to increasing the range of tools available to plant pathologists. Base editing, to date permitting C to T and A to G transitions in plants, is more limited in scope but has recently emerged as a readily available alternative for certain editing projects (Zhang et al., 2017; Hua et al., 2018).

The long term success of CRISPR/Cas9 technology in plant protection is dependent on new scientific knowledge. CRISPR/Cas9 technology can only be used if one knows which gene(s) to modify and which modification(s) to carry out in these genes in order to render plants resistant to disease. When pathogen resistance is achieved by the knock-out of one or several genes, inactivating mutations can easily be provoked by CRISPR-mediated specific DNA break and activation of the cell's error prone DNA repair, based on NHEJ. In this case, CRISPR can be used to target and inactivate a single gene or large gene families, both through single gRNA which matches several targets, or by multiplexing the system by introducing several gRNAs simultaneously. On the contrary, when specific allelic variants are involved in resistance, CRISPR-DNA break can be coupled with the less frequent cell repair mechanism based on HDR. The DNA template for HDR should be introduced into the cell together with the effector nuclease. This permits the introduction of a custom-designed sequence into the genome. The use of HDR, compared to NHEJ, can indefinitely expand the possibility of the type of mutations inserted by CRISPR, as any sequence can be inserted into a site of choice. Nevertheless, HDR is still technically challenging due to its low efficiency, the difficulty of having a selective marker and the lack of multiplexing protocols. These are aspects that will need to be improved if CRISPR applications are to expand in plant breeding. Despite the recent judgment of the Court of Justice of the European Union issued that organisms created using genome editing techniques are to be regulated as GMOs (Callaway, 2018), anyhow continuous efforts in plant pathology are necessary, in order to identify and characterize the genes involved in plant pathogen interactions. For example, the past decade was marked by the discovery of hundreds of effector molecules that are synthesized by different classes of pathogens and transferred into the host cell. A major challenge is to identify the host proteins targeted by these effectors and to characterize the underlying genes, which are one of many possible targets for future genome editing approaches. New knowledge does not necessarily have to stem from the crop species of interest. For example, the targeted mutagenesis of wheat *TaMLO* was based on knowledge of another crop, barley, where *Hvmlo* mutant varieties have provided good protection against powdery mildew that has not yet broken down, and the modification of *TaERF1* exploited knowledge from the model species *Arabidopsis*. These examples perfectly illustrate the added value of genome editing, which permits the enlargement of the gene pool of a crop species beyond all the available natural variability, by means of the transfer of knowledge acquired in other crops or model species.

AUTHOR CONTRIBUTIONS

VGB contributed by writing and editing the major part of the review. AL, AM, and PR organized and prepared some of the parts of this review. VB and PR critically revised the manuscript. AM and AL contributed to the design of the work's layout and were responsible for obtaining final approval from the other contributors.

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