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A New Approach in Management Against Plant Fungal Disease: Host Induced Gene Silencing

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Abstract

Plant pathogenic fungi may cause crop losses that affect the world economy. Although one of the most effective ways to combat plant pathogens is a chemical control, alternative methods have become necessity as a result of environmental pollution and residue problems caused by pesticides used in agriculture. The mechanism of RNA interference (RNAi) has been developed to completely prevent or decrease the production of protein which is an expression of a specific gene. Due to the degeneracy of mRNA chain which is complementary of double-stranded RNA (dsRNA) entered into cells is prevented the production of protein. RNA silencing is very important for many organisms and microorganisms. This natural phenomenon can be exploited to control agronomically relevant plant diseases, based on the demonstration that *in vitro* feeding of dsRNA can signal Post transcriptional gene silencing (one of the RNA silencing methods) of target genes in various plant pests and pathogens, such as insects, nematodes and fungi. In other words, as well as determining a function of specific gene and developing of new plant various, RNA silencing was also begun to use for developing resistant plant varieties against biotic and abiotic factors by the suppression of gene expression. This biotechnological method, termed host-induced gene silencing (HIGS), has emerged as a promising alternative in plant protection because it combines high selectivity for the target organism with minimal side effects, as compared with chemical treatments. In recent years, the significant developments related to the use of HIGS in management against plant pathogenic fungi (*Puccinia striiformis* f.sp. *tritici*, *Blumeria graminis*, *Fusarium verticillioides* etc.) was obtained. In this review, it is mentioned from the mechanism of HIGS and studies related to the use against plant pathogenic fungi.

Keywords: dsRNA, HIGS, mRNA, PTGS, fungi, pathogen

INTRODUCTION

Every year in the world, about 31-42% of the products produced due to diseases, harms and weeds are destroyed. When total loss is considered to be 36.5%, of which 14.1% is estimated from plant diseases, 10.2% from insects and 12.2% from foreign grasses (Agrios, 2005). Due to various problems such as rapid population growth, climatic changes, decreasing of water resources day by day, the need for food has become unable to supply. For this reason, the loss of crops caused by diseases, pests and weeds should be minimized. Therefore, the struggle is an inevitable. One of the most effective ways to combat plant pathogens is to use resistant varieties. The use of resistant varieties is at the forefront as an economic application.

However, it can take many years to obtain resistant varities with traditional breeding methods. For this reason, some researchers think that modern breeding methods (cloning, characterization, genetic transformation of resistance genes) can be used for such problems (İmriz et al. 2015). It is necessary to include some biotechnological applications such as gene silencing in

breeding programs in order to obtain high quality yields and to train disease resistant plants (Mmeka et al. 2014).

Gene silencing or gene inactivation which is a modern breeding method is occured in all eukaryotes from yeasts to mammals and it is a regulatory mechanism affecting gene expression (Gündoğdu & Çelik, 2009; Baumberger & Baulcombe, 2005). This mechanism is actually a natural process and is used to defend living organisms against foreign nucleic acid molecules (such as virus nucleic acids, transposons) (Gündoğdu & Çelik, 2009). Gene silencing is named differently depending on the species. It is termed co-suppression in plants, RNA interference (RNAi) in animals and quelling in *Neurospora crassa* (Duan et al. 2012). The phenomenon of gene silencing plays a role in cellular defense by protecting a plant or animal cell against the invasion of mobile genetic elements (Aras et al. 2015).

RNAi silencing is a natural event in eukaryotic organisms, and is also used in a variety of biotechnological systems to suppress the expression of endogenous genes by using synthetically produced non-coding RNAs (ncRNAs) which is 21-28 nt in length (Ruiz-Ferrer & Voinnet, 2009). In other words, RNAi silencing, a post transcriptional gene silencing (PTGS) mechanism, occurs in the presence of double-stranded (dsRNA) molecules that are complementary to a gene. In this way expression of the target gene is reduced or completely eliminated due to the messenger-RNA (mRNA) degrade (Armas-Tızapantzı & Montıel-Gonzalez, 2016). After all these developments, the interest of the scientific world has focused on RNAi, examining the functions of genes and determining the functions of genes for which we do not know how to function.

In Figure 1, RNAi mechanism is explained. During the RNAi mechanism, the sequence RNA complementary to the target mRNA binds to the significant sequence of the mRNA on the RISC factor (RNA-Induced Silencing Complex), a nuclease-active RNA multi-protein complex. The gene silencing is controlled by the RISC factor. The mRNA which interacts with the protein named 'Argounate' in the RISC factor is recognized and cleaved by the enzyme 'Dicer' which is a ribonuclease in the RNase -III family and thus the gene silencing occurs. The RNAi mechanism, in other words the gene silencing mechanism, is carried out by two types of molecules in eukaryotic organisms (Aras et al. 2015). Gene silencing is carried out by molecules called small RNA molecules, which are classified in different forms such as short interfering RNAs (siRNA), microRNAs (miRNAs), tRNA-derived RNA fragments (tRFs) and Piwi-interacting smallRNAs (piRNAs). In plants, siRNA and miRNA are the best known and studied species of these molecules (Cristiano & Dean, 2012).

Gene silencing mechanisms are based on the degradation of the target mRNA using siRNAs or shRNAs or the suppression of the translation of a specific mRNA using miRNAs (Keefe, 2013). Although miRNAs and siRNAs are very similar to each other, there are some differences between them.

While miRNA is responsible for regulating endogenous genes, siRNA is responsible for maintaining genomic integrity. miRNA precursor is single stranded RNA (single strand: ssRNAs) in hairpin structure and siRNAs precursor are long dsRNAs (Aras et al. 2015).

More recently, artificial microRNA technology has also emerged to block gene expression in plants (Ossowski et al. 2008). Artificial microRNA and siRNA technology is used to block gene expression in plants. In recent years, the use of vectors producing intron-containing hairpin RNA constructs in RNAi studies has increased in plants (Xu, 2010).

RNAi mechanism was first explored by Napoli and colleagues in 1990 by transferring genes encoding color to petunia. In this study, it was attempted to obtain darker purple petunias by promoting chalcone synthase (*chs*) genes. But the result is not as expected. As a result of silencing of the endogenous genes synthesizing the *chs* gene , petunias, which are either white or somewhat white or purple, are obtained instead of dark purple colored petunias (Napoli et al. 1990). This is called co-suppression or post transcriptional gene silencing (Armas-Tızapantzı & Montıel-Gonzalez, 2016). Fire and Mello (1998) injected dsRNA into the gonads of *Caenorhabditis elegans*, indicating that target genes were silenced.

RNA silencing is important for the investigation of the functions of genes. Suppression of gene expression through silencing of RNA has become important not only in researching gene function, but also in fighting plant diseases (Yin & Hulbert, 2015). In addition to viruses, organisms living in or interacting with the host, such as bacteria, nematodes, insects and parasitic plants, are also sensitive to small RNAs (sRNA) produced by the host and targeting foreign transcripts.

This method, called 'Host Induced Gene Silencing' (HIGS), has begun to be seen as a hope light in combating plant diseases. The genes chosen as targets for silencing are important genes responsible for pathogenic or virulence that are required for plant pathogens to survive. Recent articles published on the use of HIGS to control fungus infections are likely pioneer of more applications (Koch & Kogel, 2014).

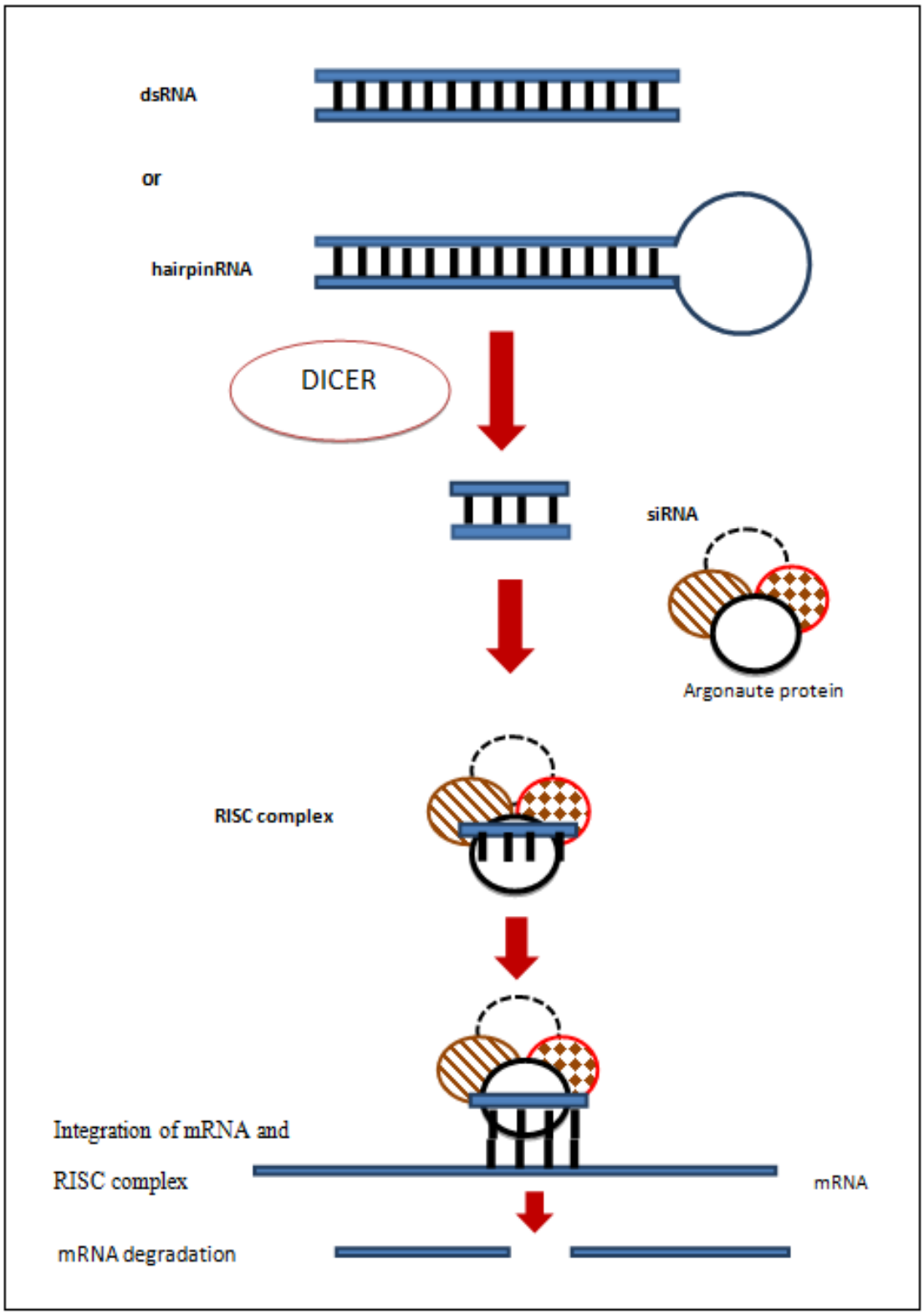

Figure 1. Mechanism of RNAi

RNAi against plant pathogenic fungi is used in two ways: (1) Directly induction of fungal genes by the host plant (HIGS) (2) indirectly induction of fungal genes by phytopathogenic viruses (VIGS) (Armas-Tızapantzı & Montıel-Gonzalez, 2016). We have focused on HIGS in this review.

Mechanism of Host Induced Gene Silencing

HIGS is an improved form of 'Virus Induced Gene Silencing' (VIGS) that allows silencing the genes of plant pathogens. HIGS is obtained by transformation of plant embryos with a vector containing a fragment of the target gene from the pathogen or a dsRNA construct (Starkel, 2011). The structure that is formed after integration with the selected target gene-vector is called vector-target gene, HIGS structure. In Figure 2, HIGS structure in the infected plant cells combines with genomic DNA and it is transformed into dsRNA molecules in consequence of the transcription of the HIGS structure. The generated HIGS structure is transferred to the plant nucleus by different gene transductions such as electroporation or agroinfiltration. Once this structure is integrated with the genomic DNA, the resulting dsRNA structure is transferred to the cytoplasm with the aid of the Exportin-5 protein. HIGS dsRNA molecules are exported from plant cells when fungal infections. Target gene regions of fungal transcripts are silenced in fungal infections. (Cristiano & Dean, 2012). How the dsRNA is processed in host plants and how these constructs are sent to pathogens from plants has not yet been fully determined (Starkel, 2011).

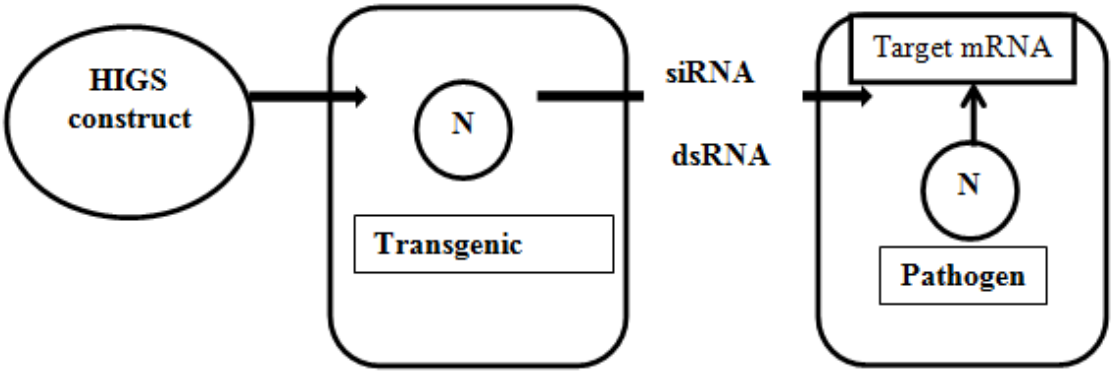

Figure 2. The mechanism of host induced gene silencing.

The Applications Related to Control of Plant Pathogenic Fungal Disease by Host Induced Gene Silencing

The principle of HIGS is based on the silencing of the genes responsible for pathogenic infection and thereby is obtained resistant plants aganist pathogens (Song & Thomma, 2016). In this way, the host directly expresses the dsRNA-forming constructs corresponding to the genes selected as the target gene in the pathogen (Andradeab et al. 2015).

Non-coding RNA (nc-RNA) structures that provide silencing of RNA, such as; small interfering RNAs (siRNA) that pass through from host to filamentous organisms, on the contrary, the repressors which play a role in silencing and pass through from filamentous organisms to the host, microRNAs (miRNA) targeting components of natural defense systems (Baumberger & Baulcombe, 2015).

When the HIGS method is used against fungal pathogens, the fungal morphology changes, the growth inhibition in the plant and most importantly the virulence decrease (Weiberg et al. 2016).

The first findings of HIGS resistance in fungi are resistance to *Fusarium verticilloides* in tobacco and to *Blumeria graminis* in cereals (Tinoco et al. 2010). It has determined that the expression of siRNA and dsRNA in barley and wheat is complementary to mRNA during protein synthesis that are expression of important fungal genes (*GTF1*, *GTF2*, *Avrak1* and *Avra10*) that play a role in hausturium formation in powdery mildews. These results show that HIGS can be used to

control diseases in plants. One of the causes of disease in wheat and other cereals is also *Puccinia striiformis* f. sp. *tritici*. In another study conducted by Yin et al. (2011), *PSTha12J12*, *PSTha5A23*, *PSTha12H2*, *PSTha2A5* and *PSTha5A1* genes which play an important role haustorium formation of *Puccinia striiformis* f. sp. *tritici* have been selected as the target gene. In this study, it was also determined that the level of protein synthesis, which is encoded immediately after infecting the pathogen, also increases in wheat. As a result of this study, it was observed that these five genes identified as target genes were silenced (Yin et al, 2011). Starkel (2011) has shown that the *CTB2* gene responsible for virulence of *Cercospora beticola* which causes significant yield losses in sugar beet, could be used as a target gene in future HIGS studies. In a study by Zhang et al. (2012), HIGS technology have used to determine the function of genes homologous to the two subunits of calcineurin (*PsCNA1* and *PsCNB1*) responsible for the development and infection of *Puccinia striiformis* f. sp. *tritici*. As a result of this study, it has been determined that there is a temporal delay in sporulation and a decrease in hypha length, number, amount of spores and size of uredia (Zhang et al. 2012).

In 2010, Tinoco et al. demonstrated that HIGS could be used in phytopathogenic filamentous fungi by showing that *Fusarium verticillioides* has a structure (*hairpin (hp) GUS*) specifically silenced GUS transcripts in races producing *β-gulukoronidase* (*GUS*) during infection. Later, it has determined that the cytochrome lanesterol 14-α demethylase (*CYP51*) gene which is required for fungal ergasterol biosynthesis, affects negatively the growth and development of mycotoxin-producing *F. graminearum* (*Fg*) by silencing through HIGS (Koch et al. 2013). *CYP51A*, *CYP51B* and *CYP51C* genes are responsible for ergasterol biosynthesis of *Fg*. It has been found that 791 nucleotides-dsRNA (*CYP3RNA*) constructs complementary to these genes inhibit fungal growth and cause significant changes in fungal morphology. Consistent with these findings, expression of *CYP3-RNA* in both *Arabidopsis* and barley has rendered that susceptible plants resist to fungal infections. Microscopic analyses has revealed that mycelium formation is restricted in the inoculation zone of the leaves expressed *CYP3RNA* and that there is almost no fungal hyphae in barley seeds inoculated *Fg*. These results have shown that HIGS can be used as an effective method to silence selected fungal *CYP51* genes as target genes to prevent pathogen. Thus, fungal mycelium formation and plant infections can be prevented. Scientists believe that, with more extensive research in the future, it is necessary to evaluate the potential for RNA to pass instead of azole group fungicides.

n barley and wheat, dsRNAs targeting fungal glucanosyltransferase genes derived from an RNA structure (barley) or BSMV-derived VIGS (wheat) have been analyzed. As a result of the analyzes made, it has determined that the symptom of *B. graminis* in barley and the formation / development of haustoria in wheat decreased (Koch & Kogel, 2014).

It has limited use of pesticide and resistant variety aganist *Sclerotinia sclerotiorum* which is one of the necrotrophic fungi. An alternative to the development of necrotrophic fungal resistance is the use of the HIGS method. In this study, chitin synthase (*chs*), which plays a role in the synthesis of chitin, has determined as the target gene. A structure of hairpin RNA has transferred to tobacco to silence the chs gene. Compared to non-transgenic plants after 72 hours from inoculation, the severity of the disease in transgenic plants was found to decrease between 55.5%- 86.7%. In transgenic plants, the silencing of the fungal *chs* gene correlates positively with the amount of siRNA. With these studies, it has been shown that the expression of the internal genes in *S. sclerotiorum* can be prevented by HIGS and tolerant plants against this pathogen can be produced (Andradeab et al, 2015).

Resistant varieties to *P. infestans*, which causes significant yield losses, is used. However, the effectiveness of these genes is diminished because of the ability to develop new races against pathogen resistance. Jahan and colleagues evaluated the HIGS strategy by identifying siRNAs complementary to the selected target gene in order to reduce the infection severity of *Phytophthora infestans*. Hairpin RNA (hpRNA) was designed using the GFP marker gene. Then this structure was

applied to the potatoes. After 72 hours, the concentration of *P. infestans*-GFP in leaf samples of transgenic plants was reduced by 55-fold, when compared to wild-type potatoes. It is demonstrated that the RNA silencing construct is functional in the pathology and can target pathogen transcripts. G protein β-subunit (*PiFPB1*), cellulose synthetase (*PiCESA2*), pectinesterase (*PiPEC*) and glyceraldehyde 3-phosphate dehydrogenase (*PiGAPDH*) in *P. infestans* are important genes responsible for the infection process (Jahan et al. 2015). Furthermore, β subunit (*PiGPB1*), an important subunit of G protein, is responsible for sporangium formation and pathogenicity (Judelson & Blanco, 2005). The *hp-PiFPB1*, hp-*PiCESA2*, *hp-PiPEC* and *hp-PiGAPDH* constructs complementary to these gene sequences were tested using transgenic methods. At the end of this study, *hp-PiGPB1* largely prevented disease development. The sequence inoculated into the transgenic potato leaves silenced the target gene post transcriptionally. This study showed that the HIGS approach is functional against *P. infestans* but the success of result is highly dependent on the target gene. This finding has shown that HIGS can be used to fight this important plant disease (Jahan et al. 2015).

Govindarajulu et al. (2015) have selected the genes that play an important role in the infection of *Bremia lactucae* as the target gene and formed the transgenic lettuce plants that express the siRNA. It has been determined that transgenic plants expressing *B. lactucae* complementary constructs to the *HAM34* or *CES1* genes inhibit expression of these genes and significantly the sporulation of *B. lactucae* (Govindarajulu et al. 2015). HIGS technology is also used to control the formation of mycotoxins.

Aflatoxin is an important mycotoxin that causes cancer that contaminates products such as peanuts. nc-RNA fragments containing negative copies of aflatoxin-encoding genes in *Aspergillus flavus* (*aflR, aflS, aflep, aflC* / *pksA* / *pksL1, pes1*) were designed and used to silence these genes in peanuts. When compared to the control, it was determined that the levels of *Aflatoxin* B_1 and B_2 in mutant strains decreased by 60-100%.

The silencing of aflatoxin-encoding genes of *Aspergillus flavus* in peanut plants shows that HIGS may be an important pathway for the destruction of mycotoxin (Arias et. al., 2015). The most important disease of tall fescue (*Festuca arundinacea* Schreb.) used as forage and grass plant is brown patch, which is caused by *Rhizoctonia solani*. Zhou et al. (2016) have identified 4 target genes (including genes encoding RNA polymerase, importin beta-1 subunit, Cohesin complex subunit *Psm1*, and a ubiquitin E3 ligase) that play an important role in fungal infection to suppress infection and experimentally designed complementary siRNA constructs to these genes. As a result of inoculation studies, it has been determined that some plants are significantly resistant to *R. solani* and that there is no resistance in plants which is no RNAi accumulation (Zhou et al. 2016).

There are no resistant varieties against *Verticillium* wilt, which is one of the diseases caused by soil-derived fungi, in many plant species. For this reason, struggle with *Verticillium* disease is very difficult. Song and Thomma tried to determine whether they could suppress *Verticillium* wilt by silencing the genes responsible for virulence in tomato and *Arabidopsis* through HIGS. In conclusion, it was determined that HIGS against *V. dahliae* is also functional in tomato and *A. thalliana*, but the achievement changes depending on the selected target gene (Song & Thomma, 2016).

In recent years, a method based on the spraying of dsRNA and sRNA targeting genes responsible for pathogen infection has been studied. This method of struggle, called SIGS, is very important for plant protection because it is environmentally friendly (Wang & Jin, 2017). Wang and colleagues have chosen genes called *Bc-DCL1* and *Bc-DCL2*, which are responsible for pathogenicity and fungal growth, as target genes to prevent *Botrytis cinerea* infection causing gray mold infection in fruit and vegetables, and applied dsRNA molecules complementary to these genes to the fruit surface. With this study, it was determined that these molecules significantly decreased Botrytis cinerea infection up to 8 days (Wang et al. 2016). Thus, an important way for the use of environmentally friendly fungicides RNA has been covered.

CONCLUSIONS

In recent years, HIGS has been emphasized in order to prevent yield losses and to cultivate durable plants. In studies up to now have shown that pathogen infection is partially or completely inhibited when appropriate target genes and nc-RNAs are used. However, no HIGS product has yet been commercialized against phytopathogenic fungi. We believe that the identification of genes that play a role in the virulence and pathogenicity of plant pathogens, the identification of silencing ncRNA constructs complementary to these genes and the increased work on applications where these constructs can be transferred more easily to plants will make these genes applicable in practice.

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