

Post-transcriptional gene silencing and its implication to the asian soybean rust- a review article

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Abstract

Soybean is one of the most economically important crops in the world. Soybean yield is severely affected by biotic agents, especially by Asian Soybean Rust (ASR). The causal agent of ASR is *Phakopsora pachyrhizi*; a biotrophic fungus that belongs to Basidiomycota. Chemical control is the most effective control method for ASR, but it is costly. In the past, many soybean varieties resistant to ASR have been developed by introgression of resistance genes (R genes) to local varieties. Since *P. pachyrhizi* isolates are highly diverse, the resistant varieties are vulnerable to newly evolved pathogen isolates. Until now there is no variety that is resistant to all pathogen isolates. Therefore, there is a need to develop new strategies other than R genes. Identification of new candidate genes that are crucial for fungi in pathogenicity may lead to new options for *P. pachyrhizi* control. Many interesting genes have been identified but their study is impaired by the biotrophic nature of rust fungi. In this regard RNAi has emerged as a reverse genetic tool. The HIGS method (Host Induced Gene Silencing) is used to down regulate fungal candidate genes *in planta*. This is an indirect method where the silencing signal is formed in the plant but it mediates RNAi in the fungus.

Highlight:

- The high diversity of *P. pachyrhizi* strains leads the resistant breakdown of all the existing soybean rust resistant varieties. Therefore, it requires the identification of new fungal candidate genes that can give the new option in the soybean rust management.
- Biotrophic nature of *P. Pachyrhizi* hampered the development of stable transformation system for study of candidate genes in fungi.
- RNAi based Host-Induced Gene Silencing (HIGS) has emerged as a successful reverse genetic tool for study of biotrophic fungi.

Keywords: Post transcriptional gene silencing, RNAi, Asian Soybean Rust, Virus induced gene silencing, Host induced gene silencing (HIGS)

Soybean importance and soybean production

The world population is growing faster than ever, but the land under cultivation and food production is not expanding accordingly. In the near future, there may be the possibility of a food crisis in the world; however, it can already be seen in undeveloped Asian and African countries. In this scenario, global food security can be achieved by adopting newly reclaimed land under food production, improving the crop yield per unit of land under cultivation by using improved varieties,

and adopting proper agronomical management. Other possibilities to overcome the danger to global food security are to reduce the losses caused by biotic agents; pest, diseases and weeds. The biotic agents cause huge losses in the yield globally; in a study it was found that the biotic factors are responsible for 42% reduction of the theoretically achievable global yield (Oerke 1994).

Apart from this, one more challenge in today's world is under nourishment. It was found that 795 million people were suffering from under



nourishment in 2014; 780 million of them lived in developing countries (FAO, 2015). The most prevailing under nourishment is Protein energy Malnutrition (PEM). Therefore, there is a need to grow more protein rich crops which are globally available. Soybean is a very good source of protein and grown worldwide, it can play a significant role towards overcome under nourishment. Soybean (*Glycine max*) is one of the economically important crops for protein and fat. It is grown worldwide, but mainly in the United States (32% of world total), Brazil (31%) and Argentina (18%) (USDA, 2016). It originated from the eastern half of North China in 11th BC (Bromfield, 1984). It belongs to family Leguminosae and is classified as oilseed crop by UN Food and Agriculture Organisation. The raw soybean is rich in protein (36.49 %), fat (19.94 %) and carbohydrates (30.16%) (USDA, 2016).

***Phakopsora pachyrhizi* and its distribution**

Soybean yield is severely affected by biotic factors. In diseases, Asian Soybean Rust causes huge economical losses and is responsible for more than 80% yield loss in favourable environmental conditions (Echeveste da Rosa, Carlos Renato 2015). It caused loss of US\$ 2 billion alone in Brazil in 2003 (Yorinori *et al.* 2005).

The causative agent of Asian Soybean Rust, *Phakopsora pachyrhizi* (Sydow & Sydow), belongs to Kingdom Fungi; Phylum Basidiomycota; Order Uredinales; Class Urediniomycetes; Family Phakopsoraceae; Genus *Phakopsora*; species *pachyrhizi* (Agrios 2005). Rust spores are windborne and can move up to 400 miles in 24 hours. *P. pachyrhizi* originated in Japan and was first reported in 1902 and was then called as *Uredo sojae* (Hennings 1903). Later it was reported in Taiwan where its current name *Phakopsora pachyrhizi* was coined by Hans and Paul Sydow in 1914 while working on *Pachyrhizus erosus* (L.). In the last century it was reported in several other countries for instance, Australia in 1934 (Goellner *et al.* 2010), India in 1970 (Bromfield 1984), Africa in 1975 (Calwell and Laing 2002) and Hawaii in 1994 (Killgore 1994).

In the last six decades there has been a dramatic change in the climate due to global warming. This leads to the invasion of new habitats by exotic bio agents. Due to long distance mobility of humans and goods, it is easier for these bio agents to establish

themselves in a new area where they have not been found before, and cause severe economic loss. For instance, *P. meibomia* that causes soybean rust was indigenous to Latin America and was not a major problem but *P. pachyrhizi* invaded Latin America from Asia and turned out to be a big problem.

P. pachyrhizi entered Latin America in recent early 2000s. It was detected in Paraguay (2001), Brazil (2002) and Argentina (2003) (Freire *et al.* 2008). In the continental USA it was detected in 2004 (Schneider *et al.* 2005). Most of the yield losses occurred in the southern states of the USA due warm and favourable climate conditions. The main reason of severe yield loss in Brazil is the favourable conditions of the rust growth. It spread quickly in the central Brazil region due to its tropical humid climate and the volunteer soybean plants after harvesting grow until the vegetative stage and act as reproduction house of spores which helps in quick spread. Apart from this, inappropriate agronomical factors such as high plant density and sowing time also play a significant role in disease outbreak.

Asian Soybean Rust disease outbreak depends on climate conditions. These conditions are well suitable in tropical and subtropical regions. Spores require temperature range 21 °C – 25 °C to germinate, with long hours of leaf surface wetness and 75%-80% humidity

P. meibomia, another species (Latin American isolate) also causes rust in soybean but is less virulent than *P. pachyrhizi* (Asian–Australian isolate) (Bonde *et al.* 2006). Based on the studies of virulence of above two species it was concluded that *P. meibomia* is not finely adapted to soybean plant (Bromfield 1984). *P. pachyrhizi* has a wide host range and affects 31 species in 17 genera, most of them are leguminous food and forage crops. Since the uredospore is frost sensitive, kudzu (*Pueraria lobata*), a weed, acts as an alternate host to overwinter (Goellner *et al.* 2010). Apart from this, under controlled conditions it can infect 60 additional species in 26 genera when artificially inoculated (Ono *et al.* 1992).

***Phakopsora pachyrhizi* life cycle and rust symptoms**

The life cycle of *P. pachyrhizi* is slightly different from typical rust fungi. It is dikaryotic in most of the life stages. The uredospore is the single source of inoculum. Teliospores and basidiospores have

been observed on several Asian hosts but their germination is still unknown in nature (Bromfield 1984). However, under controlled laboratory conditions teliospore germination to basidiospore has been observed (Saksirirat and Hoppe 1991b). There is not much known about its sexual life cycle and therefore it is unknown if *P. pachyrhizi* is autoecious or heteroecious; spermatia and aceiospores are also unknown for *P. pachyrhizi* (Bromfield 1984).

Uredinia (fruiting bodies) produce uredospores and release them via ostiole in dry conditions. Uredospores are lightweight and travel through air currents and spread to a long distance from the place of their origin. As shown in figure 1, in favorable environmental conditions, at least 6 hours long wet leaf surface and temperature 21 °C – 25 °C, the uredospore germinates with a single germ tube and forms a funnel shaped structure called appressorium (Koch *et al.* 1983). The appressorium attaches to the epidermal cells layer and penetrate across it by forming penetration hypha. The penetration leads to the death of epidermal cell, and forms into primary hypha and reach to inter cellular space. From the primary hypha, secondary hyphae develop by septum and branching, and move along the intercellular space of mesophyll cells. Haustorium mother cells developed from the secondary hyphae in the intercellular space, reach to mesophyll cells, and penetrate by forming a haustorium. The first haustorium occurs after 24-28 hours of inoculation. Hereafter, fungal myceliums colonize all the mesophyll cells and complete its life cycle by developing new uredinia (Koch *et al.* 1983). From primary infection to the production of new uredospores can take up to 9 days.

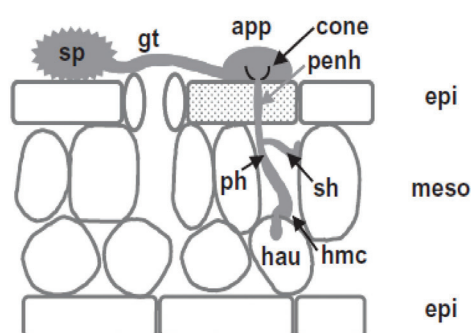


Figure 1 Discription of *P. pachyrhizi* infection: Germinating uredospore (sp), germ tube (gt), appressorium (app), appressorial cone (cone), penetrating hypha (penh), epidermal cells (epi), primary hypha (ph), secondary hyphae (sh), haustorial mother cell (hmc), mesophyll cells (meso), haustorium (hau) (Goellner *et al.* 2010).

The disease symptoms are visible on stems, pods, petiole but mainly on leaves. The first symptom occurs along the major leaf veins and appears in lower canopy. Brown-reddish colored lesions first appear on the lower surface of leaves and then cover the whole leaf surface. The lesion growth usually initiates at the leaflet base near the petiole. Since the leaflet base holds the water for longer, this is highly suitable for uredospore germination. Rust pustules are fruiting bodies that break into numerous uredospores. As the disease progress, number of lesions increases and older lesion turns to black colour and produce teliospore but there germination doesn't occur in nature. When whole leaf turns to dark yellow colour then it leads to defoliation (Rupe 2008).

Control measures for soybean rust

Plant diseases are managed by three basic control measures; Cultural, Chemical and Genetic (Resistance). Cultural methods involve improved agronomical practices according to the ecology of the pathogen for instance, alteration in row spacing, crop rotation, change in seed rate, change in tillage frequency, mulching, change in fertilizer dose and time of fertilizer application. Few of them are useful; early sowing with short duration variety avoids the rust by harvesting before arrival of rust or insignificant effect on yield. Early sowing in such a way that, the reproductive stage coincides with dry period which is not suitable for spore germination. Wider row spacing in lower canopy allows less humidity and slows down the disease incidences (Rupe 2008).

In the current situation, the chemical control is the only best option to control soybean rust. But it is an expensive tactic and requires multiple spray and different chemicals at each time. Apart from this, selection of right chemicals and right time of application is important. There are many chemicals present in the market for soybean rust and most of them can be categorized in three main groups; chloronitriles, strobilurins, and triazoles (Rupe 2008). Chloronitriles and strobilurins are preventive fungicides and must be applied before the occurrence of disease incidence. The mode of action of chloronitriles is multiple site activity. It has local effects and is not absorbed by the plant leaves. (Miles *et al.* 2007).



Strobilurin inhibits the germ tube growth and must be applied before rust infection occurrence. It is absorbed by the leaf tissue and has systemic effect which remains up to two weeks after spray (Rupe 2008). It is not effective in newly developed leaves so frequent spraying is required for effective disease control.

Triazols are sterol inhibitors and interfere with fungal cell membrane synthesis. They are curative fungicides and, have systemic effect. Therefore, they are effective in the presence of fungal infection. Triazols are effective up to 3-4 weeks after spray and also effective in newly developed leaves to some extent.

The type of fungicide used determines the number of sprays required to rust control. Generally, triazoles are considered as relatively long lasting, also required at least two sprays in a season. Depending on the disease pressure the number of sprays can be increased. In some locations of Brazil, five sprays are required to control the rust epidemics caused by early and heavy rust inoculation before the flowering (Rupe 2008).

By and large; the chemical control of disease is an expensive method and not suitable for all farmers around the world, particularly small land holding farmers and farmers with low economic condition in developing and undeveloped world. The other useful method could be the adoption of rust resistance variety.

Disease resistance in plants

A plant has two types of resistance; one is R genes mediated resistance, which is race specific and prone to be overcome by pathogens and second is polygenic, which is not a race specific resistance and relatively long lasting (Gill *et al.* 2015). To promote the disease, a pathogen delivers effector molecules in plant cells. Pathogen associated molecular patterns (PAMPs) are recognized by extracellular pathogen recognition receptors (PRR). PAMPs include bacterial flagelin, xylanases, ergosterol, cold-shock protein, oligogalacturonides, cold-shock protein and lipopolysaccharides. This response is known as PAMPs triggered immunity which involves different types of plant defence mechanism, for instance, production of reactive oxygen species (ROS) and mitogen-activated protein

kinase (MAPK) cascades. There are some effector molecules which avoid the recognition by plant PRR and propagate the infection called effector triggered susceptibility (ETS). In response to ETS plants activate effector triggered immunity (ETI) in which host plant R-proteins interact with pathogen avirulence (Avr) proteins. It leads the local and systemic acquired resistance by synthesis of salicylic acid (SA) (Jones, Dangl 2006).

New approach for disease control in plants

In this scenario, there is a need to find the new tools of resistance different from R-gene mediated resistance. It can be interference with the fungal cell metabolism by targeting the genes responsible for synthesis of substances required for pathogenicity. In this context, several pathogenicity genes have been identified but their study has been impaired by the obligate biotrophic nature of rust fungus. All the obligate biotrophic fungi such as *P. pachyrhizi*, require living host tissue to complete their life cycle. The absence of a stable transformation system for obligate biotrophic fungi has hampered the understanding of their life cycle and pathogenicity. However, few transformation methodologies for these fungi have been developed; for instance, biolistic transformation in *Uromyces fabae* (Djulich *et al.* 2011), transformation in *Erysiphe graminis* by using a gold-partical gun (Chaure *et al.* 2000) and transformation in *Plasmopara halstedii* by electroporation and mechanoperforation method (Hammer *et al.* 2007). But all the methods are not up to the mark, and have many disadvantages and undesirable results such as low transformation efficiency, variable results according to the physiology of fungi, produce multiple copies of foreign genes and high cost (Rivera *et al.* 2014).

In this regard, the concept of RNA-induced gene silencing has emerged as a useful tool for study of candidate genes in host-pathogen interactions. It is based on the RNA interference (RNAi) mechanism which degrades the mRNA copies of candidate genes. The first study ever conducted on the role of antisense oligodeoxynucleotide in gene silencing was on *Rous Sarcoma Virus* (Zamecnik, Stephenson 1978). It gave a new area of research which can be exploited further. Since then, several studies were done in this field which gave rise the development of different tools (Tab 1).

Table 1 Post -Transcriptional Gene Silencing Tools (Langlois 2003)

Tools	Mechanism
Antisense oligodeoxynucleotide (AS-ODN)	Induction of RNase H
Antisense RNA (AS-RNA)	Protein-dependent cleavage
2'-O-methyl phosphorothioate antisense oligodeoxynucleotide (AO)	Inhibition of splicing (Exon skipping)
DNAzymes	Protein-independent catalytic cleavage
External guide sequences (EGS)	RNase-P mediated cleavage
Morpholino antisense (MF-AS)	Inhibition of translation
Micro RNAs (miRNA)	RNAi (inhibition of translation)
Peptide nucleic acids (PNA)	Inhibition of translation
Phosphorothioate oligodeoxynucleotide (PS-ODN)	Induction of RNase H
Ribozyme (RBZ)	Protein-independent catalytic cleavage
Small interfering RNA (siRNA)	RNA interference (RNAi) (cleavage)
Small hairpin RNA (shRNA)	RNAi (cleavage)

RNA-induced gene silencing is called post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNA-interference in animals (Waterhouse, Helliwell 2003). However, the terms RNAi and PTGS are frequently replaced by each other to describe RNA-induced gene silencing.

Mechanism of RNAi

As described in figure 2, the RNAi mechanism is initiated in the cell upon the presence of double stranded RNA (dsRNA) in the cytoplasm. The dsRNA can be introduced exogenously, by a plant virus (produced dsRNA during replication by RNA dependant RNA polymerase) or by self-complementary single-stranded hairpin RNA (hpRNA). The dsRNA is then recognized by the enzyme dicer (an RNase-III-like enzyme) present in the cytoplasm and cleaved into double-stranded short fragments of length 19-25 nucleotides. These short fragments with 2 nucleotide overhang are

called siRNA. Then, siRNA undergo through a siRNA protein complex carrying helicase enzyme, helicase leads the unwinding of siRNA into two strands; guide strand and passenger strand. The guide strand binds to the RNA-induced silencing complex (RISC) while the other strand is degraded. The RISC-guide strand complex binds to the mRNA sequences complementary to the guide strand and promotes cleavage by Argonaute, a component of RISC, and hence degrades the mRNA.

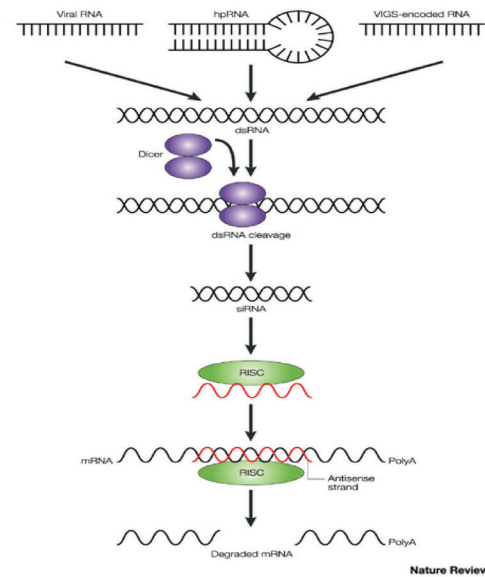


Figure 2: Mechanism of RNAi (Waterhouse, Helliwell 2003)

RNAi was reported many years before the concept was clearly understood. It was reported by Napoli and Jorgensen while working on the chalcone synthase (*CHS*) gene in petunia (*Petunia hybrida*) plant, which they hypothesised as “cosuppression” of *CHS* (Napoli *et al.* 1990). A similar phenomenon was reported in 1992 when homologous RNA sequences were introduced in *Neurospora crassa*, a red bread mold that belongs to Ascomycota, and the effect was called “quelling” of endogenous gene (Romano, Macino 1992). In animals, it was first reported in roundworm (*Caenorhabditis elegans*) by Guo and Kemphues where they observed the *par-1* gene suppression after introduction of antisense RNA to *par-1* gene (Guo, Kemphues 1995).

Based on the previous observations of cosuppression and quelling, in 1998 Fire and Mello conducted an experiment on *Caenorhabditis elegans*, and came up with a clear explanation of the role of single stranded RNA (antisense and sense) and double

stranded RNA in RNAi gene silencing (Fire *et al.* 1998).

RNAi was considered a powerful mechanism not only for functional genomics but also for disease control. In 1993 one such study showed that the tobacco plant transformed with *Tobacco Etch Virus* coat protein sequences, was found resistant to the *Tobacco Etch Virus* infection. However, the typical viral symptoms were visible after inoculation, but the plant recovered to healthy state after 3 to 5 weeks of inoculation (Lindbo *et al.* 1993). This phenomenon led the development of Virus Induced Gene Silencing (VIGS) when target gene sequence is delivered with virus vector.

Mechanism of VIGS

In plants, VIGS was first demonstrated by Monto Kumagai and colleagues in 1995, well before the concept was developed. An endogenous plant phytoene desaturase (PDS) gene, an enzyme involved in carotenoid biosynthesis, was introduced in the cDNA *Tobacco Mosaic Virus* clone; upon infection with recombinant virus, the plant showed photo bleaching symptoms which indicated the PDS gene was silenced (Kumagai *et al.* 1995). The plant cell infected with recombinant virus triggers the RNAi mechanism and degrades the viral mRNA, along with that it also degrades the endogenous gene sequences inserted in the viral RNA. It can further be illustrated in figure 3 by an example of *Barley Stripe Mosaic Virus* (BSMV) (Lee *et al.* 2012).

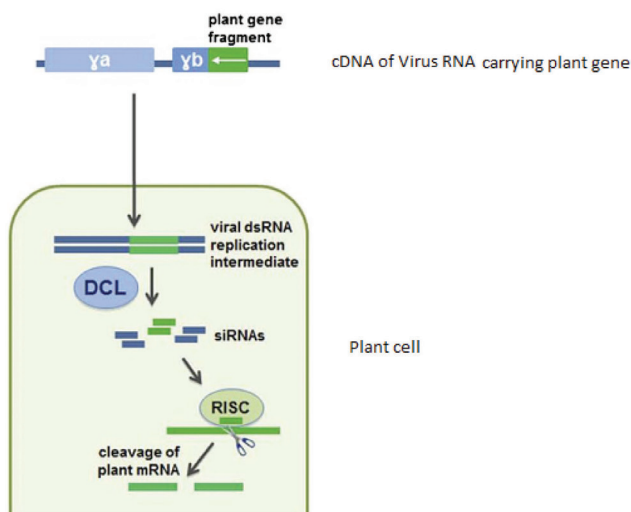


Figure 3 Mechanism of BSMV mediated VIGS (Lee *et al.* 2012)

Apart from the functional genomics, VIGS have one more application; Host Induced Gene Silencing (HIGS).

Mechanism of HIGS

HIGS can be exploited in plant pathology for study genes in fungi, especially obligate biotrophic fungi. In a study it is hypothesised that, in plant-fungus interaction there is an exchange of RNAi signals between plant and fungus via extrahaustrial matrix (EHM) along with nutrient uptake (Nowara *et al.* 2010). This hypothesis was further supported by a study which proved the exchange of mRNA and small non-coding RNA between host and parasite in interactions such as *Cuscuta pentagona* or *Tryphisaria versicolor* (Westwood *et al.* 2009). Therefore, siRNA carrying the fungal target gene can move from plant cell to fungal cell via extrahaustrial matrix and triggers the RNAi in fungal cells. In the *Blumeria graminis*-barley pathogen system, Nowara and colleagues have demonstrated HIGS, where they successfully silenced the Avr10 effector gene in fungus. This study clearly indicates the movement of siRNA from the plant cell to the fungus cell (Nowara *et al.* 2010). However, the mechanism of siRNA movement across the extrahaustorial matrix is still not clear. HIGS is illustrated further in figure 4 by an example of Barley Stripe Mosaic Virus (BSMV) (Lee *et al.* 2012)

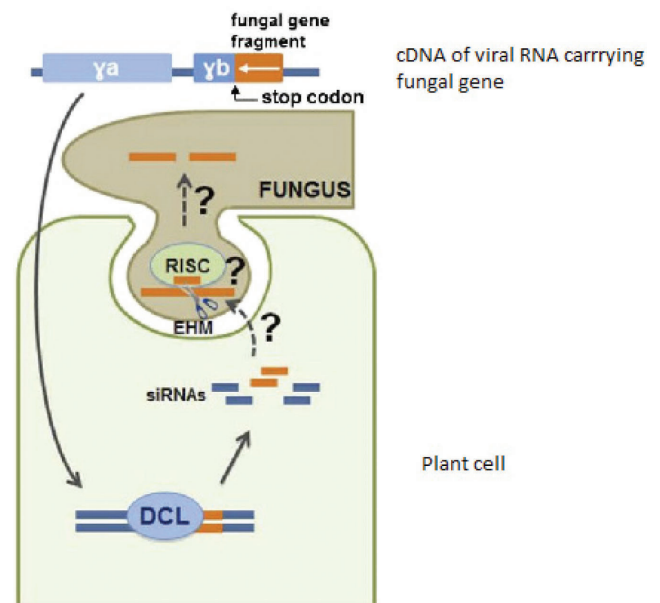


Figure 4 Mechanism of BSMV mediated HIGS (Lee *et al.* 2012)



Applications of RNAi

RANi can be used as a potential measure for disease control. There have been many studies conducted on RNAi in fungi, which indicate its prospect in fundamental study of fungal genome during host pathogen interaction and as a novel strategy to disease control. For instance, Three fungal genes; MAPK, calcineurin and cyclophilin, have been silenced by BSMV mediated HIGS in *Puccinia triticina*-wheat pathosystem. The HIGS plants showed disease suppression, indicating the crucial role of silenced genes in disease development (Panwar *et al.* 2013). Similar results were observed with BSMV mediated gene silencing in *P. striiformis* (Yin *et al.* 2011).

Apart from this, several genes have been functionally analyzed by RNAi in plants as well. For instance, 11 genes were identified conferring the *Rpp2* mediated resistance to Asian Soybean Rust by *Bean Pod Mottle Virus* (BPMV) mediated VIGS (Pandey *et al.* 2011). Similarly five candidate genes were identified conferring *Rpp4* mediated resistance to *P. pachyrhizi*. (Meyer *et al.* 2009). There are many more examples, which show that RNAi is an effective tool for functional genomics. For instance, silencing of putative hydrolase (*ACT72*), a host-selective ACT-toxin in *Alternaria alternata* showed disease suppression (Miyamoto *et al.* 2008); less virulence was observed in *Fusarium graminearum* where transcription factor 6 (*Tri6*) was silenced (McDonald *et al.* 2005), and *Phytophthora infestans* silenced with G-protein β -subunit encoding gene (*Pigpb1*) was unable to sporulate (Latijnhouwers, Govers 2003).

CONCLUSION

Virus induced gene silencing was shown to be a powerful reverse genetic tool. This technique is relatively new, and many fungal candidate genes have been silenced effectively with this technique. But in soybean rust this technique has not been exploited and not so many genes analysed with this tool. This technique is very powerful and can substitute any transformation system of biotrophic fungi. The selection of the candidate gene is critical for analysing the silencing effect. In HIGS, the candidate gene must be relatively highly expressed in haustoria. Since, these are in direct contact with host cells and receive the silencing signals. In a study it was found that genes, which

are constitutively expressed show insignificant gene silencing compared to genes, which are highly expressed in haustoria (Yin *et al.* 2011). The best choices for HIGS are the genes, which are highly and solely expressed in haustoria, and cannot be substituted by the genes in the other cells. The candidate genes selected for this system must be analyzed *in planta*. The expression pattern and specificity of genes should be verified by RT-qPCR. Only genes that are specific to the haustoria and expressed relatively higher should be selected for gene silencing in HIGS system.

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