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Overlap extension PCR to anneal multiple DNA fragments for high-throughput double stranded RNAi vector construction

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ABSTRACT

RNA interference is a sequence dependent gene silencing mechanism with huge potential in agriculture. Virus disease management has become a daunting task due to the lack of availability of resistant cultivars. In this scenario, RNAi mediated viral resistance has acquired importance. RNAi can be employed to silence the viral genes conferring virus resistance. Transformation of the host with an RNAi vector synthesizing hairpin RNA is a prerequisite. It is highly desirable to develop multiple virus resistance due to the fact that, viruses counter the host RNAi by employing silencing suppressors there by leading to resistance breakdown. This can be addressed by targeting all the commonly infecting viruses along with viral slicing suppressors. This increases the complexity of RNAi vector construction as multiple fragments are involved to develop an ihp construct. OE(Overlap-Extension)-PCR is the method of choice due to its several advantages such as no extraneous sequences are incorporated unlike as in restriction based fragment joining and it is also simple and economical compared to other methods. In this experiment, we demonstrate the use of OE-PCR in the construction of an intron containing hairpin RNA synthesizing vector targeting seven genes from four viruses viz. that commonly to tomato and chilli for imparting multiple virus resistance viz. capsicum chlorosis virus, groundnut bud necrosis virus, cucumber mosaic virus and chilli veinal mottle virus. Seven viral gene fragments were joined together in to 1.64kb fragment by OE-PCR. The complete stretch was assembled into the pBI121 and mobilized in to Agrobacterium for transformation.

KEY WORDS: OVERLAP PCR, RNA INTERFERENCE, VIRUS RESISTANCE, AGROBACTERIUM

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) and chilli (*Capsicum annum*) are commercially important vegetable crops grown globally. Virus management in these crops has been challenging in recent years due to intensive cultivation and spurt in the insect vectors population resulting in virus disease outbreak (Sastrosiswojo 1995; Umamaheswaran *et al*., 2003). Viral diseases caused by some major viruses like Tospovirus, Cucumovirus and Potyvirus results in immense economic loss in these crops productivity. Tospoviruses (capsicum chlorosis virus and groundnut bud necrosis virus) are negative single-stranded RNA virus, belongs to *Bunyaviridae* family and containing three genomic segments L, M and S, transmitted by thrips (Haan *et al*., 1990, 1991, Krishna Reddy *et al*., 2008). Cucumovirus (cucumber mosaic virus-CMV) is a positive sense single-stranded RNA virus belonging to family *Bromoviridae* (Palukaitis *et al*., 1992). Potyvirus (ChiVMV-chilli veinal mottle virus), belongs to the family *Potyviridae*, is a single molecule which is linear, positive-sense, single-stranded RNA (ssRNA). Potyvirus and cucumovirus are transmitted by insect vector aphids in a non-persistent manner (Berger *et al*., 2005). Current virus disease management involves prophylactics, roguing and insect vector control. Non availability of the resistance gene pools in most of the cultivated crops poses a serious hurdle in resistant breeding. In this regard RNA interference is highly promising in imparting virus resistance (Simon-Mateo and García 2011, Duan *et al*., 2012; Li *et al*., 2106; Jia *et al*., 2017).

RNA interference is a natural antiviral defense system, degrades the RNA molecules in a sequence-specific manner and it is activated in response to double-stranded RNA formed during viral replication (Voinnet *et al*., 2001). RNAi mediated gene silencing involves, formation of double-stranded RNA, so called “hairpin” RNA (hpRNA), formed by annealing of the reverse complementary sequence at one half of the RNA, which is recognized by and activates plant RNAi machinery leading to the degradation the viral RNA, finally plants recovering from viral infection. The expression of virus-specific dsRNA as hairpin structures renders the plants resistant against virus infection (Bonfim *et al*., 2007). In plants, intron-mediated hairpin RNA (ihpRNA) by incorporating an intron as a spacer sequence between the sense and antisense fragments results in higher gene silencing efficiency in the transgenic plants (Wesley *et al.,* 2001). Moreover, field level virus resistance is achieved by targeting multiple viruses in a single chimeric construct (Bucher *et al*., 2006; Zhang *et al*., 2011; Thu *et al.,* 2016; Hameed *et al.,* 2017).

Therefore, a cost efficient, high-throughput system for designing and making of ihpRNA constructs is in great demand. To achieve broad spectrum resistance, there is a need to target multiple virus genes, to join these fragments the selection of restriction enzymes may not be possible in case multiple fragment splicing due to the limited choice and incompatibility of restriction enzymes and also the introduction of extraneous non-target sequences might interfere with the silencing efficiency. Due to these difficulties, OE-PCR is the method of choice to join multiple fragments.

OE-PCR is a technique used to synthesize artificial genes, joining one or more DNA segments together to form a new genetic combination (Li *et al*., 2008) The OE-PCR was first described by (Higuchi *et al*., 1988). The basic principle of OE-PCR is that individual DNA fragments are designed to have a short stretch of their 3’ ends to be complimentary and these overlaps are extended in PCR to splice the two fragments in to one (Harton *et al.,* 1989), the initial PCR fragments then generate overlap gene fragments that can be used as new templates for another PCR to form a full length fragment (Heckman et al., 2007; Wäneskog and Bjerling 2014; Hussain and Chong 2016).

Targeting multiple viruses is important in aiming for RNAi based robust and durable resistance. In this study, RNAi gene construct against broad spectrum of viruses was designed based on the conserved sequences from four different viruses commonly infesting tomato and chilli *viz.,* Capsicum chlorosis virus-nucleocapsid (CaCV-N), Groundnut bud necrosis virus-Nucleocapsid and Non-structural proteins (GBNV-N and NSs), Cucumber mosaic virus-2b and Coat protein (CMV-2b and CP) and Chilli veinal mottle virus-helper component proteinase and coat protein (ChiVMV HC-Pro and CP). We used OE-PCR to join all seven fragments in a single stretch to construct hpRNAi-MVR binary vector for plant transformation to impart multiple virus resistance in tomato and chilli. This is the first report by using OE-PCR for joining seven gene fragments in ihpRNA construct for plant virus resistance.

MATERIALS AND METHODS

Selection of target gene sequences for dsRNA construct

Based on the literature survey, we have selected major virus diseases affecting tomato and chilli in Southern India, viz. GBNV, CaCV, CMV, ChiVMV diseases have caused a drastic yield loss in India. To develop broad spectrum ihpRNA vector, we have selected the above four viruses, from these 7 crucial genes were selected to express hairpin RNA in transgenic plants. The sequences

of these genes were retrieved from GenBank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). BioEdit-multiple sequence alignment using Clustal W (Hall 1999) was carried out and highly conserved regions were selected for dsRNA design from the alignment of about 100-150 viral sequences reported. The selected conserved regions of CaCV-N gene (255bp), GBNV-N gene (255bp) and GBNV-NSs gene (261bp) from Tospovirus, CMV 2b (232bp) and CMV CP gene (214bp) from Cucumovirus and ChiVMV HC-Pro (209bp) and ChiVMV-CP (221bp) from Potyvirus respectively. The detailed GenBank accession for the selected genes included in the (table 1). Off-target effect minimization was carried out using dscheck and Genome BLAST analysis to select highly conserved regions.

Overlap Primer designing and amplification of desired gene fragments

The clones of viral genes were obtained from the Division of Plant pathology, ICAR-IIHR, Bengaluru. The overlap primers were designed for the individual fragments of fragment S1, S2, S3, S4, S5 and S6 and it generates overhang region of 10-15bp to join the next fragment. No restriction enzymes were incorporated between these fragments. Restriction sites of *Bam*H1/*Sac*1 and *Sex*A1 were added in 5' region of S1 fragment and 3' region of S7 fragment to form ihpRNA construct forming sense: Intron: Antisense orientation. All individual fragments were amplified using PCR. All PCR reactions were carried out in 25µl reaction contains 50ng of template, the PCR mix contains 1x Phusion HF buffer 3% DMSO, 200 µM DNTPs, 0.5 µM PCR primers and 1U Phusion® High-Fidelity DNA Polymerase (NEB, USA). The PCR conditions were as follows: initial denaturation for 30 seconds at 98 °C, followed by 35 cycles of denaturation 10 seconds at 98 °C, 30 seconds at 55 °C annealing, 10 seconds at 72 °C extension and the final extension 30 minutes at 72 °C.

The amplified products were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany), and ligated into PTZ57R/T cloning vector by using T4 DNA ligase (Thermo scientific, USA). The ligated products were transformed in to *E.coli* (DH5α) using heat shock method. The transformed clones were sequenced by Sanger Sequencing method (Eurofins Genomics India Pvt Ltd, Bangalore). The confirmed clones were used further for overlap PCR.

Joining of fragments by Overlap PCR:

Three rounds of overlap PCR were carried out to join all 7 fragments. The PCR parameters for the modified Overlap PCR is as follows: PCR was carried out in a 50µl reaction contains 50ng of purified individual fragments, the PCR mix contains 1x Phusion HF buffer 3% DMSO, 200 µM DNTPs, 0.5 µM PCR primers and 1U Phusion® High-Fidelity DNA Polymerase (NEB, USA) the PCR mix contains no primers. The overlap PCR as follows: initial denaturation for 1 minute at 98 °C followed by extension at 72 °C for 15 minutes, pause/hold the programme at 72 °C or place the tubes in ice, then add the end primers and resume the programme followed by 35 cycles of denaturation for 10 seconds at 98 °C, annealing for 30 seconds at 60 °C, extension for 30s/kb at 72 °C and final extension for 30 minutes at 72 °C. The above PCR conditions were followed to join all fragments.

In First round of overlap PCR, the fragments of S1+S2 were joined by using F1 and R2 primers, S4+S5 fragments were joined by using F4 and R5 primers and the fragments of S6+S7 were joined by using F6 and R7 primers respectively. An aliquot of all post PCR products were confirmed on agarose gel, the confirmed products were purified by NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The **Second round** overlap PCR was carried out by joining the fragments of S1+S2 with S3 using F1 and R3 primers and fragments of S4+S5 with S6+S7 were joined using F4 and R7 primers. An aliquot of post PCR products was used for confirmation of amplification and the rest was used for purification. Likewise, **the final** round PCR was continued by joining the fragments of S1+S2+S3 with S4+S5+S6+S7 using F1 and R7 primers (Fig 1 and 2). The *Bam*H1/*Sac*1 restriction sites were incorporated in the F1 primer and *Sex*A1 restriction site were incorporated in R7 primer, used to form Sense and Antisense fragments. A complete stretch 1.647kb fragment was purified and ligated in to PTZ57R/T cloning vector (Thermo scientific, USA) using T4 DNA ligase (Thermo scientific, USA) and the ligated product was transformed in *E.coli* (DH5α cells). Plasmids were isolated using alkaline lysis method and three clones were sequenced for confirmation of the fragments joined by OE-PCR.

The complete stretch containing all three fragments of 7 genes in sense and antisense orientation interspersed by PDS intron was released using *Bam*H1 and *Sac*I restriction sites, this fragment was purified and ligated into the binary vector pBI121 between CaMV 35S promoter and NOS terminator using *Bam*H1 and *Sac*I sites. The ligation of sense-intron-antisense fragments in pBI121 binary vector were confirmed by restriction digestion using *Bam*HI and *Sac*I. The resulting plasmid was designated as hpRNAi-MVR. The RNAi expression cassette (hpRNAi-MVR) was electroporated into *Agrobacterium tumefaciens* strain EHA105, the electroporated cells were grown on selectable antibiotics kanamycin 100mg l^{-1} and rifampicin 25mg l^{-1} containing

Table 1. List of primers used in this study F1 primers containing Bam H1/Sac1 restriction sites indicated in italics. Overlap regions indicated in bold letters. R7 primer containing SexA1 restriction site indicated in italics.

Primer Name	Primer sequence (5' –> 3')	Segment size in bp
F1	AGTGAggatccAGTGGAAAGTATAAGTTCTGTG	
F1	AGTGAgagctcAGTGGAAAGTATAAGTTCTGTG	
R1	TTGACACCTTCTCCAGAGAAACCAATACATAACATATCC	255
F2	TTCTCTGGAGAAGGTGTCAA	
R2	TGAAGGTCCAGTCATCTGGATTTGGTTATGCATTGTGAA	261
F3	CAGATGACTGGACCTTCAA	
R3	CCGTCGATTCTTCTTGTGAGCCAGAGGGAATATAATTGGT	255
F4	CTCACAAGAAGAATCGACGG	
R4	GCGACAGGACTCTAATCATTACCAGCGAACCAATCCG	232
F5	TTAGAGTCCTGTCGCAACAG	
R5	TGTTGCTCTTCCACCAGCTTCTTATCGAACTCAGTG	214
F6	GGTGGAAGAGCAACAAGCGA	
R6	AAGGTTAACAACAGCCCACGCTTTCCCCATATGA	209
F7	GCTGTTGTTAACCTTGAACACCT	
R7	AGATaccwggtTCCATCATAACCCAATAACC	221

LB media and the clones were confirmed by PCR and Restriction digestion. Glycerol stocks were prepared for the confirmed clones and stored in -80 °C for further use.

RESULTS AND DISCUSSION

PCR amplification of seven individual fragments by using proof reading Phusion DNA Polymerase yielded the expected amplicon sizes as assessed by agarose gel electrophoresis and sequencing. The expected amplicon sizes were CaCV-N gene 255bp, GBNV-NSs gene 261bp, GBNV-N gene 255bp, CMV-2b gene 232bp, CMV-CP gene 214bp, ChiVMV-HC pro gene 209bp and ChiVMV-CP gene 221bp respectively.

Overlap extension PCR was carried in 3 successive rounds of PCR reactions to efficiently complete the joining of all the seven fragments (Fig. 1).

In first round of OE-PCR, the S1+S2, S4+S5 and S6+S7 fragments were successfully joined separately

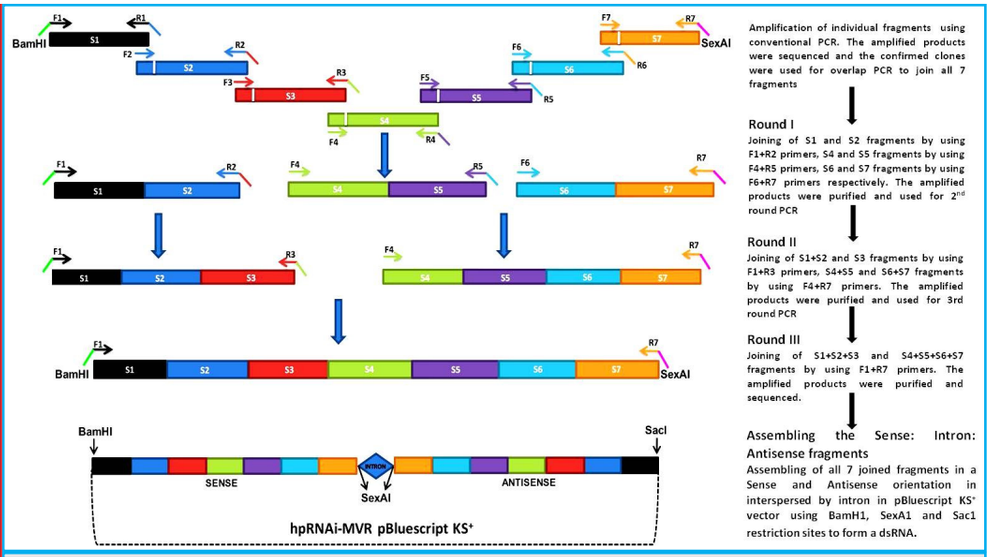

FIGURE 1. Overview of overlap extension PCR method

with the expected size if about 516bp, 446bp and 430bp. The amplified PCR products were purified and these eluted products were subsequently used for next round of OE-PCR. In second round OE-PCR, S1+S2+S3 and S4+S5+S6+S7 were joined successfully and the fragment sizes were 771bp and 876bp. Third round OE-PCR, assembled all seven fragments by combining fragments obtained from the second round OE-PCR. The size of the final fragment was about 1647bp. Sequencing revealed that there were no errors or mutations/ mismatches after at splicing of all the 7 fragments (Fig. 2).

The amplicon represents the results of final round overlap PCR, for sense fragment, *Bam*H1 forward primer

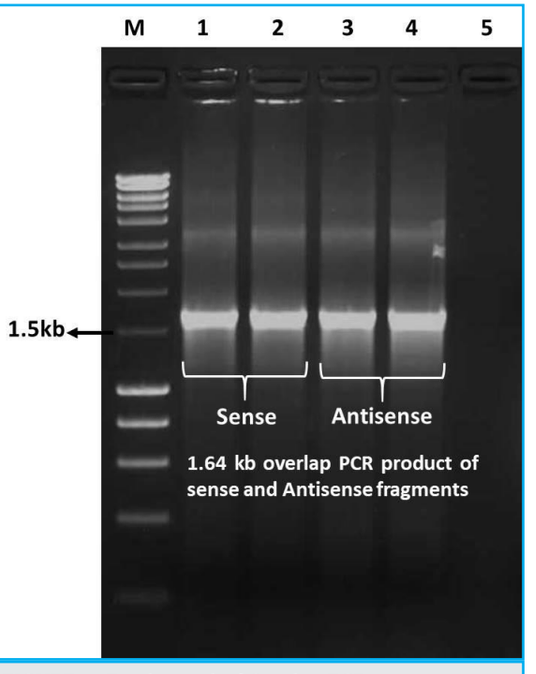

FIGURE 2. Final round of Overlap PCR.

and SexA1 reverse primer (F1+R7) were used and for Antisense fragment *Sac*1 forward primer and SexA1 reverse primer (F1+R7) were used. The gel picture depicts that the expected amplicons of both sense and antisense fragments. M. HyperLadder 1kb; 1, 2. Final overlap PCR product of Sense fragment of about 1.64kb; 3,5. Final overlap PCR product of Antisense fragment of about 1.64kb; 5. No template control (NTC).

To form dsRNA, the complete stretch of 1.647kb fragment in sense and antisense orientation with the intervening 0.25kb of pds intron were ligated and cloned, the plasmids were purified and the orientation of sense, intron and antisense fragments was confirmed by respective restriction digestion and release of fragment of expected size (Fig. 3).

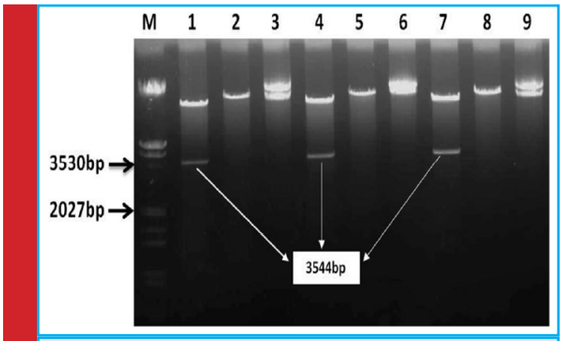

FIGURE 3. Restriction digestion of hpRNAi-MVR binary vector to confirm the stretch of Sense, Antisense and Intron fragments:
M : Lambda DNA/EcoRI+HindIII Marker
Lane 1,4,7 : Digested the hpRNAi-MVR binary vector with BamH1 and Sac1 restriction enzymes to confirm the complete stretch of Sense, Antisense and Intron fragments.
Lane 2,5,8 : Digestion of hpRNAi-MVR binary vector with BamH1
Lane 3,6,9 : hpRNAi-MVR binary vector un digested

DISCUSSION

Biotic stresses like viral diseases pose serious threat to crop production and estimated economic loss ranks second only to the other pathogens (Simon-Mateo and Garcia 2011). Efficient virus diseases control measures are not available and current virus management strategy in most cases are limited to insect vector control and roguing. Development of virus disease resistant lines is highly desirable and RNAi has a direct bearing in the development of genetically engineered virus resistant crops (Chicas and Macino 2001). Imparting virus resistance has been proven effective in various plant species by using hpRNAi constructs (Ammara *et al.,* 2015; Jia *et al.,* 2017; Kumar *et al.,* 2017).

Towards this, in this experiment we have developed a gene construct targeting multiple viruses commonly infecting two important Solancaeous crops, chilli and tomato by employing OE-PCR. Targeting single virus may not be sufficient as most often, the viral infections are mixed under field conditions, to overcome this problem, and also to minimize the resistance breakdown by a mechanism employed by viruses to overcome host induced viral gene silencing, known as "suppression of silencing" targeting multiple gene segments of virus including the respective silencing suppressors could therefore result in more durable and robust virus resistance to broad spectrum of virus infections under field conditions. In this regard, we have selected viral silencing suppressors like NSs gene for Tospovirus (Goswami

et al., 2012; Zhai *et al*., 2014), 2b gene for Cucumovirus (Du *et al*., 2014; Nemes *et al*., 2014) and HC-Pro gene for Potyvirus (Torres-Barcelo *et al*., 2010; Sahana *et al*., 2014) for broad spectrum virus resistance. The viral suppressors plays an important role, it protects the viral genome against RNA silencing and acts as RNA silencing suppressor (Lakatos *et al*., 2006). Hc-Pro gene from potyvirus blocks PTGS at tissue level, CMV 2b from cucumovirus prevents systemic silencing (Brigneti *et al*., 1998). NSs gene from tospovirus regulates the suppression of the viral movement and host defense mechanism (Lokesh *et al.,* 2010). Many researchers have reported resistance to multiple virus infections (Bucher *et al*., 2006; Zhang *et al*., 2011; Thu *et al.,* 2016; Hameed *et al.,* 2017). In this experiment, we have selected seven fragments to target seven viral genes, and this renders the development of gene construct a tedious and complex task as the commonly employed restriction digestion and ligation of fragments gets unwieldy as the number of fragments to be joined is more. Thus, we have employed OE-PCR successfully to accomplish the assembly of hpRNAi construct.

Splicing of two fragments of DNA, referred as overlap extension PCR established by (Yolov *et al.,* 1990), this method has many advantages like joining of many fragments of about 10.8kb using *pfu* proofreading polymerase (Shevchuk *et al.,* 2004). Majority of commercial kits and long PCR protocols are mainly based on Taq polymerase thus cannot be used for overlap extension PCR, because Taq leaves a single A-overhang at 3' region. This leads to disrupt the primer binding at overlap regions during overlap extension PCR (Sambrook *et al.,* 2001). The overlap extension PCR has many applications like, joining of 26 synthetic oligos of 54nt of about 969bp with 5 rounds of sequential overlap extension PCR (Zhang *et al.,* 2013). Many modified methods of OE-PCR have been applied in many genetic engineering areas, such as fusion of long length DNA fragments (Fujii *et al.,* 2013; Engel *et al.,* 2013), assembly of longer gene fragments (Benders *et al.,* 2010; You *et al.,* 2012).

Here, we developed a broad spectrum, multiple virus resistant hpRNAi-MVR vector by targeting 7 genes from 4 common virus groups for both tomato and chilli crops viz., CaCV N gene, PBNV N and NSs genes from Tospovirus, CMV 2b and CP gene from cucumovirus and HC-Pro and Polyprotein from ChiVMV. We have successfully employed the overlap extension PCR method to splice multiple gene fragments without using any restriction sites and adaptors etc. as the addition of non-specific sequences in the gene construct would have affected the RNAi efficacy due to its sequence dependence. The use of OE-PCR is highly desirable in the development of RNAi constructs involving splicing of multiple fragments. Simplified and efficient gene construct assembly for RNAi would aid in the development of virus resistant crops in avoiding crop yield loss.

CONCLUSION

Seamless joining of multiple fragments to create single fragment without using any restriction sites or ligase is a critical step in RNAi. Here, we demonstrated the application of overlap extension PCR to join seven viral gene fragments to develop hpRNAi-MVR construct useful for imparting virus disease resistance in both tomato and chilli. Virus diseases drastically reduce the crop yield and quality and are difficult to manage and only the long term solution to this is to develop virus resistant crop lines. Development of broad spectrum hpRNAi construct for commonly infecting viruses is one of the important aspects. Here, we developed an hpRNAi-MVR vector that is useful in developing multiple virus resistant tomato and chilli employing OE-PCR. Demonstrate that OE-PCR is a method of choice in RNAi gene construct for virus resistance as it is simple, fast and economical, can join multiple fragments in less time. This approach can be employed in developing gene constructs for durable, multiple virus resistance in other important crops.

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