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Genome Engineering for Nematode Management

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ABSTRACT: Nematodes, parasitizing crop plants, are one of the key biotic stressors that cause substantial yield loss to the global food production system. According to a conservative estimate, PPNs cause an annual yield loss of 173 billion US dollars globally in diverse crops. Understanding the basis of nematode-host interaction along with functional genomic studies on plant parasitic nematodes has helped in designing molecular strategies for nematode management. Engineering resistance against plant parasitic nematodes by developing transgenic plants through transferring the R gene, or host-delivered dsRNA molecules targeting crucial nematode genes are seen as academic successes but have not translated into any product that can be cultivated because of the non-availability of permissions from the biosafety regulators of scientific bodies in the country. CRISPR/Cas9 gene editing technology will transform agriculture as newer varieties can be produced at a very fast pace with easy and cheap protocols. Nematode resistance can also be engineered in crop plants using this technology.

Keywords: CRISPR/Cas 9, genome editing, nematode

INTRODUCTION

Nematodes are envisaged as the most plentiful creatures inhabiting the planet Earth. One square meter of the top layers of soil may have up to 100 million nematodes (Peterson and Luxton, 1982). In soil, nematodes may parasitize crop plants or predate on microorganisms (Blaxter and Koutsovoulos, 2015). Plant parasitic nematodes (PPN) feed on plant tissues of all kinds of crops (horticultural, cereal, feed, oilseed, pulse, fibre, spice, ornamental) and forest trees (Schmitt and Sipes, 1998; Williams *et al.,* 2021*).* Most of the PPNs are root feeders and suck cell sap using their stylet, thereby impacting the efficient use of water and nutrients by the crop plants. PPN feeding affects crop yields and threatens the world's food security (Bernard *et al*., 2017*).* More than 4100 species of PPNs have been recorded (Decraemer and Geraert, 2006), which causes an annual loss of about US$173 billion to agriculturally important crops globally (Elling, 2013).

Plant parasitic nematodes have evolved to survive in varied ecosystems existing on Earth. This survivability characteristic makes nematode control a challenging task. It is pragmatic to reduce or manage the nematode population significantly below the economic threshold level. Several approaches like the use of chemicals, crop rotation, resistance and biological control strategies have been implemented to manage PPNs but each of the approaches has its own advantages and disadvantages. Chemicals are very effective and fast in reducing the nematode population but are detrimental to public health and the environment and that is why the use of many chemicals has been banned or restricted. Eating food with pesticide residues can cause serious health illnesses in humans as well as other life forms (Singh *et al.,* 2020). Crop rotation with plants that are not good hosts of the predominant nematode species can reduce the nematode population in the field, but many a times farmer may not opt for such crops in view of their economic viability. Cultivating nematode-resistant crops is one of the best

options of management but there are very few such options available amongst the commercially cultivable crops against the important PPNs. The use of nematode natural antagonists (fungi, bacteria, and other microorganisms) is another environment-friendly option for the control of PPNs but is expensive, slow in reducing the nematode population and many a-times the biological organism may not naturally establish in the soil. In view of the above-stated scenarios, there is a dire need to find new green molecules or use molecular and biotechnological approaches to transfer/engineer resistance in crop plants against PPNs.

R-GENE ENGINEERING

Understanding the nematode-host interaction at the molecular level has helped researchers to develop/design new strategies for their management. DNA recombinant technology allows the movement of genes across species barriers. Experiments conducted to transfer nematode resistance genes across the same or different plant species/genera have yielded mixed results. The root-knot nematode resistance gene of tomato (*Solanum lycopersicum*), Mi-1.2 (Milligan *et al*., 1998), also exhibits resistance against two insects, (i) the potato aphid (*Macrosiphum euphorbiae*, Rossi *et al*., 1998), and (ii) whitefly (*Bemisia tabaci*, Nombella *et al*., 2003). The gene arbitrates a hypersensitive reaction at the initial stage of giant cell formation. Transgenic aubergine (*S. melongena*) plants having the *Mi-1.2* gene showed resistance against *Meloidogyne javanica,* but were susceptible to *Macrosiphum euphorbiae*; whereas susceptible transgenic tomato lines of the same gene exhibited resistance against insects and root-knot nematode (Goggin *et al*., 2006), suggesting that *Mi-1.2* could only confer nematode resistance in plants belonging to the Solanaceae family but the potato cyst nematode (PCN) resistance gene of tomato, *Hero A* (Earnst *et al*., 2002), when transferred to potato didn't show any resistance to *Globodera rostochiensis* and *G. pallida* (Sobcjak *et al*., 2005). This could mean that possibly some more genetic elements are needed for the complete expression of the R gene in related species. Apart from transferring R genes across genera or species, for generating resistance against PPNs, nematode toxin-producing genes can also be used for engineering resistance in host plants. Insect pest-resistant cotton, eggplant, soybean, and sweet corn having Cry protein genes of *Bacillus thuringiensis,* are being successfully cultivated in several countries but no such product has been released for nematode management. Few Bt proteins *viz*., Cry14, Cry5B, Cry6A against root-knot nematodes (Li *et al*., 2007; Li *et al*., 2008; Ravari and Moghaddam, 2015), Cry14Ab against the soybean cyst nematode (Kahn *et al.,* 2021) and Cry31Aa against the rice white tip nematode (Liang *et al.,* 2022) have been found to provide resistance.

RNAi-BASED RESISTANCE ENGINEERING

Another technology-driven approach that has proved effective in reducing nematode populations is RNA interference (RNAi). It is a gene-silencing technology that is sequence-specific and homology-dependent. Host-delivered RNAi has been put to use for resistance generation against the PPNs. A dsRNA construct for the specific nematode target gene is developed by cloning a part of the gene cDNA in sense and antisense orientation separated by an intron or spacer region and transferred into the host plant. Self-complimentary hairpin structures are formed on transcription of the sense and antisense strands (Smith *et al.*, 2000). PPNs can directly ingest these dsRNA molecules or siRNA molecules while feeding on the transformed plants (Bakhetia *et al.,* 2005; Dutta *et al.,* 2015a). Several important genes having role in nematode parasitism and development have been targeted for resistance engineering against root-knot nematode, *Meloidogyne incognita* in our and NIPB laboratories (Dutta *et al.,* 2015b; Kumar *et al.,* 2017; Banerjee *et al.,* 2017a, Banerjee *et al.,* 2017b;

Banerjee *et al.,* 2018; Kohli *et al.,* 2018; Joshi *et al.,* 2019; Joshi *et al.,* 2020; Koulagi *et al.,* 2020; Kumar *et al.,* 2022). The use of root-specific or feeding tissue-specific promoters instead of constitutive promoters can limit the production of the dsRNA in the host roots or the galls. Patents for (i) nematode-induced root-specific (Jain *et al*., 2024) and (ii) nematode-induced gall-specific (Jain *et al*., 2023) promoters have been awarded to ICAR-National Institute for Biotechnology along with our lab at ICAR-IARI, New Delhi. Various levels of reduction in the root-knot nematode population have been observed in the above-cited works. In spite of no protein formation of the incorporated transgene in the RNAi-based resistance engineering approach, very few RNAi GM crops have been commercially released.

A new technology of exogenous dsRNA application for pest management has been developed by scientists which doesn't fall under the ambit of GMO regulations and may have a bright future. It is also termed as spray-induced gene silencing (SIGS). The SIGS has been demonstrated for virus management (Mitter *et al.,* 2017a; Mitter *et al.,* 2017b); insect management (San Miguel and Scott, 2016; Jain *et al.,* 2019; Worrall *et al.,* 2019); and fungal pathogens (Koch *et al.,* 2016; McLoughlin *et al.,* 2018; Hofle *et al.,* 2020). However, no reference is available for SIGS for managing plant parasitic nematodes. SIGS, in principle, is suitable for sustainable agriculture as it has proven very effective in pest management and also has a minimal environmental footprint.

GENOME EDITING TECHNOLOGY

Genome editing technology has tremendous potential for use in plant and animal sciences. Its use in agriculture can bring a paradigm shift by developing crops that are highly productive, pest and pathogen-resistant, drought and heat/cold-tolerant, nutritionally rich and with improved commercial properties (shelf life, seedless fruits, colour, appearance, flavour *etc*.). Great impetus is being observed in the scientific community of the country in the area of genome editing research after the Ministry of Environment, Forest and Climate Change order vide OM F. No. C-12013/3/2020-CS-III, dated March 30, 2022. The order exempted the genome-edited plants in the categories of Site-Directed Nuclease 1 and Site-Directed Nuclease 2, which are free of exogenously introduced DNA, from biosafety assessment. Genome editing can be processed by using tools like the CRISPR-Cas system, TALENs, and Zinc finger nucleases.

The Clustered Regularly Interspaced Short Palindromic Repeats/crisper-associated protein 9 is the full form of the acronym CRISPR/Cas9. It is the most promising and used genome editing technology in the present time. Accurate gene mutations are created by initiating site-specific double-stranded DNA breaks (DSBs). CRISPR was brought to light and characterized by Ishino *et al.* in the year 1987, but the term was devised by Jansen *et al.* in 2002. CRISPR-Cas9-based genome editing is the simplest, most versatile and precise method of genetic manipulation which banks on the increasingly available pangenomes and whole-genome DNA sequences of the crop plants. Genome editing can accelerate the release of varieties superior to the elite varieties because mutations can be directly introduced in the elite or commercial varieties (Lowe *et al.,* 2016; Debernardi *et al.,* 2020), eliminating the need for repeated backcrossing, thereby reducing nearly two-thirds of the time taken for developing a better variety.

A short noncoding guide RNA (gRNA) and a nuclease, CRISPR-associated protein 9 (Cas9) constitute the CRISPR-Cas9 system. A target-specific CRISPR RNA (crRNA) and an auxiliary trans-activating crRNA (tracrRNA) are the two constituents of the gRNA. The 5¹crRNA complementary base pair component helps the gRNA to identify the target sequence of the gene of interest and it binds with the target DNA by way of the

protospacer adjacent motif (PAM). The Cas9 protein has two lobes, the recognition (REC) lobe and the nuclease (NUC) lobe and requires sgRNA to function. As the name suggests, the REC lobe, having two domains REC1 and REC2, is responsible for binding guide RNA, and the NUC lobe is responsible for nuclease activity because of the interactive domains of RuvC, HNH, and PAM. When the Cas9 protein is added to a cell, it fastens up with the gRNA and then glides along the strands of the target DNA till it locates and attaches to the 20-base pair-long sequence of the target gene that matches part of the gRNA sequence (spacer sequence). Then a double-stranded break (DSB) at a site 3 base pair upstream to PAM is made by the Cas9. The complementary strand is cleaved by the HNH domain of Cas9, and the non-complementary strand of target DNA is cut by the RuvC domain to produce predominantly blunt-ended DSBs. Two cellular repair mechanisms, the Non-homologous end joining (NHEJ), and homology-directed repair (HDR) play an active role in mending the DSBs. NHEJ cellular repair mechanism facilitates the joining of DSBs by an enzymatic process and is active in all the phases of the cell cycle but is error-prone. It may yield small arbitrary insertion or deletion (indels) at the cleaved site, thereby creating premature stop codon or, a frameshift mutation leading to the loss of gene function. HDR, on the other hand, is mostly active in the late S and G2 phases of the cell cycle but is very specific as it uses the homologous DNA template for repair (Liu *et al.,* 2018; Yang *et al.,* 2020). CRISPR/Cas9 technique has been widely accepted because of its high degree of flexibility and specificity in cutting and pasting DNA on a large scale at a very low cost.

NEMATODE MANAGEMENT BY GENOME EDITING

Plant parasitic nematodes have very close interrelation with their host plants and manoeuvre the host metabolic apparatus to their self-advantage. Nematode host parasitism studies indicate up-regulation of genes that promote and sustain susceptibility (S genes) and down-regulation of genes that are associated with host defence mechanisms. Mutation or functional loss of the S gene can limit/reduce the ability of the nematode to parasitize on the host plant. Knocking out the susceptibility (S) genes or genes that promote nematode parasitism, with CRISPR/Cas9 technology, in crop plants looks to be a feasible approach to PPN management. Such induced mutations in the S genes, tend to be long-lasting and will impact their expression in the host system and hence may provide resistance of a certain degree against the feeding nematode.

In our lab, we used the genome editing tool CRISPR/Cas9 to modify two host susceptibility genes, WRKY45 and KRP6, associated with the formation of *Meloidogyne incognita* feeding-site complex in *Arabidopsis* plant. Nematode bioassays demonstrated a significant reduction in gall formation and the development of adult females in the mutated lines compared to wild-type plants. The KRP6.1 and WRKY45.1 edited *Arabidopsis* lines exhibited a reduction in the number of galls by 62.14 and 46.16 per cent, respectively. The decrease in the adult female development was 60.39 per cent in the KRP6.1, and 53.33 per cent in the WRKY45.1 transformed lines (Neeraj, 2023). Several genes conferring or supporting nematode susceptibility in host plants have been identified and are well documented in a review by Dutta *et al.* (2023). These genes facilitate (i) nematode penetration, (ii) induction, (iii) maintenance of the feeding sites for sustained food supply, and (iv) negative regulators of immune signalling. All such genes can be potential targets for genome editing; however, each such gene may yield a variable degree of engineered resistance against PPN. As of date, no single specific S gene which controls complete nematode parasitism in any host crop against any plant parasitic nematode has been reported in the literature. Hence, expecting absolute resistance against a plant nematode by using this technology will be

a far-fledged dream. Published research in nematology suggests improved nematode resistance in the CRISPR/Cas9 edited plants.

CRISPR/Cas9 system is a very powerful tool of genome editing having immense potential for applications in agriculture, veterinary and medicine. In spite of it being a very safe and easy-to-use technology, some concerns regarding efficiency, specificity, delivery and stability may still need to be improved further, the genome editing components included. Several ways to ameliorate the specificity of the CRISPR/Cas9 system have been proposed by many researchers. Computational algorithms may help envisage the specific designing of the gRNA sequences and also address the off-target risks involved in the technology. An in-depth understanding of the plant-nematode interactions at the gene level shall lead to wider and more effective use of this technology for the management of the plant parasitic nematodes.

CONCLUSION

CRISPR/Cas9 technology being easy to use, cheap, specific and effective, has a very promising future. Editing the target cells' genomes of the commercially viable crop varieties and to further improving them, makes it a unique strategy for crop improvement. The technology can be applied to enhance crop yield, improve nutrition, resist biotic or abiotic stresses, *etc*. It can credibly be envisioned that CRISPR/Cas-based genome editing will come into being as an indispensable breeding technology for crop improvement. The technology is still in its infancy and the real potential shall be visible in the forthcoming years as delivery systems improve and become error-free. The possibilities of using CRISPR/Cas9 system are innumerable and will depend on the available knowledge and the imagination of the researchers but ethical, legal, biosafety and social issues should always be kept in mind. CRISPR/Cas9 technology can revolutionize agricultural research and has the potential to solve the food and nutritional security of the ever-growing human and livestock population on our planet.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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