Project Title: Improve soybean resistance to nematodes

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Abstract

As the most destructive pest disease for soybean, The soybean cyst nematode (SCN) costs huge losses every year. This project is expected to generate a SCN resistant soybean variety using the artificial miRNA technology, which is the next generation RNAi technology. We have generated a soybean variety expressing an artifical miRNA construct. In collaboration with Dr. Loren Giesler, We evaluated the SCN resistance of this variety. This soybean soybean variety displayed improved SCN resistance. However, in subsequent generation, the SCN resistance was reduced due to transgene silencing. To solve this problem, we have designed new generation synthetic vector that expresses four siRNAs targeting four different genes of SCN. This synthetic strategy is expected to provide more strong resistance and reduces the gene silencing encountered for transgenes. The vector has been transformed in Dr. Tom Clemente lab. Once the transgenic plants are obtained, we will evaluate their SCN resistance and determine if this strategy is effective to improve soybean SCN resistance.

In addition, we also identified the miRNAs that are responsive to SCN infection, which has the potential to identify important regulator controlling soybean's responses to SCN infection and lead to a better understanding of mechanisms used by soybean to fight against SCN.

Introduction

The soybean cyst nematode (SCN), *Heterodera glycines,* is one of the most destructive pests affecting soybeans in the world. Annual yield losses in soybeans due to SCN have been estimated at about $1.5 billion in the U.S. alone (Koenning and Wrather, 2010). SCN is now becoming an increasing problem in Nebraska because at least 18 counties in eastern Nebraska have established SCN. SCN management heavily depends on the resistant soybean varieties. In order to retain the usefulness of SCN resistant varieties over the long term, it is essential to alternate planting resistant varieties that represent different sources of SCN resistance. However, the number of available varieties of SCN resistance is limited, which has caused the adaption of SCN to existing SCN resistant varieties (Davis and Tylka, 2000; Klink and Matthews, 2009). This proposed research targets these gaps and has the potential to benefit soybean production in Nebraska as well as other states.

An emerging strategy to engineer resistance is RNA interference (RNAi), which involves a process triggered by ~21-24 nucleotide small RNAs to specifically repress gene expression (RNA silencing) (Li et al., 2016). SCN is able to uptake small RNAs generated from soybean and therefore the plant-originated small RNAs are able to silence the SCN genes using SCN RNA silencing machinery (Govindarajulu et al., 2015). This founding provides the opportunity to improve soybean resistance to SCN by generating small RNAs targeting essential genes for SCN viability in plants (Govindarajulu et al., 2015). In fact several research has been performed to improve soybean resistance to SCN by overexpressing long-dsRNA. However, the long-dsRNA generates a population of small

RNAs, which often silence non-target gene (called off-target effect) and may cause undesirable effect on soybean growth (Govindarajulu et al., 2015). The short-hairpin RNA (shRNA) technology is an improved technology, as it only produces a single small RNA limiting the off-target effect and reduces the negative effect on soybean.

Results

Generation of transgenic soybean plants expressing an amiR1 targeting a ribosomal gene of SCN.

The amiR1 targeting a ribosomal gene (hg-rps-23) of SCN was designed according to (Schwab et al., 2006). Hg-rps-23 is an essential gene for SCN. Repression of this gene is expected to limit the development of SCN. The result DNA fragment was inserted into pPTN200 such that the expression of amiR1 was driven by the consititutive 35S promoter (Figure 1A). This vector was transformed into the soybean variety Throne. After transgenic plants were obtained, the expression of amiR1 in T0 plants was confirmed by northern blot (Figure 1B).

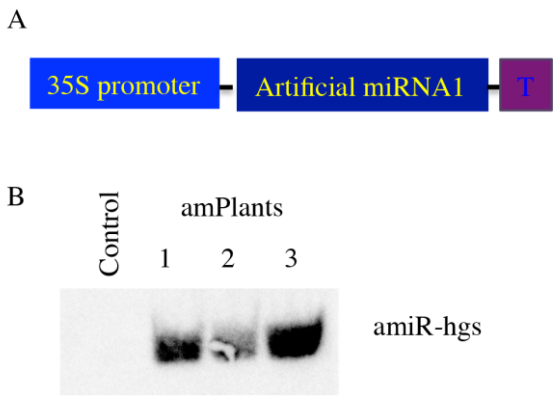

Figure 1. (A) diagram of amiRNA vector. (B) Detection of amiR1 in transgenic soybean plants by Northern blot

Transgenic soybean plants expressing amiR1 displayed improved resistance to SCN infection in T1 plants.

T1 seeds harvested from To plants was then planted for SCN resistance assay. One month after SCN challenge (1000 eggs per plant), Visual inspection of the roots at the completion of the bioassays revealed differences between negative controls and transgenic roots. As expected the susceptible variety of soybean Williams has average 140 nematodes per plant while the resistant variety PI8778, PI437654 and Peiking have less than 20 nematodes per plants (Figure 2). The non-

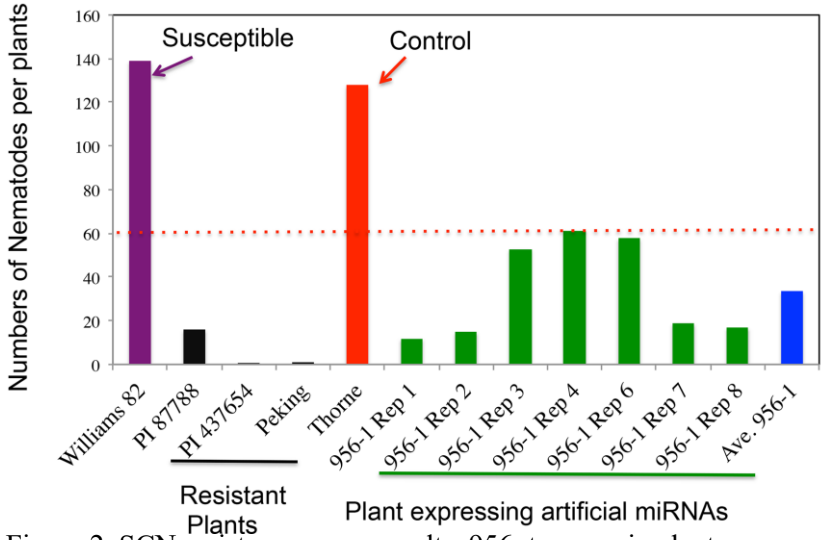

Figure 2. SCN resistance assay results. 956: transgenic plants

transgenic control plants have ~ 130 nematodes per plants (Figure 2). In contrast, among seven tested transgenic plants expressing amiR1, four contained less than twenty SCNs (similar to the resistant variety PI8778) (Figure 2). The other three has SCNs ~ 50 in the root (Figure 2). These results suggested that the reduction of ~60% to 80% SCN compared with the control plants, suggesting that artificial miRNA could be a valuable approach to help against SCN.

Transgenic soybean plants expressing amiR1 displayed improved resistance to SCN infection in T2 plants.

Next, we used T2 plants (next generation) to evaluate the sustainability of SCN resistance. One month after SCN challenge (1000 eggs per plants), we visual examined the amount of SCN in PI8778 (resistant), Williams (susceptible), Throne (control) and two transgenic lines. SCN amount in PI8778 and Williams are 15 and 112 respectively (Figure 3). The number of SCN in throne are ~110 per plants. In contrast, the numbers of SCN in two transgenic lines are ~90, represent ~19% reduction (Figure 3). However, compared with T1, the SCN resistance was reduced. Consistent with this observation, the levels of amiR1 seemed to be reduced. We suspected that the expression of amiR1 may be silenced due to transgene silencing.

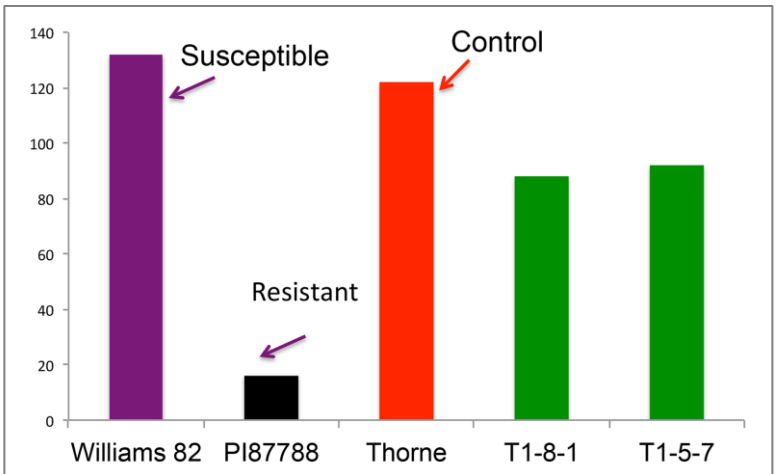

Figure 3. SCN resistance of T2 (T1-8-1; T1-5-7) plants. Number indicates average of 15 plants.

Development of synthetic RNAi technology.

Due to transgene silencing, the SCN-resistance is greatly reduced in the offspring. Thus, to apply the amiRNA to improve SCN resistance, we need to overcome transgene silencing. The silencing of RNA Polymerase III (pol III) promoter is rarely reported. Thus, Pol III has the potential to eliminate the effect of transgene silencing. In addition, Pol III

Figure 4. Diagram of synthetic RNAi vector. Pro: pol III promoters. T: Terminator

can drive the expression of short RNAs that form a short hairpin (shRNA) to produce a singe small RNA, which is equitant to amiRNAs. This technology is less explored in plants. We have isolated four different Pol III promoters, which enable us to produce a synthetic RNAi vector expressing four different shRNAs at the same time (Figure 4). We designed four shRNAs, which target Chorismate mutase that is a critical enzyme for amino acid biogenesis, a ATP synthase subunit, a cysteine protease and a ATP citrate lyase. All these genes have been identified as essential genes for SCN survive. Killing these genes in

SCN will limit the growth of SCN. The resulted shRNAs together with their promoters have been cloned into pPTN200 (Figure 4). The soybean transformation is ongoing in Dr. Clemente's Laboratory.

Identification of miRNAs that potentially regulate soybean resistance in soybean. miRNAs have emerged as an mechanism used by plants to cope with various biotic stress. Plants often up- or down-express certain miRNAs when facing biotic stresses Thus, in order to identify miRNAs that are potentially regulate SCN resistance, we analyze the alteration of miRNA expression in responses to SCN infection through illumina deep sequencing. Two biological replicaes were performed. After removing adaptor sequences, filtering out low quality reads and cleaning up sequences derived from adaptor–adaptor ligation, we mapped the sRNA sequences to known miRNA sequences and compared the abundance of miRNAs in SCN infected plants with that in control plants. We were able to identify 20 miRNAs with elevated expression (more than 1.8 fold) and 6 miRNAs with reduced expression (Table 1).

Up-regulated miRNAs	Fold (Rep1)	Fold (Rep2)	Down-regulated miRNAs	Fold (Rep1)	Fold (Rep2)
miR1511	1.8	2	miR1507	0.4	0.45
miR1531	2	2.3	miR1520c	0.38	0.45
miR169o/r	7.6	7	miR156z	0.65	0.5
miR319a	2.1	1.9	miR162	0.4	0.36
miR319b	2.2	1.9	miR164	0.36	0.32
miR319e	2.2	1.8	miR166e	0.5	0.6
miR319h	2.1	1.8			
miR319j	2.2	1.8			
miR319l	1.7	1.7			
miR319m	2.2	1.9			
miR399	1.8	1.8			
miR4360	3.6	4.6			
miR4397	2.8	2.5			
miR4997	1.6	1.8			
miR5037a	17	12			
miR5037b	5	4			
miR5037c	14	9			
miR5044	8	20			
miR862a	13	22			
miR862b	8	22			

Table 1. miRNAs with altered expression after SCN infection. To calculate Fold change, normalized readings in SCN infected root were divided by those in control plants.

Conclusion.
In summary, we have shown that amiRNA is an effective strategy to against SCN. However, transgene silencing may limit its application. We are in the process to develop a technology to overcome this obstacle. In addition, we identified miRNAs that showed

altered expression in response to SCN infection. These miRNAs may have potential roles in regulate soybean responses to SCN infection.

References

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