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The Effect of Plant Elicitor Peptide on Nematode Infected Soybean

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The Effect of Plant Elicitor Peptide on Nematode Infected Soybean

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Abstract

Soybean (*Glycine max*), an important economic crop native to East Asia, is grown worldwide for its edible beans. Soybean plants are vulnerable to a wide range of diseases, and nematodes are among the worst pests of soybean. The southern root-knot nematode (*Meloidogyne incognita*) and soybean cyst nematode (*Heterodera glycines*) are both notable nematode pests of soybean, with the soybean cyst nematode causing more than 30% of total yield loss in the US. Numerous attempts have been made to control soybean nematodes over the past several decades, and genetic engineering technology has received a lot of attention in recent years. Plant elicitor peptides (Peps) are endogenous polypeptides consisting of amino acids that can trigger innate immune defense in plants and are present in various plant species, including soybean. *Gm*Pep3 are plant elicitor peptides present in soybean and the gene *GmPROPEP3* regulates the synthesis of *Gm*Pep3 in soybean. In this study, two independent nematode bioassays were conducted to investigate whether *Gm*Pep3 treatment and the insertion of *GmPROPEP3* in susceptible soybean cultivar Magellan could enhance soybean defense towards the southern root-knot nematode and the soybean cyst nematode, and whether different treatments on soybean have any effect on soybean growth. According to the results, the *Gm*Pep3 treatment and the insertion of *GmPROPEP3* in Magellan have no negative effect on soybean growth. However, whether the treatments have any positive effects on soybean growth needs further investigation. The root-knot nematode bioassay results suggested that there were no significant differences in egg number between treatment groups, while the soybean cyst nematode result indicated that both transgenic lines were resistant to soybean cyst nematodes. However, the data presented in this study is insufficient, and future experiments are necessary in order to support the current conclusion.

Acknowledgments

First, I would like to thank Dr. Fiona Goggin for giving me an opportunity to join her lab and study in an area that I knew almost nothing about. I would like to thank my committee members Dr. Ashley Dowling, Dr. Ken Korth, and Dr. John Rupe for their help with my thesis and defense. I'm grateful to all the lab members that have worked with me, especially to Dr. Jiamei Li, who taught me and helped me a lot with my research. I would also like to thank Mr. Devany Crippen for his assistance with my nematode experiments. Special thanks to my best friend Natalie Hambuchen for her support during the hard times. Thanks to all my other friends who have listened to me and cheered me up when I ran into difficulties. Finally, thanks to my parents who have supported me to study what I want to study.

Dedication

I dedicate this thesis to my best friend Natalie Hambuchen from Conway Arkansas. We have known each other for eight years since 2014 when I was an exchange student in Conway. Natalie has helped me so many times over the years, that without her, I would not be here today.

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I. Introduction

Nematodes can be agricultural pests that cause tremendous yield loss of crops worldwide. Plant-parasitic nematodes, for example, southern root-knot nematode (*Meloidogyne incognita*) and soybean cyst nematode (*Heterodera glycines*), can be devastating agricultural pests. The estimated yield loss caused by nematodes is 12.3% ($157 billion dollars) worldwide (Singh *et al*., 2015). Plant-parasitic nematodes infect a variety of crops, for example, soybean (*Glycine max*), one of the most important economic crops globally. Southern root-knot nematodes and soybean cyst nematodes are the most important nematode pests on soybeans, and people have been working for years to control them. Plant elicitor peptides are plant signaling molecules endogenous in higher plants. Recent studies have discovered that plant elicitor peptides can regulate pathogen resistance responses, which have the potential to be used to control plant parasitic nematodes in the future (Yamaguchi & Huffaker, 2011). This thesis focuses on the application of plant elicitor peptides forthe control of soybean cyst nematode and root knot nematode on soybean.

Nematodes

The nematodes, also known as roundworms, belong to phylum Nematoda. The word nematode originates from Latin, nemat- means “thread”, and -odes means “the nature of”. Nematodes are typically 5-100 μm thick, and 0.1 to 2.5 mm long. Free-living nematodes can reach up to 5 cm, while the size of parasitic nematodes varies. The largest parasitic species can reach over 1 m, and the smallest species can only be seen through a microscope (Ruppert et al., 2004). Nematodes live in a wide range of environments, and they have successfully adapted to nearly every ecosystem. They are very common in freshwater, marine and terrestrial environments, and often outnumber other organisms in both species diversity and abundance.

Around 30,000 species have been described, however, the estimated extant nematode diversity is more than one million (Blaxter, 2016).

Plant-parasitic Nematodes

Nematodes can parasitize both animals and plants, and some parasitic nematodes can have a huge economic impact worldwide. Over 4100 plant-parasitic nematode species have been described (Decraemer and Hunt, 2006), and damage caused by plant nematodes is estimated at US$80 billion per year (Nicol *et al*., 2011). However, the number is likely to be underestimated, since growers in developing nations may be unaware of plant-parasitic nematodes.

Despite their wide variety of interactions with hosts, plant-parasitic nematodes all possess hollow, protrusible stylets, used for feeding or entering the host. Plant-parasitic nematodes can be ectoparasitic or endoparasitic. The ectoparasitic species feed on the root, but do not enter the root, while endoparasitic species feed inside the root. Some migratory ectoparasitic nematodes migrate through the soil and only use roots as an ephemeral food resource as they encounter them. On the other hand, migratory endoparasitic nematodes migrate through host tissue after entering the host, which can cause extensive damage. The most damaging plant-parasitic nematodes include root knot nematodes (*Meloidogyne spp.*), cyst nematodes (*Heterodera* and *Globodera* spp.), and root lesion nematodes (*Pratylenchus spp.*) (Van Megen *et al*., 2009).

Root-knot Nematodes

Root Knot Nematodes belong to the genus *Meloidogyne*, a relatively small but important polyphagous group of plant pathogens. *Meloidogyne* means apple-shaped female, which describes the morphology of adult females . Root knot nematodes have a worldwide distribution and their hosts include nearly every species of higher plants. They typically reproduce and feed within the plant root, which can induce small to large galls that are also known as root knots, one

of the oldest known nematode diseases of plants. As an endoparasitic nematode, root knot nematodes disrupt the plant physiology, which may reduce crop yield and product quality, and thus cause economic damage.

Life Cycle and Behavior

Root Knot Nematodes, like all plant parasitic nematodes, have a stylet to feed on host plant cells. The females are sedentary and globose at maturity, ranging in length from 400 to 1000 micrometers. The complete life cycle from egg to egg typically takes three to six weeks depending on nematode species. Root-knot nematode eggs that are enclosed in gelatinous egg sacs can be found on the surface of galled roots, or sometimes within the galls. within the egg after embryogenesis, gives rise to the second stage juvenile (J2). Temperature stimulates the hatching of Meloidogyne J2 from eggs, while stimulus from plant roots is not required. Yet, root diffusates can sometimes stimulate hatching (Karssen & Moens, 2013).

When infective J2 leaves the egg mass, they can be found freely in the soil. J2 can also survive in the soil in a quiescent state for an extended period before infecting nearby galled roots or entering new roots. While J2 are in soil, they consume the food stored in the intestine. Thus, infectivity will be reduced as they spend more time out of the root, since infectivity is related to food reserve. Plant roots can emit attractants that attract J2 and J2 accumulate behind the root cap, where cell elongation happens. Apical meristems, points where lateral roots emerge, and penetration sites of other J2 can also attract J2. However, little is known about the mechanism of attraction. While many organic and inorganic compounds excreted from roots may influence the nematodes, carbon dioxide is the most important factor that attracts plant-parasitic nematodes to the root area (Karssen & Moens, 2013).

Host Response to Parasitism

Juvenile root knot feeding can cause morphological and physiological changes in plants. Feeding sites that are established in the phloem or adjacent parenchyma are called giant cells, which are highly specialized cellular adaptations. While giant cells are established, root tissues around the nematode undergo hyperplasia and hypertrophy, causing characteristic root galls that are usually associated with *Meloidogyne* infections. Galls normally form 1 or 2 days after J2 penetrate the roots, and their size is related to host plant, the number of J2, and the nematode species (Karssen & Moens, 2013).

Species *Meloidogyne incognita*

Meloidogyne incognita, also known as the southern root-knot nematode, is one of the most common root-knot nematodes. It has a wide distribution in temperate regions and can also be found in greenhouses. *Meloidogyne incognita* is also able to reproduce on a wide range of plants, and it is estimated that more than 3000 plants, including both monocotyledon and dicotyledon plants, can be infected (Perry & Starr, 2009).

Cyst Nematodes

The cyst nematodes refer to a group of plant-parasitic nematodes in which the females can form cysts. Different species of cyst nematodes can affect different types of crops, including cereals, rice, potato, and soybean. After fertilization and production of embryonated eggs, the female cyst nematodes become sedentary and the body wall starts to tan and dry. The eggs enclosed by the cyst can survive for extended periods until a suitable host is growing nearby. The cyst is able to persist in soil for many years in the absence of a host, and this ability to survive for a long time makes them of great economic importance in agricultural situations (Karssen & Moens., 2013).

Life Cycle and Behavior

A cyst can contain up to 400 eggs and each egg contains a J2. After hatching from the cyst formed by the female nematode, the J2 moves through the soil and invades a host root. It then moves through the root to find a feeding site, where it feeds and develops. Juveniles develop either into females or males, with females saccate and rupture the root and vermiform males that leave the root to locate a female to mate. Generally, coarse-textured soil favors hatching and invasion of the root system by providing suitable conditions for aeration and nematode migration. The J2 leaves the cyst through either a natural opening or the neck where the female has broken away.

Heterodera glycines

Heterodera glycines, also known as the soybean cyst nematode, has a host range including legume and weed species (Venkatesh *et al*., 2000). It is widely distributed in regions where soybean is produced. The yield loss caused by *H. glycines* in soybean is greater than any other soybean pest, which makes it an agricultural pest of concern (Wrather, 2010).

Soybean

Soybean, *Glycine max*, is a species in the family Fabaceae native to East Asia and it is widely grown throughout the world for its edible bean. In 2019, the United States was the world's second largest soybean producer, with annual soybean production of 97 million tons, contributing almost one third of the world's total soybean production of 334 million tons (FAOSTAT, 2019). The majority of soybeans are grown in subtropical and temperate zones in North and South America and East Asia. Three countries in North and South America (United States, Brazil, and Argentina) constitute almost 80% of the world's total soybean production (FAOSTAT, 2019). The soybean planted acreage of the United States was 85.3 million acres in

2020, with an estimated 54 percent of total production being exported (USDA, 2021). Since soybeans can be grown in both high and low latitude, the soybean maturity group is used to classify different varieties of soybeans. Different maturity groups of soybeans have different developmental periods , for example, MG 0 varieties, with shorter developmental periods, are best adapted to North Dakota and northern Minnesota (Scott & Aldrich, 1970), while MG 6 varieties are better suited to most of the southern states (Zhang *et al*., 2007).

Nematode Damage on Soybean

Estimated yield loss of soybean caused by nematodes was9884.8 thousand metric tonnes worldwide in 2006, with soybean cyst nematode causing 7192.9 thousand metric tonnes yield loss (Wrather, 2010). The southern root-knot nematode can cause dramatic root symptoms and galls are formed on the root system as a result of nematode feeding. On the other hand, above ground symptoms caused by southern root-knot nematodes are not unique to it. Above ground symptoms include wilting, stunting, and other nutrient deficiency symptoms, since the galled roots limit the ability of the host plant to absorb water and nutrients. Because nematodes move slowly in the soil, above ground symptoms often appear on clusters of plants, with infection radiating gradually outward from an initial point of infection (Mitkowski & Abawi, 2003).

Both above ground symptoms and below ground symptoms caused by soybean cyst nematodes are not specific. Infected roots are dwarfed or stunted, and often have fewer nitrogen-fixing nodules. Above ground symptoms can be mistaken for damage from compaction, iron deficiency chlorosis and other nutrient deficiencies, drought stress, herbicide injury, or other plant diseases. The only unique symptom is the presence of adult female cysts on the soybean roots. And the only accurate way to diagnose soybean cyst nematodes in the field is the observation of adult female cysts (Tylka, 1994).

Control of Plant-parasitic Nematodes

The significant yield loss caused by plant-parasitic nematodes has received a lot of attention for decades, and different attempts have been made to control plant-parasitic nematodes, which includes the use of chemical nematicides, agronomic control strategies, the physical method of soil solarization, the application of plant-derived formulations, and biocontrol. Chemical nematicides like methyl bromide are effective, but the use of methyl bromide can cause stratospheric ozone depletion (Sasanelli *et al.*, 2021). In order to prevent further environmental damage caused by chemical nematicides, many countries have banned the use of particular chemical nematicides. Other approaches alone all have their own restrictions before Integrated Pest Management (IPM) wasput into practice.

Control of Nematodes on Soybean

Despite the use of synthetic nematicide to control nematodes on soybean, other control methods that are environmentally safer have been applied to control soybean nematodes. Researchers have discovered that root extracts can be used to control root-knot nematodes on soybeans (Adegbite & Adesiyan, 2008). Root extracts of Siam weed, Neem, Castor bean, and Lemongrass all exhibited inhibition of egg hatch and larval mortality when exposed to root-knot nematode eggs. Fungi can also be used to control soybean nematodes (Chen and Liu, 2005). Two species of *Hirsutella*, capable of infecting and parasitizing a wide range of invertebrates and pathogens, were able to control soybean cyst nematodes in greenhouse studies. Another method of nematode control is to use resistant cultivars of soybeans. Genetic engineering has been used to create genetically modified crop variants for decades. Cultivars of soybeans that are herbicide and pathogen resistant have been on the market since the 1990s (Owen & Zelaya, 2005, Ishiwata & Furuya, 2020), but nematode resistant cultivars are still under study.

Transgenic Soybean

Genetically modified soybeans were introduced into the U.S. market by Monsanto in 1996, and the cultivation of genetically modified soybeans is now worldwide (Bawa & Anilakumar, 2013). Although transgenic soybeans that are nematode resistant are not available on the market at this point, researchers have made progress towards putting it into practice. For example, a transgenic soybean with nematode-active *Bacillus thuringiensis* (Bt) Cry protein, shows a significant reduction in soybean cyst nematode populations. The Bt Cry protein, Cry14Ab, is known to cause damage to the nematode intestine (Khan *et al.*, 2021). Other transgenic soybeans have also been proven to be nematode resistant: instead of the use of toxin to kill nematode, it over expresses a plasmid membrane protein *GmDR1*, which can trigger broad-spectrum immunity in soybean and reduce nematode populations (Ngaki *et al*., 2020). Using toxins generated by crops to control pests can be effective in theshort term, however, studies have suggested that pests can also develop resistance towards Bt crops. For example, pink bollworm has developed resistance towards multiple toxins, which enables its survival on Bt cotton that produces multiple toxins (Fabrick, 2015). Thus, the use of plant innate immunity is the next step of nematode control in soybean.

Plant Elicitor Peptides

Plant elicitor peptides (Peps) are a DAMP that trigger immune response in plants. DAMPs refer to damage-associated molecular patterns that are mainly cytosolic proteins, peptides, nucleotides, and amino acids. When cells undergo pathogen invasion, DAMPs are released from damaged cells to interact with pattern recognition receptors (PRPs) to induce innate immune response in plants (Hou *et al*, 2019).

Peps are 23-36-amino-acid-long sequences generated from the carboxyl terminal of longer

pro-peptide precursors. A pro-peptide precursor is an inactive peptide that can be turned into an active form by post-translational modification. Plant elicitor peptides are widely distributed defense signaling molecules and play an important role in broad-spectrum defense against insects and pathogens (Tavormina *et al.*, 2015).

Plant Elicitor Peptides in Plant Defense

A discovery 30 years ago first revealed that an endogenous polypeptide, consisting of 18 amino acids, can activate defensive genes in tomatoes. This peptide signal systemin, isolated from tomato leaves, was able to promote proteinase inhibitor accumulation, volatile emission, and is critical to resistance against lepidopteran herbivores in tomato (Pearce, 1991). However, peptides with analogs in other plant families remained elusive for a long time. In 2006, a 23-aa peptide from *Arabidopsis*, *At*Pep1, was found to be able to trigger innate immune response by activating transcription of the defensive gene defensin and the synthesis of H_2O_2 (Huffaker *et al*., 2006).

Plant elicitor peptides trigger multiple defensive pathways in plant immunity. In *Arabidopsis thaliana*, *At*Peps regulate expression of pathogen defensive genes associated with salicylate, jasmonate, and ethylene signaling pathways (Huffaker & Ryan, 2007). Figure 1 demonstrates the amplification model of defense response by plant elicitor peptides. Further studies have indicated that orthologues of *At*Peps occur in various plant families, including crops such as maize, wheat, rice, and soybean. Thus, Peps in other plants have the potential to mediate innate immunity against pathogens (Huffaker *et al*., 2012). For example, researchers have discovered that a member of the maize (*Zea mays*) plant elicitor peptide family, *Zm*Pep3, regulates defense responses against herbivores (Huffaker *et al*., 2012).

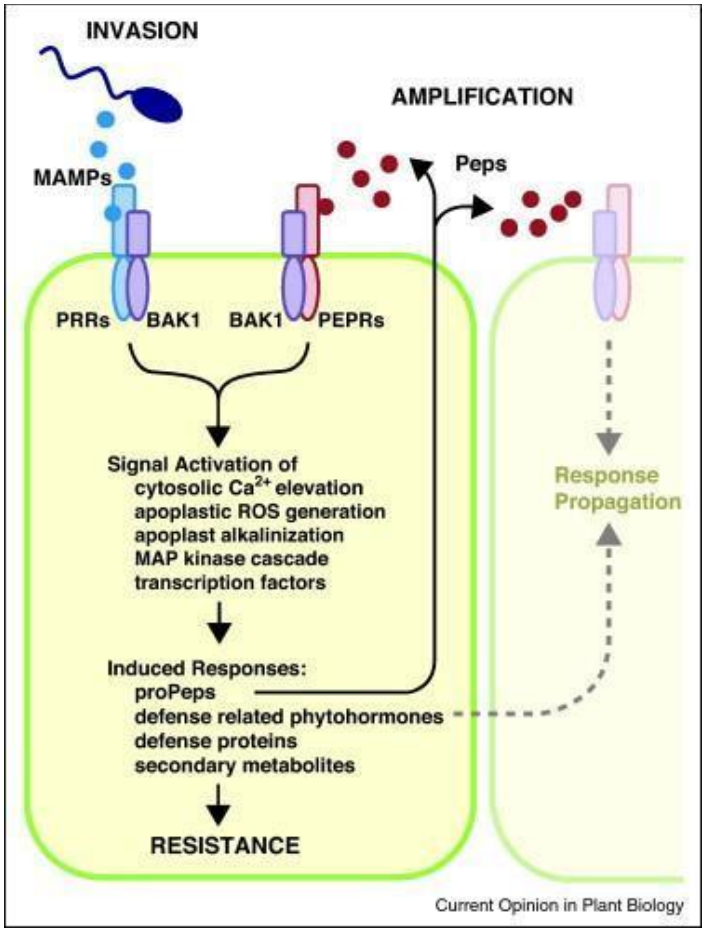

Figure 1. Plant elicitor peptides defense response amplification model (Yamaguchi and Huffaker, 2011).

_Gm_Peps in soybean

Six putative plant elicitor propeptide (*PROPEP*) genes were predicted from the soybean genome in 2010: *GmPROPEP1* (on chromosome 10), *GmPROPEP2* (chromosome 20), *GmPROPEP3*, *GmPROPEP4* and *GmPROPEP5* (grouped within a 7-kb region of chromosome 13), and *GmPROPEP6* (chromosome 4) (Schmutz *et al*., 2010). The genes are predicted to release 23-residue elicitor peptides. In the *Gm*Peps, *Gm*Pep6, *Gm*Pep1, *Gm*Pep2 and *Gm*Pep3 show distinctive similarity to each other, while *Gm*Pep4 has more similarity with *Gm*Pep5 than with any other *Gm*Pep. This finding suggests that *Gm*Peps can be classified into three groups,

with each group having the possibility to be involved in different functions.

Expressions of the six *GmPROPEP* genes were examined in different plant tissues including roots, leaves, flowers, seeds, nodules and pods, from healthy, uninfected soybean plants (Severin *et al.*, 2010). Three of the six genes were transcribed, which indicates constitutive expression in the absence of pests, pathogens, and wounding. *GmPROPEP1* was expressed in all tested tissues, *GmPROPEP4* was expressed in seeds, and *GmPROPEP6* was expressed in seeds and roots at a very low level. Researchers decided to focus on *GmPROPEP1*, *GmPROPEP2*, and *GmPROPEP3* in subsequent experiments because these three genes constitute a distinct subgroup based on sequence homology but differ in whether they are constitutively expressed or not. Besides, a previous study indicated that exogenous application of *Gm*Pep3 to soybean foliage could induce the production of volatile organic compounds (VOCs), which is associated with the recruitment of the natural enemies of herbivores (Huffaker *et al*., 2012).

A study published in 2017 assessed whether *Gm*Peps could limit nematode infection (Lee *et al*., 2017). RKN and SCN reproduction were analyzed on plants that had received seed treatments with *Gm*Pep1, *Gm*Pep2, and *Gm*Pep3. Results indicate that all three *Gm*Peps can significantly reduce nematode reproduction in both nematode species compared with water treated controls.

Plant Elicitor Peptide and Plant Growth

Several studies have suggested that plant elicitor peptides have a neutral or positive effect on plant growth. No significant effect was found on shoot or root biomass of above- and below-ground biomass of plants treated with water and *Gm*Peps (*Gm*Pep1, *Gm*Pep2, and *Gm*Pep3) were compared without nematode infection. Further experiments have indicated that when infected by RKN, *Gm*Pep1 treated plants have significantly greater aboveground than that of water treated

plants, suggesting that plant elicitor peptide can reduce the damage of nematodes caused on plants (Lee *et al*., 2017). Other experiments have drawn similar conclusions. Pretreatment of a potato plant elicitor peptide *St*Pep1 on potato have no marked effect on above and below ground weights of *Meloidogyne chitwoodi* infected plants (Zhang and Gleason, 2020).

Objectives of the Study

I decided to further investigate whether a susceptible cultivar of soybean with the introduced *GmPROPEP3* gene has the capability to reduce the population of soybean nematodes. I also wanted to further confirm that other *Gm*Pep3 treated susceptible cultivars of soybean are resistant to soybean nematodes. Finally, I focused on improving the germination method of soybean seeds after treating GmPep3.

Thus, the main objectives of this study were:

1) To confirm whether *Gm*Pep3 treated susceptible cultivar is resistant to the southern root-knot nematode (RKN) and the soybean cyst nematode (SCN)

2) To investigate whether transgenic soybeans expressing GmPROPEP3 are resistant to RKN and SCN

3) To examine whether *Gm*Pep3 treatment and *GmPROPEP3* insertion have any effect on plant growth

4) To modify the current germination method after *Gm*Pep3 treatment

II. Material and Methods

Experiment Material

The University of Missouri Plant Transformation Facility introduced *GmPROPEP3* overexpression construct using *Agrobacterium tumefaciens* mediated transformation method into a cultivar Magellan (PI 595363) (Schapaugh *et al*., 1998), a high-yielding cultivar susceptible to RKN and SCN. The CaMV 35S promoter, derived from Cauliflower mosaic virus, facilitates the expression of GmPROPEP3 in transgenic soybeans (Kay *et al*., 1987). The *bar* gene was used as a selective and screenable marker in transgenic soybeans (D'Halluin *et al*., 1995). The T1 seeds were obtained from independent transgenic events (T0 plants), and the empty vectors (EV) transformed from the T1 seeds were also obtained. Empty vectors used as a control do not express *GmPROPEP3* but do express the *bar* gene. Transgenic seeds used in the experiments were the T1 seeds propagated from the T0 seeds after genotyping. Three transgenic lines were tested in two independent nematode assays: ND21-2 and ND21-4 were tested in the RKN assay, while ND21-2 and ND15-1 were tested in SCN assay. ND21-2 and ND21-4 were previously tested to have significantly higher expression of *GmPROPEP3* (Dr. Galla, personal communication). Race 5 SCN and RKN originally collected from soybean in Arkansas were reared in a glasshouse at Shult Agricultural Research & Extension Center (SAREC). The experiment design was random, with one replicate. All plants were nematode inoculated.

Seed Treatment

Two batches of GmPep3 (PSHGSVGGKRGSPISQGKGGQHN), synthesized by Biomatik Corporation (Cambridge, ON, Canada), were applied to soybean seeds in two independent nematode bioassays. The RKN bioassay received GmPep3 treatment with lot number GT70297-91SP180146-3 received in February 2018. The SCN bioassay received GmPep3

treatment with lot number P201210-YS417154 received in January 2021. Soybean seeds (*Glycine max* cv. Magellan) were imbibed in Petri dishes at room temperature (24 °C) overnight in a solution of 0.05% Tween 20 and 1 μm of GmPep3. Seeds for control were treated with 0.05% Tween 20 and water only.

Seed Germination

In the RKN assay, overnight treated seeds were transferred to germination paper (Crepe Type Seed Germination Paper, GSM: 80 – 120) manufactured by Nissy Enterprise and placed in zipper bags in a growth chamber for 48 hours (16-h light/8-h dark photoperiod, 30°C). In SCN assay, seeds after overnight treatment were transferred directly to vermiculite and placed in the same growth chamber. Germination was defined as a plant that survived after transplanting.

In the RKN assay, seedlings after 48 hours incubation were transferred to vermiculite in a glasshouse at SAREC (16-h light/8-h dark photoperiod, 21-27°C) until cotyledons are fully expended. Seedlings around 1 week old from both assays were transferred from vermiculite to autoclaved pure sandy loam in 250ml clay pots for future growth. Plants were fertilized with slow-release fertilizer (Miracle-Gro® All Purpose Continuous Release Plant Food). The early growth of soybean has a few stages: the VE (Emergence) occurs 5 to 21 days after planting, followed by the VC stage when unifoliate leaves have unrolled. The unifoliate leaf node is the first node, it can also be used as a reference point to count the vegetative stages. All other true leaves formed later are called trifoliate leaves, with three leaflets borne on long petioles. For example, a soybean reaches V1 stage when its first trifoliate leaves are formed (Sadras & Calderini, 2020).

Nematode Bioassay

When soybeans reached V1 stage, an estimate of 5000 fresh nematode eggs suspended in

water were inoculated in soil via two holes per plant about 3cm deep. Infection levels were measured 5 weeks after inoculation for the SCN assay, and 7 weeks after inoculation for the RKN assay. For the RKN assay, nematode eggs were extracted from roots using 10% bleach and sieving (Hussey & Baker, 1973). For the SCN assay, cysts were separated from the root system after roots were washed using a 250 μm sieve (Ithal *et al*., 2007). Numbers of cyst and nematode were counted on a small glass petri dish under a microscope. For the SCN assay, cysts were crushed after counting to obtain nematode eggs.

Genotyping *GmPROPEP3* in Transgenic Lines

Leaf tissues were collected for DNA extraction when plants were about 5 weeks old. DNA for all transgenic plants in bioassays were extracted (Healey *et al*., 2014). 100mg of leaf tissue at 5 weeks old were collected. 500μl of CTAB Extraction Buffer was added, and the mixture was homogenized and transferred to a 65°C bath for 30 minutes. After centrifuging the homogenate for 5 minutes at 14,000 x g, the supernatant was transferred to a new tube with 1μl of RNase solution A and incubated at a 37°C bath for 30 minutes. An equal volume of chloroform was added to each sample and centrifuged at 14,000 x g for 1 minute to separate the phases. The upper aqueous phase was transferred to a new tube and DNA was precipitated by adding 0.7 volume cold isopropanol and incubated at -20°C for 15 minutes. The samples were then centrifuged at 14,000 x g for 10 minutes, pellets were washed using 500μl ice cold 70% ethanol, and dried. 50μl TE buffer was used to dissolve each DNA sample. The quality of DNA samples was checked using a spectrometer (BioTeK, Winooski, VT, USA). PCRs were performed using the *bar* gene as a selective marker with SYBR Green PCR Master Mix (Thermo Fisher Scientific, Warrington, UK) to confirm the existence of *GmPROPEP3* in all transgenic lines. The PCR conditions were 95 °C for 5 min, followed by 40 cycles of 40 cycles of 95 °C for 15 s, 55

°C for 30 s and 72 °C for 60s, and 72 °C for 5 min. The primer pairs for the *bar* gene were BarF TGCACCATCGTCAACCACTA and BarR ACAGCGACCACGCTCTTGAA. Electrophoresis was performed with 1% agarose gel.

Quantification of *GmPROPEP3* in Transgenic Lines

Leaf and root tissue were collected to analyze gene expression in transgenic line ND 15-1. Magellan, empty vector, and ND 15-1 were germinated in soil and grown under glasshouse condition (16-h light/8-h dark photoperiod, 21-27°C), roots and leaves were collected at V1 stage and flash frozen in liquid nitrogen at −80 °C and stored at −80 °C. RNA extraction was performed using method described in (Jordan-Thaden *et al.*, 2015). Bio-Rad T100 PCR Thermal Cycler was used to synthesize cDNA, under 95 °C for 5 min, followed by 40 cycles of 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 60s, and 72 °C for 5 min. Superscript III reverse transcriptase and oligo-dT primers were used to generate cDNA. The qPCR was performed with Bio-Rad CFX Connect and the PCR conditions were 95 °C for 15 min, then 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Data from *GmPROPEP3* were normalized to the expression levels of the endogenous control *Translational elongation factor 1 subunit β (ELF1b)* (Glyma02g44460). The primer pairs used for RT-qPCR were *GmPROPEP3* (NM_001248158), forward (5′-CTCGCCTATTGGGAAACCTT-3′); reverse (5′-TCAACCCTAGCCTCGTCATT-3′); *ELF1b* (Glyma02g44460), forward (5′-GTTGAAAAGCCAGGGGACA-3′); reverse (5′-TCTTACCCCTTGAGCGTGG-3′). Primer efficiency was calculated using $E = 10^{[-1/Ct\ slope]}$ (Rasmussen, 2000), and relative gene expression was calculated using a method from Pfaffl (2001).

Statistical Analysis

All experiments were analyzed using JMP Pro 16 (SAS Institute, Cary, NC, USA). ANOVAs were performed to detect differences among genotypes and treatments. Student's *t*-tests were used to make pairwise comparisons between treatments.

III. Results

The Root-knot Nematode Assay

Genotyping results of the Root-knot Nematode (RKN) assay (Figure 2). Forty-nine plants were screened, numbers marked in different colors indicate different genotypes and numbers in white were non-transgenic plants used for control. Broad band under the thin band (if present) is a byproduct of the PCR reaction. Sample 19 with a faint band and 47 with no band were excluded from the RKN assay and further statistical analysis.

Stem height of each genotype in the RKN assay (Figure 3) differed significantly (df=4, F=5.26, P=0.001), stem height of *Gm*Pep3 treated Magellan was significantly higher than other genotypes in the experiment. There was no significant difference in the aboveground dry weight (df=4, F=0.78, P=0.54) and the root dry weight (df=4, F=1.91, P=0.12) among treatment groups (Figure 4). In the root-knot nematode reproduction analysis (Figure 5), there was no significant difference in the number of eggs per plant (df=4, F=1.33, P=0.27) and the number of eggs per plant/ root dry weight (df=4, F=1.69, P=0.16).

Germination Rate

Two germination methods were used to germinate the plants in the RKN assay and the SCN assay. The total germination rate (total seeds germinated/ total seeds treated) of plants in the RKN assay is 52% and the total germination rate of plants in the SCN assay is 83%.

The Soybean Cyst Nematode Assay

Genotyping result of the SCN assay (Figure 6). 49 plants were screened, numbers marked in different colors indicate different genotypes, numbers in white were non-transgenic plants used for control. Negative control (-) with no template was contaminated, thus genotypes of samples could not be determined.

Stem height of each genotype in the SCN assay (Figure 7) differed significantly according to ANOVA (df=4, F=6.15, P=0.0003). There was no significant difference in the aboveground dry weight (df=4, F=0.47, P=0.76) and the root dry weight (df=4, F=1.61, P=0.18) among treatment groups (Figure 8). In the root-knot nematode reproduction analysis (Figure 9), there was significant difference in the number of cysts per plant (df=4, F=4.35, P=0.0034) and the number of eggs per plant (df=4, F=4.77, P=0.0019). ND21-2 and ND15-1 had significantly lower numbers of cysts than that of other genotypes.

***GmPROPEP3* expression in ND15-1**

Relative gene expression of *GmPROPEP3* was analyzed in Magellan and ND15-1. The average gene expression of *GmPROPEP3* is 1 in Magellan, and 7.83 in ND15-1. The gene expression of *GmPROPEP3* in Magellan was set as a standard, and the gene expression of *GmPROPEP3* in ND15-1 was 7.83 times higher than that of Magellan.

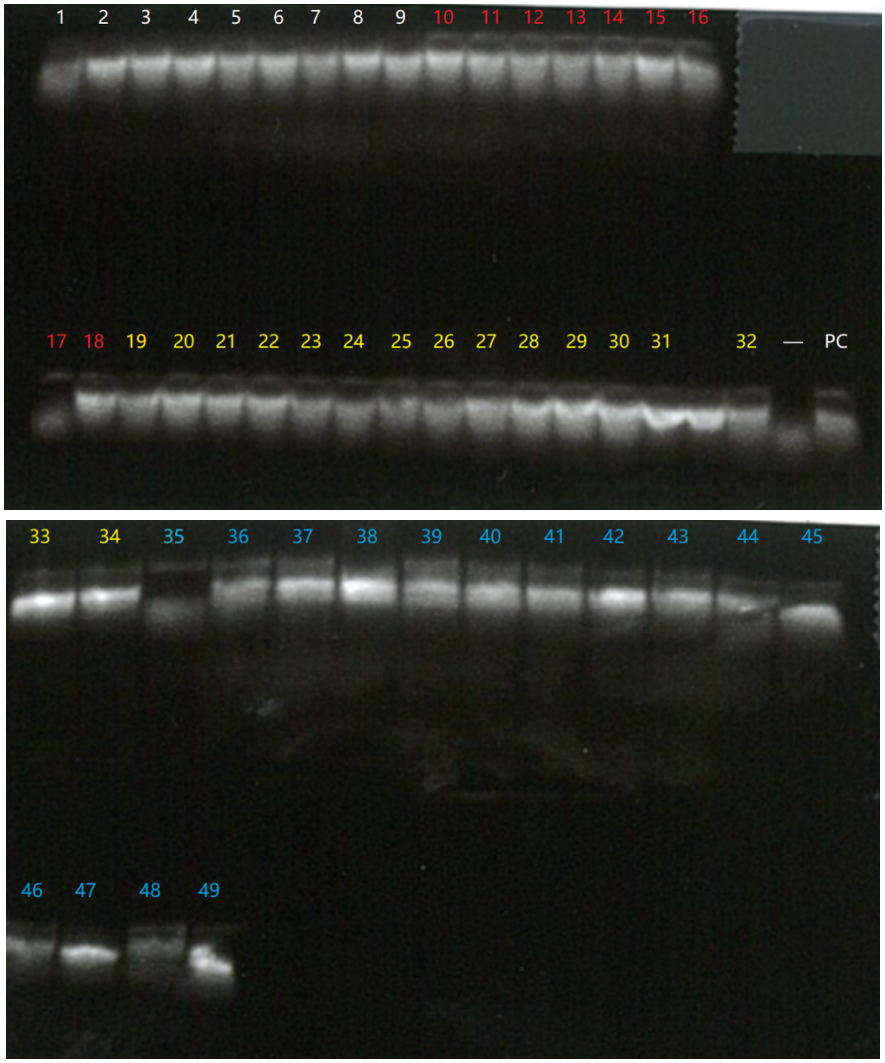

Figure 2. Screening soybeans from the RKN assay for *GmPROPEP3*. Samples marked white were non-transgenic soybeans (1 to 3 were Magellan, 4 and 5 were Magellan+GmPep3, and 6 to 9 were Osage). Samples marked in red were empty vector YZ7-1, samples marked in yellow were ND21-2, and samples marked in blue were ND21-4. Minus (-) indicate no template control. PC is a positive control, which contains a template from previously screened transgenic soybean with a positive result.

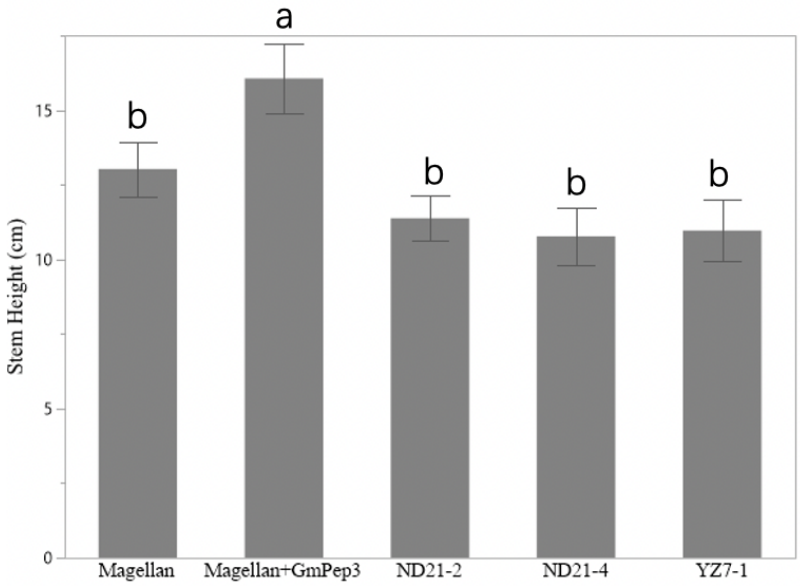

Figure 3. Stem height of the RKN assay (n=13 for Magellan, n=14 for Magellan+GmPep3, n=15 for ND21-2, n=15 for ND21-4, and n=9 for YZ7-1). Transgenic lines overexpressing *GmProPEP3* (ND21-2, ND21-4), an empty vector control line (YZ7-1), untransformed controls (cv. Magellan), and untransformed controls that received a 1 µM GmPEP3 seed treatment (Magellan + GmPep3) were inoculated with 5,000 root-knot nematode eggs. Error bars represent the standard deviations. According to One Way ANOVA, there were significant differences in stem height among treatment groups in the RKN assay (df=4, F=5.26, P=0.001). Treatments labeled with the same letter are not significantly different according to Student's t-tests.

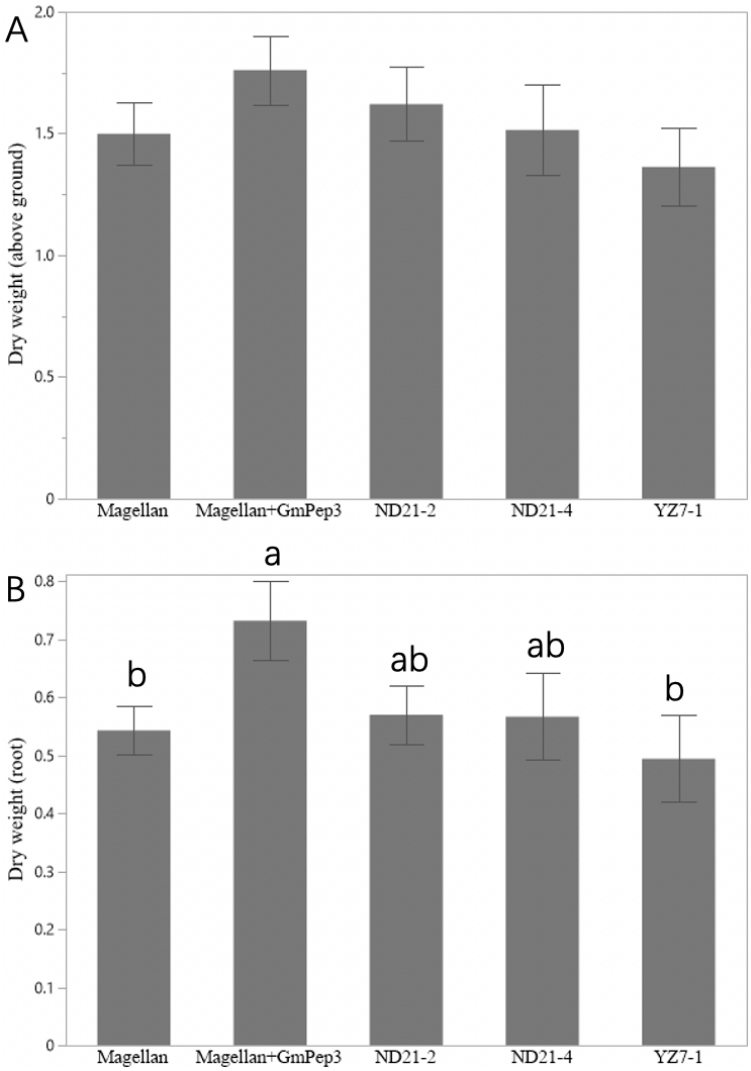

Figure 4. Above ground dry weight (A) and root dry weight (B) of the RKN assay (n=13 for Magellan, n=14 for Magellan+GmPep3, n=15 for ND21-2, n=15 for ND21-4, and n=9 for YZ7-1). Transgenic lines overexpressing *GmProPEP3* (ND21-2, ND21-4), an empty vector control line (YZ7-1), untransformed controls (cv. Magellan), and untransformed controls that received a 1 µM GmPEP3 seed treatment (Magellan + GmPep3) were inoculated with 5,000 root-knot nematode eggs. Error bars represent the standard deviations. According to One Way ANOVA, there were no significant difference in dry weight (above ground) among treatment groups in RKN assay (df=4, F=0.78, P=0.54), there were no significant difference in dry weight (root) among treatment groups in RKN assay (df=4, F=1.91, P=0.12). Treatments labeled with the same letter are not significantly different according to Student's t-tests.

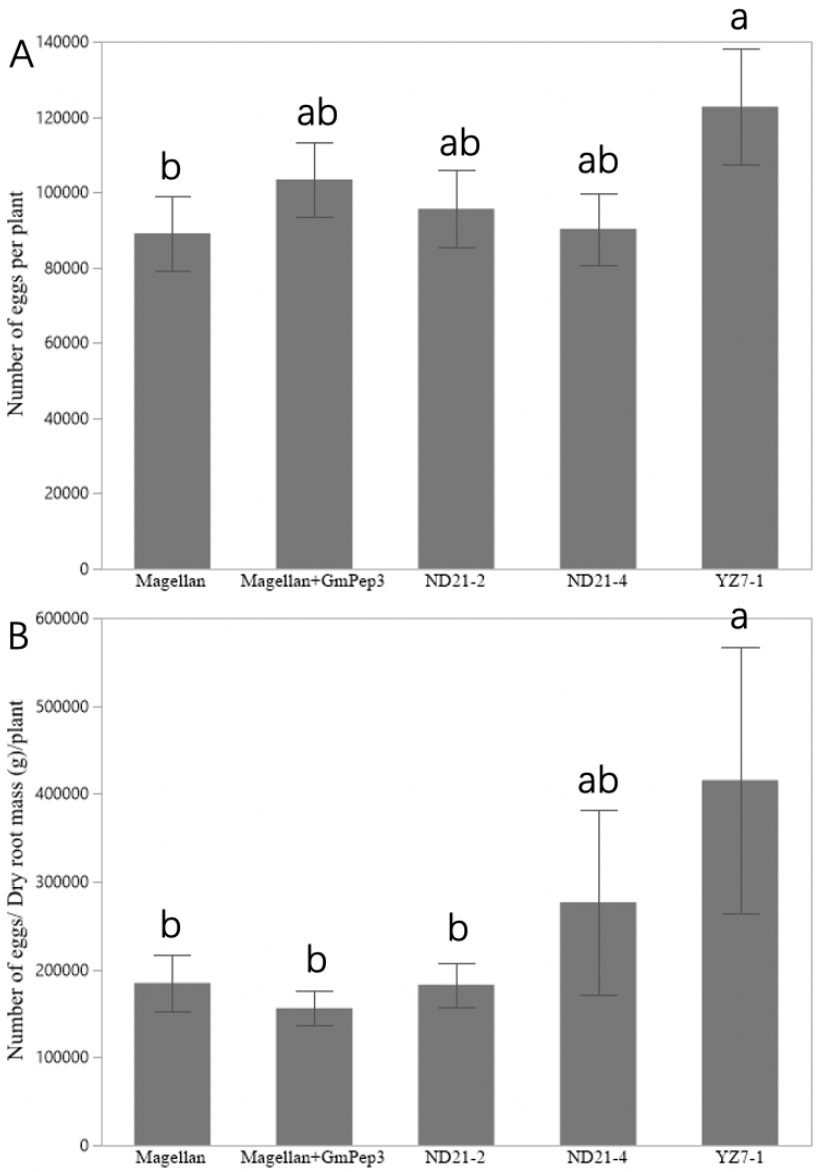

Figure 5. Root-knot nematode reproduction. Transgenic lines overexpressing *GmProPEP3* (ND21-2, ND21-4), an empty vector control line (YZ7-1), untransformed controls (cv. Magellan), and untransformed controls that received a 1 µM GmPEP3 seed treatment (Magellan + GmPep3) were inoculated with 5,000 root-knot nematode eggs. Egg numbers (A) were counted 5 weeks after inoculation and number of egg/dry root mass (B) were calculated (n=13 for Magellan, n=14 Magellan+GmPep3, n=15 for ND21-2, n=15 for ND21-4, and n=9 for YZ7-1). According to One Way ANOVA, there was no significant difference in egg numbers (df=4, F=1.33, P=0.27) and number of egg/dry root masses (df=4, F=1.69, P=0.16). Treatments labeled with the same letter are not significantly different according to Student's t-tests.

Table 1. Germination rate of soybeans of the RKN assay. Total represents the total number of seeds in all genotypes. Number of seeds germinated include all the seeds germinated successfully and number of seeds treated include all the seeds receiving overnight water/*Gm*PEP3 treatment. Percentage of germination is the decimal representation of number of seeds germinated/number of seeds treated.

	Magellan	Magellan+GmPep3	ND21-2	ND21-4	YZ7-1	Total
Number of seeds germinated/ Number of seeds treated	13/20	14/20	16/30	16/30	9/30	68/130
Percentage of germination	65%	70%	53%	53%	30%	52%

Table 2. Germination rate of soybeans of the SCN assay. Total represents the total number of seeds in all genotypes. Number of seeds germinated include all the seeds germinated successfully and number of seeds treated include all the seeds receiving overnight water/*Gm*PEP3 treatment. Percentage of germination is the decimal representation of number of seeds germinated/number of seeds treated.

	Magellan	Magellan+GmPep3	ND21-2	ND15-1	YZ7-1	Total
Number of seeds germinated/ Number of seeds treated	20/20	20/20	15/20	17/20	11/20	83/100
Percentage of germination	100%	100%	75%	85%	55%	83%

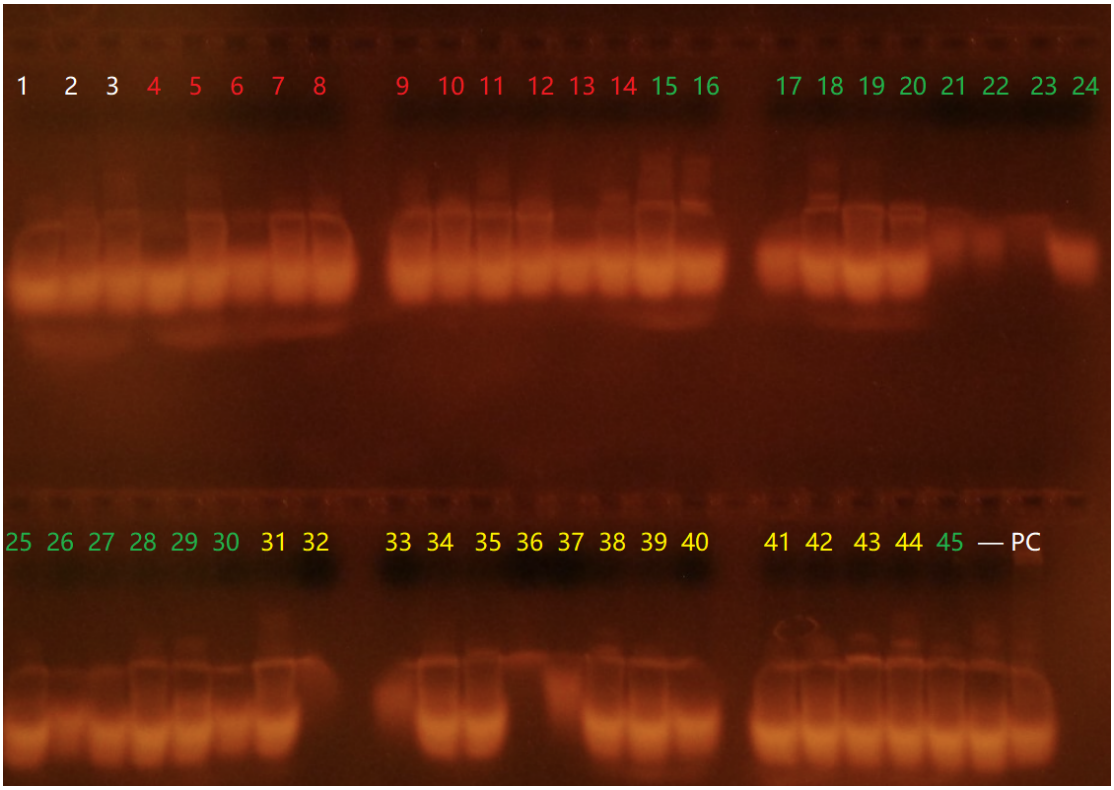

Figure 6. Screening soybeans from the SCN assay for *GmPROPEP3*. Samples marked white were non-transgenic soybeans (1 was Magellan, 2 was Magellan+GmPep3, and 3 was Lee). Samples marked in red were empty vector YZ7-1, samples marked in yellow were ND15-1, and samples marked in blue were ND21-2. Minus (-) indicate no template control. PC is a positive control, which contains a template from previously screened transgenic soybean with a positive result.

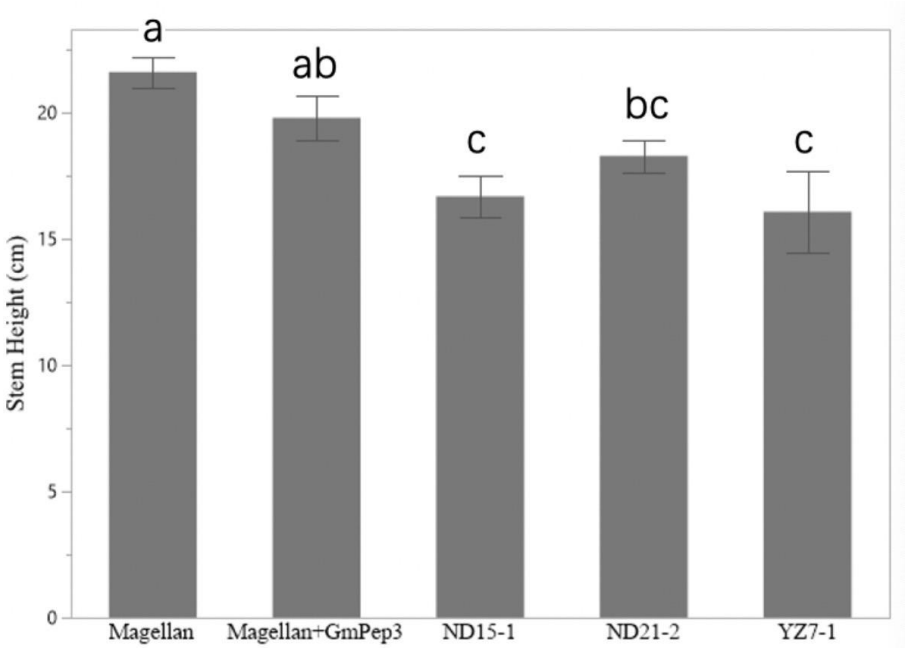

Figure 7. Stem height of the SCN assay (n=15 for Magellan, n=15 for Magellan+ GmPep3, n=15 for ND21-2, n=17 for ND15-1, and n=11 for YZ7-1). Transgenic lines overexpressing *GmPROPEP3* (ND21-2, ND15-1), an empty vector control line (YZ7-1), untransformed controls (cv. Magellan), and untransformed controls that received a 1 μM GmPEP3 seed treatment (Magellan + GmPep3) were inoculated with 5,000 soybean cyst nematode eggs. Error bars represent the standard deviations. According to One Way ANOVA, there were significant differences in stem height among treatment groups in the SCN assay (df=4, F=6.15, P=0.0003). Treatments labeled with the same letter are not significantly different according to Student's t-tests.

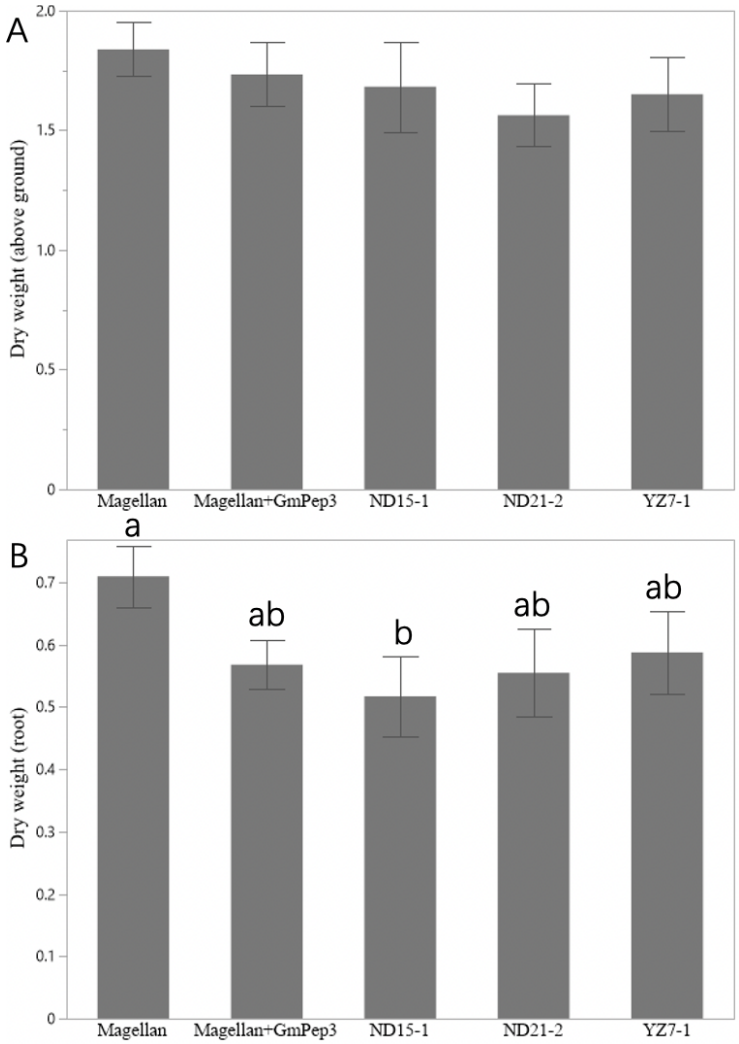

Figure 8. Above ground dry weight (A) and root dry weight (B) of the SCN assay (n=15 for Magellan, n=15 for Magellan+ GmPep3, n=15 for ND21-2, n=17 for ND15-1, and n=11 for YZ7-1). Transgenic lines overexpressing *GmProPEP3* (ND21-2, ND15-1), an empty vector control line (YZ7-1), untransformed controls (cv. Magellan), and untransformed controls that received a 1 µM *Gm*PEP3 seed treatment (Magellan + GmPep3) were inoculated with 5,000 soybean cyst nematode eggs. Error bars represent the standard deviations. According to One Way ANOVA, there were no significant difference in dry weight (above ground) among treatment groups in SCN assay (df=4, F=0.47, P=0.76), there were no significant difference in dry weight (root) among treatment groups in the SCN assay (df=4, F=1.61, P=0.18). Treatments labeled with the same letter are not significantly different according to Student's t-tests.

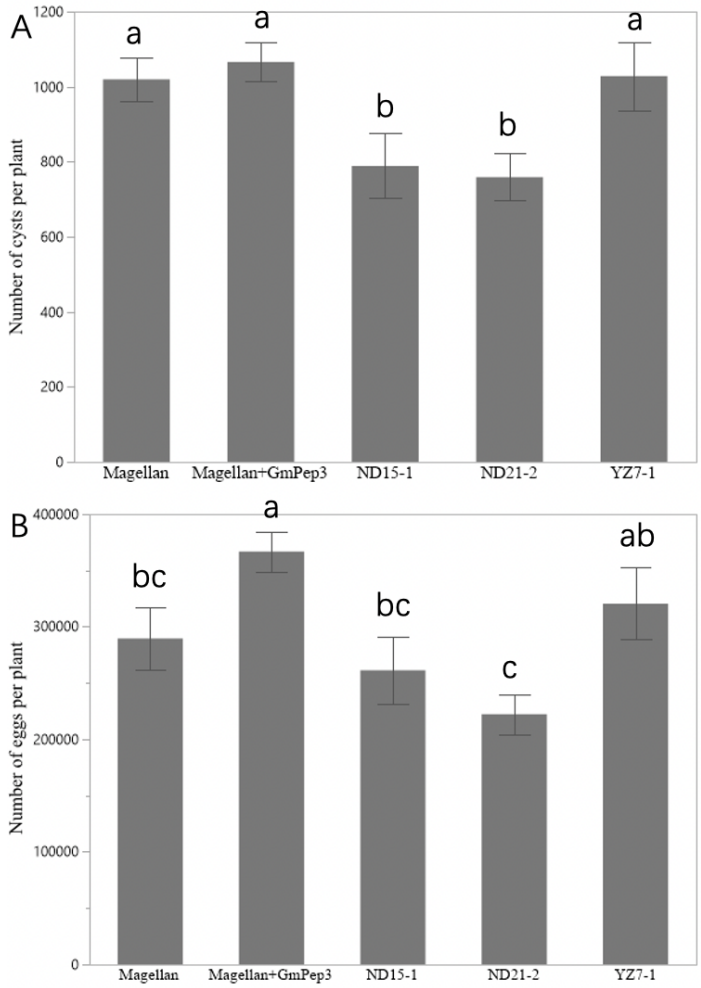

Figure 9. Soybean cyst nematode reproduction. Transgenic lines overexpressing *GmProPEP3* (ND21-2, ND15-1), an empty vector control line (YZ7-1), untransformed controls (cv. Magellan), and untransformed controls that received a 1 µM GmPEP3 seed treatment (Magellan + GmPep3) were inoculated with 5,000 soybean cyst nematode eggs. Egg numbers (A) were counted 5 weeks after inoculation and number of egg/dry root mass (B) were calculated (n=15 for Magellan, n=15 for Magellan+ *Gm*Pep3, n=15 for ND21-2, n=17 for ND15-1, and n=11 for YZ7-1). According to One Way ANOVA, there were significant differences in cyst numbers (df=4, F=4.35, P=0.0034) and egg numbers (df=4, F=4.77, P=0.0019). Treatments labeled with the same letter are not significantly different according to Student's t-tests.

Table 3. Relative expression of *GmPROPEP3* in ND15-1

Genotype	Number of samples	Average expression
Magellan	2	1
ND15-1	3	7.83

IV. Discussion

In the RKN assay, the stem heights among different genotypes differed significantly (p=0.001) (Figure 2). According to ANOVA, *Gm*Pep3 treated plants were significantly higher than other treatment groups, including Magellan, ND21-2, ND21-4, and YZ7-1. However, stem heights of the two transgenic lines ND21-2 and ND21-4 expressing *GmPROPEP3* were not significantly higher than stem heights of Magellan and empty vector YZ7-1. The aboveground dry weights and root dry weights of the RKN assay were not significantly different among treatment groups (p=0.54, p=0.12). According to the statistics, *Gm*PEP3 treatment could promote plant growth in height, but not in aboveground or root mass in the RKN experiment. Transferring *GmPROPEP3* into plants had no positive or negative impact on plant growth. In the SCN assay, the stem heights among different genotypes differed significantly according to ANOVA (p=0.0003) (Figure 7). The stem heights of genotype Magellan were significantly higher than stem heights of ND15-1 and YZ7-1. The stem heights of *Gm*PEP3 treated Magellan were also significantly higher than that of ND15-1 and YZ7-1. However, no significant differences were found in stem heights between Magellan and *Gm*PEP3 treated Magellan. Despite the difference in stem heights in the SCN assay, no significant differences were found in aboveground weights and root dry weights among treatment groups (p=0.76, p=0.18). The patterns of stem heights in the RKN assay and the SCN assay are different, suggesting that more experiments need to be conducted to form a conclusion.

According to the nematode assay of the RKN experiment, no significant differences were found in egg numbers (p=0.27) and egg number/dry root mass (p=0.16) among treatment groups. There were no significant differences in the number of eggs and the number of eggs/dry root mass among treatment groups. The egg number/ dry root mass ratio indicates the number of eggs

present on one unit of root - the higher the ratio is, the more susceptible the plant is towards root-knot nematodes. In the SCN assay, significant differences were found in cyst numbers ($p=0.0034$) and egg numbers ($p=0.0019$) in different treatment groups. The cyst numbers and egg numbers of ND21-2 and ND15-1 were significantly higher than other treatment groups, including Magellan, *Gm*PEP3 treated Magellan, and YZ7-1. According to the results, the transgenic lines ND21-2 and ND15-1 demonstrate resistance towards soybean cyst nematodes. To further support the result, more SCN assays need to be conducted with similar results.

A germination paper was used to germinate seeds in the RKN assay, and the germination rate of plants in the RKN assay was 52%, 68 seeds were germinated among 130 treated seeds. To improve the germination rate in further nematode bioassays, a new germination method was applied to seeds in the SCN assay. According to Martins *et al*. (2012), the ipe seeds using vermiculite as a substrate for germination had the highest germination rate compared to soil, sand, and paper rolls . The seeds were transferred directly to vermiculite to germinate after *Gm*PEP3 or water treatment. The result indicated that the germination rate has improved from 52% to 83%, a total of 83 seeds were germinated among 100 treated seeds.

In the SCN genotyping experiment, the PCR result indicated that the control without DNA template contained DNA, suggesting contamination in previous processes. However, after repeating the PCR and gel electrophoresis with new reagents, the water control was still contaminated. It is possible that the primers were contaminated during previous handling.

Future Studies

In both RKN and SCN assays, only treatment groups with nematode inoculated were included in the bioassay. Whether *Gm*PEP3 and the insertion of *GmPROPEP3* have any effect on soybean growth is unknown. In future experiments, controls of soybeans without nematode

inoculation should be added, including Magellan, *Gm*PEP3 Magellan, transgenic lines, and empty vector YZ7-1.

Besides, the *GmPROPEP3* expression result of soybeans in the nematode bioassay should be included in future experiments. The *GmPROPEP3* level of a plant reflects its ability to synthesize *Gm*PEP3, though indicates its potential to resist nematodes. Combining *GmPROPEP3* expression analysis with greenhouse nematode bioassays helps to better understand the pattern of resistance in transgenic and PEP treated plants.

What's more, it is important to determine the copy numbers of *GmPROPEP3* in transgenic lines. Researchers have discovered that copy numbers of a gene affect gene expression. Multiple research has indicated that variation in gene copy number can result in changes in gene expression. Small differences in gene copy number can cause large-scale changes in gene expression (Mileyko, Joh, & Weitz, 2008). Research in tobacco has demonstrated that multiple copies of the inserted gene can result in gene silencing (Li *et al*., 2002). Thus, determining the number of copies of *GmPROPEP3* and selecting plants with the least possible number of copies of *GmPROPEP3* can stabilize the *Gm*Pep3 expression in transgenic lines and reduce the variables in future experiments.

Finally, after obtaining desirable greenhouse experiment data, the next step is to conduct field experiments of nematode bioassays. In the greenhouse experiments, the plants were not allowed to live until harvest, and the impact of nematodes on yield is unknown. Besides, field experiments can best simulate natural growth conditions, thus conducting field experiments can obtain data on plants in different conditions. For example, whether the *GmPROPEP3* expression is affected in drought or the presence of other insect herbivores.

V. Conclusion

Genetic engineering in agriculture has been a hot area for decades. As the global population still grows, genetically modified (GM) plants that take advantage of natural genetic variation can be modified for different uses. The overuse of pesticide in agriculture has already demonstrated the pitfalls of reliance on chemicals and that GM plants that are pest resistant are the future of agriculture. Instead of using toxin synthesized by plants to eliminate pests, innate immune responses in plants can also be utilized in pest control, without the accumulation of toxin resistance.

This study found that inserting *GmPROPEP3* in soybean may have no negative effect on soybean growth. The SCN nematode bioassay result suggested that the transgenic lines are likely to be nematode resistant. However, without the genotyping data, the bioassay data itself is not sufficient to drive a conclusion that soybeans carrying *GmPROPEP3* are resistant towards nematodes.

The data provided in this study is limited, but provides insights to future study. The design of the experiments can be improved by adding a control group without nematode infection, in order to compare the effect of nematode on soybean growth. The copy number of *GmPROPEP3* should be determined since the copy number of the inserted gene can affect gene expression. Field experiments should also be designed to investigate transgenic soybeans' response to nematodes in application.

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VII. Appendix

The nematode eggs were preserved in a sucrose solution of 20 ml at 4°C after extraction from root tissue. 100 μl of the nematode egg solution were pipetted on a watch glass with 12 sections of equal area. After the eggs were evenly distributed, nematode eggs on three sections of the watch glass were counted under a microscope. The number of eggs on three sections were averaged and multiplied by 12 and then by 200 to get the total estimated number of nematode eggs in one sample.

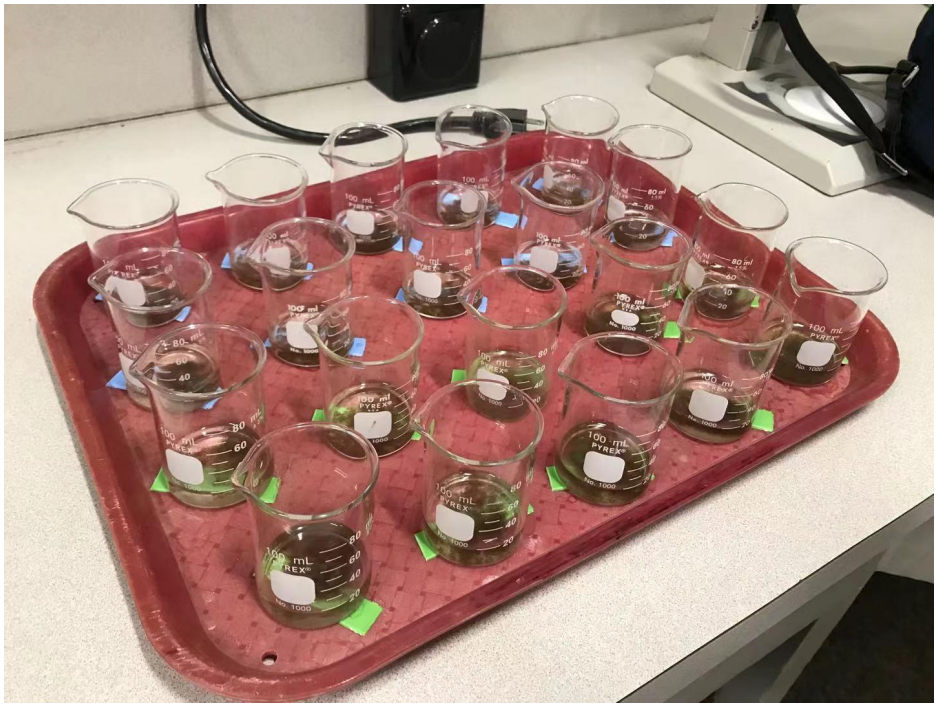

Figure A1. Nematode eggs preserved in sucrose water ready to be screened. Photo by the author.

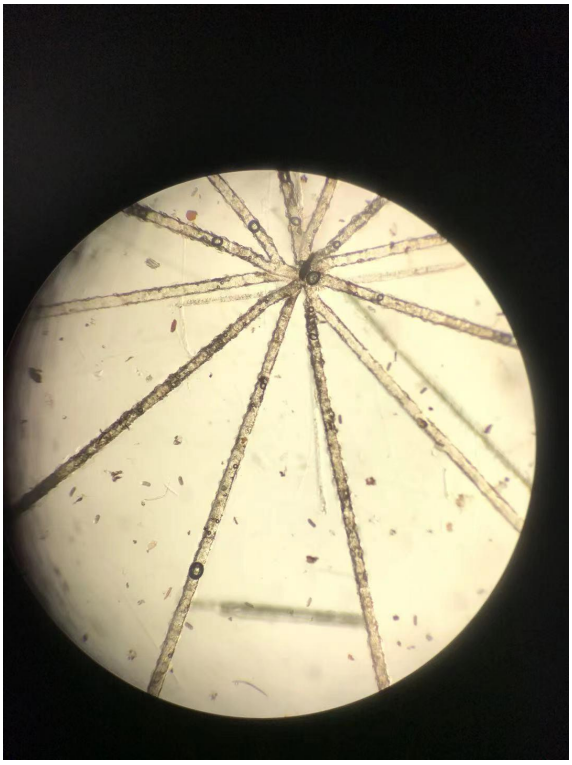

Figure A2. Nematode eggs on a watch glass under a microscope. The kidney shaped dots are the nematode eggs. Photo by the author.

Figure A3. Mr. Crippen watering plants from the RKN bioassay in the greenhouse. Photo by the author.