Analysis of two host genes required for induction of root-knot nematode feeding sites

(ネコブセンチュウが栄養摂取部位の誘導に要する二つの宿主遺伝子の解析)

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Arshana Nor NOORUL AMIN

Division of Bio-systems Sustainability,
Graduate School of Agriculture,
Hokkaido University,
Sapporo, Japan.

ABSTRACT

Root-knot nematodes are plant parasites that cause widespread loss in agriculture worldwide. Host infestation by root-knot nematodes is comprised of three stages. During the first invasion stage, nematode juveniles penetrate the root and migrate to target cells in the root tip. This is followed by the induction stage, when the nematode induces host cells to adopt a new enlarged "giant cell" morphology specialized for nutrient transfer. The nematode then feeds permanently from these giant cells for its remaining life-cycle. The induction stage is the key step that determines parasitic success and thus the development of effective control strategies requires an in-depth understanding of this process. However, the molecular mechanism(s) by which root-knot nematodes induce feeding sites remains unknown. In this study, two host genes were analysed to provide insight on the molecular mechanisms for feeding site induction. The first gene, *FIE*, was selected in a hypothesis-based approach for genes that may drive the global reprogramming process, whereas the second gene, *ASTRAY*, was selected based on infestation phenotype observed in mutant plants.

1. Functional study of the FIE Polycomb subunit during feeding site formation

In plants, the Polycomb Repressive Complex 2 (PRC2) regulates global gene expression during developmental transitions. This study therefore tested the hypothesis that the plant PRC2 complex is used by root-knot nematodes to direct the reprogramming of giant cell fate during the induction stage. The role of PRC2 was tested by perturbing expression of the complex core subunit gene, *FIE*. An inducible RNAi vector system was used for temporal knockdown of *FIE* expression during root-knot nematode invasion and induction stage. Inducer treatments were established for use with nematode infestation assays and the inducer vector was confirmed to be effective at depleting *FIE* levels in a temporal manner. A transgenic hairy root system was successfully implemented for testing and optimisation, although poor nematode infestation was observed. Nevertheless, all knockdown lines obtained showed a phenotype with at least 50% reduction in number of enlarged nematodes, indicative of a defect in the induction stage following inducer treatment. This was consistent with a role for FIE in feeding site development and stable independent transgenic plant lines have now been successfully generated that will facilitate future in-depth studies.

2. A robust system for quantitative analysis of root-knot nematode infestation

One limiting factor in understanding the molecular mechanisms of feeding site development has been the lack of a standardised quantitative assay, which should also enable downstream analyses and be free of any other biological sources of variability. As part of this study, a new robust and highly reproducible assay method has now been developed that solves these problems. This includes a new approach for preparation of high quality axenic nematode populations and optimised infestation conditions for the selected host plant *Lotus japonicus*, a model legume. In addition, an improved compatible hairy root transformation protocol was established facilitating mechanistic studies. The strategies developed here are also readily applied to different plant hosts.

3. Characterisation of the *astray* mutant defective in feeding site induction

The absence of a plant loss-of-function mutation that disrupts feeding site development has been a major factor in studying the associated molecular mechanisms. During this project a *L. japonicus* symbiosis mutant, *astray* (Gifu ecotype), was identified as having a possible defect in root-knot nematode infestation and the genetic nature of this mutation was characterised. Using a combination of genetic crosses and the above-described hairy root assay strategy, it was found that the *astray* mutation was a recessive loss-of-function trait that restricts infestation prior to completion of feeding site induction. This was associated with reduced invasion, indicating that ASTRAY has an essential function during both the invasion and induction stages. Unlike that observed for symbiosis, this phenotype was constitutively displayed in both plate and soil culture. A similar loss-of-function *astray* allele was isolated from the MG-20 ecotype by targeted mutagenesis screening, however, surprisingly the *astray* mutation in this different genetic background did not display the nematode phenotype. These results suggested that *ASTRAY* is an essential host gene for root-knot nematode infestation and that this role may have remained hidden due to the presence of parallel genetic pathways for root development.

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TABLE OF CONTENTS

ABBREVIATIONS

bp	base pairs
CDK	cyclin-dependent protein kinases
cDNA	complementary DNA
CYC	cyclin
DEX	dexamethasone
DNA	deoxyribonucleic acid
FIE	fertilization independent endosperm
J2	second stage juvenile
me	methylation
me2	dimethylation
me3	trimethylation
PcG	Polycomb group
PCR	polymerase chain reaction
PRC	Polycomb Repressive Complex
RNA	ribonucleic acid
RT-PCR	reverse-transcription PCR

1. GENERAL INTRODUCTION

1.1. Root-knot nematodes are parasites that infest plants

Plant parasitic nematodes are small worm-like animals that live primarily in the plant root throughout their life cycle (Bridge & Starr, 2007). Nematodes are a major problem in agriculture as they attack nearly all plant species. Total worldwide loss of plant yield caused by nematode infestation is estimated to be about 5%. Characteristics of nematode-infested plants are deformed root growth, stunted plant development and susceptibility to other diseases. There are several kinds of nematodes and one group of these is the sedentary endoparasites, which are primarily made up of root-knot nematodes (*Meloidogyne* species) and cyst nematodes (*Globodera* and *Heterodera* species). Root-knot nematodes are about 400 μm in length and about 15 μm in width.

A unique feature of endoparasitic nematodes is that they induce a permanent feeding site within plant roots that consist of enlarged plant cells. In the case of root knot nematode, this feeding site is made up of giant cells, whereas for cyst nematode, it is syncytia. Giant cells are host cells that are induced to become enlarged by the nematode, whereas syncytia is attained through cell wall dissolution of neighbouring cells, both resulting in a multinucleated feeding site that provides all the nutrients required by the nematode to complete its life cycle. At present, the exact molecular mechanism(s) involved in the induction of these feeding sites still remains unknown. There are similarities between root knot nematodes and cyst nematodes in terms of their life cycle, but the mechanism involved in the establishment of feeding site is different between these two nematodes. In this study, the focus is on root-knot nematodes. The major species of *Meloidogyne* are *M. arenaria*, *M. hapla*, *M. javanica* and *M. incognita*. With all these four species combined, it is capable of infesting all crop plants.

The typical life cycle of a root-knot nematode involves the invasion of a root tip by a second stage juvenile (also known as J2) and the establishment of a permanent feeding site (Figure 1.1). The J2 selects specific cells and injects unknown compounds into these cells, triggering changes within these cells. The J2 then matures through the third (J3) and fourth (J4) juvenile stages, to become an adult female. At this stage, eggs are

produced within a gelatinous mass that are visible on the root surface. The juveniles can also mature into adult males, exiting the root as vermiform nematodes. Since most root-knot nematode species are parthenogenetic, the male nematodes are unnecessary as the female nematodes are able to complete the life cycle and produce eggs without fertilization (Mitkowski & Abawi, 2003).

The formation of giant cells are believed to be induced by nematode secretions that contains effectors responsible for triggering feeding site formation. These secretions mainly originate from pharyngeal glands (two subventral and one dorsal) excreted through the stylet, but secretions from chemosensory amphids, cuticles, excretory/ secretory system and rectal glands could also be involved in processes such as invasion during early host-parasite recognition; in nematode-host interaction by disguising the invading parasite; and for producing cell wall-degrading enzyme (see review by Vanholme *et al.,* 2004). Examples of secretions include nucleosome assembly protein that regulate gene expression related to morphological changes observed in formation of giant cells (Bellafiore *et al*., 2008) and 16D10 that has been shown to interact with a plant SCARECROW-like transcription factor, which is believed to be involved in plant growth and development, and to stimulate host root proliferation (Huang *et al.,* 2003; Huang *et al.*, 2006).

1.2. Early changes related to induction of giant cells

Upon infestation by root-knot nematodes, plant cells undergo several changes. One of the earliest observation was made by Jones and Payne (1978), in which they observed cell division in host cells. Cell plate alignment proceeded normally but cytokinesis was not successful, hence leading to formation of multi-nucleated cell. As summarized by Caillaud *et al*. (2008a), some of the key changes that takes place in giant cells are altered gene regulation; cell cytoskeleton re-arrangement; extensive and meticulous remodeling of cell wall for cell expansion; involvement of plasma membrane transporter genes; and repression of defense genes.

A global view on differential regulation of genes in uninfested and infested host plant has also been obtained using plant microarray technology. In *A. thaliana*, it was found

that 3,373 genes were differently regulated between infested (galls) and uninfested roots (Jammes *et al*., 2005); whereas in tomato, 3,882 genes were differently regulated (Uehara *et al*., 2006). However, Barcala *et al*. (2010) found that only 1,161 genes were differently regulated when giant cells were compared to surrounding vascular cells. This huge difference is because giant cells only form a small portion of cells in a gall, thus when assessing gene expression of a gall, it includes giant cells and also surrounding non-giant cells. Apart from that, an increase in cell size, nuclei and DNA content in giant cells have suggested that initial induction of giant cell formation involves manipulation of host cell development at the chromosome level, particularly manipulation of the cell cycle (Jones & Payne, 1978; Wiggers *et al.,* 1990; Starr, 1993). Mitotic abnormalities such as persistent chromatin bridges were also observed (Wiggers *et al.,* 1990).

The exact mechanism on how these controlled reprogramming events occur still remains unknown. However, global change in gene expression and developmental changes that are observed in these giant cells indicate that the root-knot nematodes are using the host regulatory mechanism to convert normal host cells into feeding cells. In order to understand this better, it is important to look at cell cycle and gene regulation mechanism. The cell cycle is divided into four phases: G_1, S, G_2 and M. In the S phase, DNA replication takes place and in M phase, mitosis takes place. Between both these stages, there are gap phases, G1 and G2, which prepares the cells to enter the next phase. Cell cycle is regulated mainly by cyclin-dependent protein kinases (CDK) and cyclins (CYC). CDKA is constitutively present during cell cycle, and they control the G_1–S and G_2–M transition points, whereas CDKB is necessary for G_2-M transition (see reviews Menges *et al.*, 2005; De Veylder *et al*., 2007).

CDKs bind to CYC to form an active CDK-CYC complex for cell cycle regulation and the different combinations regulate cell cycle progression. CYCA is mainly present during S to M phase, whereas CYCB is present during G_2-M transition and M phase; and CYCD regulates G_1-S transition phase. The activity of CDK-CYC complexes can be inhibited by CDK inhibitory proteins such as interactor/inhibitor of CDK/Kip-related protein (ICK/KRP) and SIAMESE/SIAMESE-related (SIM/SMR) families. Other

regulators of cell cycle includes retinoblastoma, E2F transcriptional factors and WEE1 (see reviews Menges *et al.*, 2005; De Veylder *et al*., 2007).

Increase in DNA content of giant cells reflects the possibility of endoreduplication as well. Endoreduplication is a DNA replication process that is achieved by cycling between G_1 and S phase, which leads to a cell with an increased amount of DNA. However, it does not undergo nuclear or cell division. Endoreduplication is a residual cell-cycle competence that takes place after mitotic cell cycle and is observed in various organs of the plant. This process is usually carried out by the plant to allow cell differentiation, cell expansion, increase in metabolic activity and fitness for survival (De Veylder *et al*., 2007). In a review by Larkins *et al*. (2001), they reported that during endoreduplication, M-phase CDKs (known as mitosis promoting factors) activity is lost and S-phase CDKs are oscillated. The reduction in activity level of CYCB, which is also a mitosis promoting factor, is apparent during this process. Regulators of endoreduplication include CDK, WEE1, *SIAMESE* genes and KRP2 (a CDK inhibitor) (Larkins *et al.,* 2001; Francis, 2007).

By using *Arabidopsis thaliana*, several cell cycle markers have been identified to be expressed during the early stages of feeding site formation. This includes *AtCDKA;1*; *AtCDKB1;1*; *AtCycA2;1* and *AtCycB1;1* (Niebel *et al*., 1996; de Almeida Engler *et al*., 1999). The use of cell cycle blockers led to arrest of giant cell development during the initial stage, confirming the involvement of these cell cycle markers (de Almeida Engler *et al.*, 1999). Vieira *et al.* (2013) have reported that KIP-related protein (KRP), which is an inhibitor of CDK, have a potential role in nematode feeding site development as well. KRP expression levels are important to maintain cell proliferation and cell differentiation and disruption of *KRP* gene expression had an effect on feeding site development, suggesting the importance of these cell cycle regulator during the early phase of feeding site induction.

1.3. Induction stage determines success of parasitism

The induction of feeding site is the key stage that determines the parasitic success of root-knot nematodes. At this stage, there is a global reprogramming of host genes

(Jammes *et al*., 2005; Uehara *et al*., 2006; Barcala *et al*., 2010). Any changes to host plant, such as suppression of defense genes also takes place during stage (Goto *et al.,* 2013). This stage also marks the lifestyle transition of root-knot nematodes, as they adapt a sedentary lifestyle as opposed to their previous migratory lifestyle (Bartlem *et al.,* 2013). Induction stage also marks the initiation of feeding site formation and its success determines the nematodes' ability to complete its life cycle. To understand the underlaying mechanism of nematode infestation, it is necessary to study the host genes required during the feeding site induction.

1.4. Project aims

The induction of feeding site is the key stage that determines the parasitic success of root-knot nematodes. This induction stage should be the target for developing sustainable and effective control methods, however the mechanisms that nematodes use for inducing these giant cells remain unclear. To elucidate the underlying mechanism, targeting genes that are necessary or show potential requirement during this stage would be crucial. The aims of this project are:

- To identify the role of the *FIE* gene during the induction stage. FIE is part of Polycomb Repressive Complex 2 (PRC2), which is involved in global reprogramming of gene expression in organisms.
- To set up a robust system for effective quantitative analysis of root-knot nematode infestation using *Lotus japonicus. L. japonicus* is a model legume plant that can support root-knot nematode infestation.
- To study the *ASTRAY* gene in *L. japonicus*. This gene was selected because *astray* mutant plants showed impaired infestation phenotype.

2. FUNCTIONAL STUDY OF THE FIE POLYCOMB SUBUNIT DURING FEEDING SITE FORMATION

2.1. Background

2.1.1. Genome-wide changes in gene expression typically occur at the chromatin level

Plant cells have plasticity that allows them to continually grow and differentiate throughout their life cycle, due to the presence of pluripotent cells in the meristems (see review by Kaufmann *et al*., 2010). This leads to development of different organ types. The switch that drives the change from meristem tissues to plant organs is well studied and is controlled by multiple environmental and internal input pathways. Once a signal is received, it triggers a cascade of changes at the gene level, leading to changes of developmental programs that controls cell and organ identity. Ectopic expression of regulatory transcription factors also has the ability to trigger reprogramming of cell fate, leading to dedifferentiation or conversion of one partially or fully differentiated cell type into another. The initiation of correct differentiation program and the suppression of the previous program is a coordinated event involving multiple transcriptional factors at various levels and genes encoding structural proteins, other signaling molecules and enzymes. There are also several feedback and feedforward loops that are involved, creating a complex transcriptional network structure.

Chromatin structure that undergo global changes is associated with the initiation of differentiation or cell reprogramming, whereas local changes is associated with cell fate specification in developing organs. Chromatin-associated proteins often repress or activate transcriptional factors that control cell identity. The end of M phase and the beginning of G_1 phase in cell cycle are important for cell fate determination because at these stages, cell fate regulators are either induced or remain repressed. To maintain the cellular memory of gene expression and cell fate changes, histone-modifying enzymes are important. Such modifications include acetylation and trimethylation (me3).

2.1.2. Epigenetic regulation of gene expression

Cells translate temporary information of pattern-generating signals into stable and persistent gene expression through epigenetic modification of the genome. Epigenetics is described as any potentially stable and heritable change in gene expression that occurs without a change in DNA sequence. There are several types of epigenetic modification that typically occur during regulation of developmental genes such as DNA methylation and histone modification (by Polycomb group proteins). DNA methylation is generally associated with gene repression and shares a parallel to the Polycomb activity. Most genes that are repressed by Polycomb undergo DNA methylation during cell differentiation (Sawarkar & Paro, 2010). However, DNA methylation does not have a widespread role in regulating developmental genes because the methylation marks are usually meiotically heritable in plants and not necessarily reset in each cell generation. There has also been few research suggesting the role of DNA methylation in controlling development. This is in contrast to Polycomb-mediated gene regulation that is able to reset their marks in between cell generations and have also been reported to be involved in development phase transitions (Schubert *et al.*, 2005).

2.1.3. Polycomb complexes control gene expression

Polycomb complexes are known to have a wide array of functions, such as stem cell fate determination; neoplastic development; cell cycle regulation; and controlled timing of development. Loss of Polycomb group (PcG) function in plants leads to inhibition of cell differentiation and the failure to progress past embryo state (Sawarkar & Paro, 2010). From *Drosophila* studies, where it was first discovered, two distinct Polycomb complexes were identified: the Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). PRC1 and PRC2 are estimated to be 2 MDa and 600 kDa in size, respectively (Guitton & Berger, 2005). The function of PRC1 is to maintain the repression elicited by PRC2. This is achieved by monoubiquitylate H2AK119 using the ubiquitin ligase dRING1 in PRC1 and methylation of H3K27 through enzymatic activity of E(Z) in PRC2 (Margueron & Reinberg, 2011).

PRC1 contains Polycomb (Pc); Polyhomeotic (Ph); Posterior sex combs (Psc); Sex combs on midleg (Scm); dRING1; and general transcriptional factors. On the other hand, PRC2 contains Extra sex combs (ESC; contains WD40 domain that is necessary for a gene repressor); Enhancer of Zeste (E(Z); has methyltransferase enzymatic activity); Suppressor of Zeste 12 (Su(Z)12; zinc-finger protein); and p55 (a histone-binding protein) (Table 2.1). PcG complexes are well conserved between organisms in terms of composition and molecular function but varies according to cell or tissue type (Guitton & Berger, 2005).

From mammalian cell studies, it was also discovered that PRC2 contains several other polypeptides such as AEBP2 (a zinc-finger protein that enhance enzymatic activity of PRC2), Polycomblikes (PCLs; functions to regulate enzymatic activity of PRC2 and for gene recruitment of PRC2) and JARID2 (a protein that is interdependent with PRC2). Due to the various components that are required for PRC2 to function at maximum activity, it is considered to be a holoenzyme (Margueron & Reinberg, 2011). Even though PRC1 and PRC2 have distinct functions, both are required to maintain gene repression. Two other PcG proteins were also characterized in *Drosophila:* PHO-repressive complex (PhoRC) and Polycomb repressive deubiquitinase (PR-DUB) (Margueron & Reinberg, 2011). In addition to that, PRC1-like complex has also been identified in mammals and plants (Hennig & Derkacheva, 2009; Kaufmann *et al.,* 2010; Margueron & Reinberg, 2011; Molitor & Shen, 2013).

While PcG proteins repress gene expression, there is another complex that works antagonistically and it is the Trithorax group of proteins. Both these complexes form a dynamic molecular switch for controlling gene expression. In *Arabidopsis*, ATX1 and ATX2 have been identified to methylate H3K4 that leads to gene activation (Saleh *et al.,* 2008). As described by Sawarkar and Paro (2010), silencing by PcG is advantageous because repression is robust; multiple intrinsic and extrinsic signals are interpreted before regulating an important developmental output.

2.1.4. Genes are silenced by PRC2 and silencing is maintained by PRC1

Developmentally regulated genes that are in a silent state are generally methylated at H3K27, which is catalysed by the E(Z) histone methyltransferase in PRC2. PRC2 can

either monomethylate (me), dimethylate (me2) or trimethylate (me3) H3K27, with H3K27me3 being a stable mark (Margueron & Reinberg, 2011). In wild type *Arabidopsis,* H3K27me is found mainly in the heterochromatin; H3K27me2 is found predominantly in heterochromatin but weakly in euchromatin; and H3K27me3 is dominant in euchromatin (Schubert *et al.,* 2005).

Several interactions required for PRC2 recruitment have been studied in *Drosophila.* Lund & van Lohuizen (2004) have reported that DNA sequences known as Polycomb response elements (PREs) are targeted by PRC2. Apart from that, RNA Polymerase II that is stalled at CpG islands (rich in C and G nucleotides) during gene transcription produce non-coding RNA (ncRNA) that can also recruit PRC2 (Guenther & Young, 2010). In addition, Margueron and Reinberg (2011) have suggested several other interactions that takes place for a successful recruitment of PRC2 in mammalian cells such as interaction of JARID2 and AEBP2 with DNA; interaction of histone-binding protein to histones; and interaction of EED (homolog of FIE in plants and ESC in *Drosophila*) with H3K27me3.

Studies with *Drosophila* have indicated that once the H3K27me3 mark has been established, it facilitates the recruitment of PC, a component of PRC1. PRC1 then maintains this transcriptional repression through the following proposed mechanisms: (1) PRC1 blocks transcription factors and chromatin remodeling factors from accessing DNA; (2) PRC1 recruits chromatin-modifying enzymes such as histone deacetylase; and (3) PRC1 and H3K27me3 blocks gene activating marks such as H3K4 methylation (Lund & van Lohuizen, 2004).

2.1.5. Silencing by Polycomb can be reversed by demethylase

Gene silencing is a reversible process. Modified histones could be passively diluted during mitosis or actively exchanged to unmodified histones (Henning & Derkacheva, 2009). However, during specific stages of development, the H3K27me3 mark decreases indicating that a specific demethylase exists to remove this epigenetic silencing. Several histone demethylases have been identified in plants. Through phylogenetic analysis, JMJ14 (in *Arabidopsis,* TAIR accession no.: At4g20400) and JMJ15 (in *Arabidopsis,* TAIR accession no.: At2g34880) have been identified for demethylation of

H3K4; and JMJ25 (in *Arabidopsis,* TAIR accession no.: At3g07610), JMJ11 (in *Arabidopsis,* TAIR accession no.: At5g04240) and JMJ706 (in rice, Os10f42690) have been identified for H3K9 demethylation (Chen *et al.*, 2011). Based on the antagonistic action on EARLY FLOWERING IN SHORT DAYS (EFS; a histone methyltransferase), RELATIVE OF EARLY FLOWERING 6 (REF6) was proposed (Chen *et al.*, 2011) and shown to be a plant demethylase for H3K27me3/me2 marks (Lu *et al.,* 2011).

Instead of a demethylation mechanism, Sawarkar and Paro (2010) have described an alternative mechanism that leads to loss of H3K27me3 mark. In lung fibroblasts, it was discovered that phosphorylated serine 28 on H3 (H3S28p) and H3K27me3 marks resulted in a double modification of the chromatin. This lead to loss of recognition by PcG proteins, and subsequently the target gene is de-repressed. However, this has not be shown in plants yet.

2.1.6. Polycomb in plants

In *Arabidopsis*, several PcG genes that are conserved in PRC2 have been identified. Homologue of E(Z) is encoded by three genes: *CURLY LEAF* (*CLF*)*, SWINGER* (*SWN*) and *MEDEA* (*MEA*). The ESC homologue in *Arabidopsis* is encoded by *FERTILIZATION INDEPENDENT ENDOSPERM (FIE)* gene and there is only one copy of this gene in *Arabidopsis*. Homologue of Su(Z)12 is encoded by *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*), *EMBRYONIC FLOWER 2* (*EMF2*) and *VERNALIZATION 2* (*VRN2*) genes. These proteins form at least three known PRC2-like complexes: EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN) and FERTILIZATION INDEPENDENT SEED (FIS). Although these complexes have distinct functions, some of them target the same genes (Ohad *et al.,* 1999; Guitton & Berger, 2005; Hennig & Derkacheva, 2009).

Initially, it was assumed that plants do not have PRC1 to stabilize silencing. Schubert *et al.* (2005) remarked that the lack of PRC1 in plants reflects the instability of the silencing mechanism in plants, hence cell fate in plants is more liable to change compared to its animal counterpart. However, of recent plant PRC1 components have been identified. Core PRC1 members can either catalyze histone post-translational modifications (“writer proteins”) or can recognize specific histone modification marks

("reader subunit"). The writer proteins can be subdivided into either RING1 or BMI1 clade. They all share a conserved N-terminal RING-domain and C-terminal ubiquitin-like domain called RAWUL; and has H2A monoubiquitination properties, similar to that observed in animals. The LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) has also been shown to have a N-terminal CHROMO-domain and C-terminal CHROMOSHADOW-domain which is always associated with Pc homolog proteins, thus confirming the homolog of Pc in plants is LHP1. Other components that were proposed to have PRC1-like functions were VRN1 and EMF1, which act downstream of PRC2 complexes. EMF1 has been reported to have remodeling activity similar to Psc. I*n vivo* protein interaction between the PRC1 components in plants are yet to be studied, so complete understanding in plants is not yet achieved (Guitton & Berger, 2005; Hennig & Derkacheva, 2009; Molitor & Shen, 2013).

2.1.7. FIE is the core protein in PRC2

FIE has a role in histone methylation; regulation of endosperm development; regulation of gene expression by genetic imprinting; reproduction and vernalization (Yadegari *et al*., 2000; Kohler *et al*., 2003; Katz *et al*., 2004; Wood *et al*., 2006). It is reported to be widely expressed in cauline leaves, stem and roots throughout the entire plant life cycle. *FIE* encodes a WD40-type protein that is a homologous to ESC (from *Drosophila*) and EED (from human), with all three containing seven repeats of WD motif. The multiple copies of WD motif allows FIE to be able to bind simultaneously to various different polypeptides, resulting in multi-protein complexes *(*Ohad *et al.*, 1999).

FIE regulates class I KNOX genes that regulate shoot meristem maintenance (such as *BREVIPEDICELLUS*, KNOTTED-like from *Arabidopsis thaliana* 2 and *SHOOTMERISTEMLESS*); MADS-box genes (such as *AGAMOUS*, *APETALA3* and *AGAMOUS LIKE 17*); and PcG SET domain proteins such as MEA (Katz *et al.,* 2004). Hence, any mutation in *FIE* leads to an altered developmental program. FIE was knocked down in *Arabidopsis* through co-suppression and its effect throughout the life cycle of the plant was observed. Knockdown of FIE lead to aberrant phenotype but allowed plants to reach maturity for analysis of the gene function. Loss of apical dominance, curled leaves, early flowering and homeotic conversion of leaves, flower

organs and ovules into carpel-like structures were some of the phenotypes that were observed. This showed that FIE was crucial for developmental processes in shoot and leaf development (Katz *et al*., 2004), but the role of FIE in root development is still unclear. There is a possibility that constitutive knockdown of FIE has more pronounced effect in the root system, hence an inducible RNAi construct would be a better option to study this gene.

FIE-silenced plants and *clf* mutants share the same leaf and flower phenotype, indicating a possibility that FIE and CLF share common function. Through yeast two-hybrid analysis, FIE was suggested to be able to form complexes with CLF. Initially, PcG proteins in plants were thought to only regulate MADS-box gene expression and not homeobox genes as seen in *Drosophila*. However, it was reported that plant PcG proteins regulate homeobox genes as well. This implies that the functional role of PcG proteins is conserved between plant and animals (Katz *et al*., 2004).

Apart from CLF, FIE also forms complex with MEA and this was confirmed through yeast two-hybrid analysis (Spillane *et al*., 2000; Yadegari *et al.*, 2000). FIE and MEA belong to *FERTILIZATION INDEPENDENT SEED* (*FIS*) class of genes. By using size exclusion chromatography, it was found that MSI1 (interacts with retinoblastoma), MEA and FIE form a complex. This complex has a molecular weight of 600 kDa and is similar to its *Drosophila* counterpart. Through binding assay, it was shown that MSI1 can efficiently bind to FIE but not to MEA. This suggested that even though MSI1 is part of the FIE-MEA complex, its interaction with MEA requires FIE to mediate the interaction (Kohler *et al*., 2003). Pull-down assays between FIE and retinoblastoma showed that these two molecules interact (Mosquna *et al.*, 2004). In the context of cell cycle, FIE interacts with retinoblastoma and subsequently, retinoblastoma controls the transition between G_1 and S phase (Ingouff *et al.*, 2005).

FIS gene negatively regulates endosperm growth and proliferation. This was supported by several findings that report *fis* mutation lead to autonomous seed development in absence of fertilization; interferes in endosperm development; and prevents mitotic domain formation. Phenotypical changes in *fis* mutant and wild-type was only detected after the embryo heart stage, whereby *fis* mutants do not undergo

arrest of proliferation and show improper differentiation. The *FIS* gene is required for endosperm molecular differentiation after cellularization. However, the *fis* mutation does not impair basic cellular processes such as growth and proliferation. It was also reported that *PHERES1* is ectopically expressed in *fis* mutant endosperm (Ingouff *et al*., 2005). *PHERES1* belongs to the type I-MADS box transcription factor family and is expressed during early endosperm development but its role is not understood, however it is a direct target of FIE-MEA complex (Guitton & Berger, 2005).

Another PcG protein that belongs to FIS is FIS2. Analogous to *Drosophila* and mouse studies, FIS2 in plants acts as a zinc-finger protein that recruits FIE. Other proteins that are bound by FIE would come together as well to form a stable complex for repression of gene transcription (Yadegari *et al.*, 2000). Mutations in *EMF2* (homolog of *FIS2*) leads to bypass of vegetative growth phase and immediate transition from embryonic stage to flowering stage, which causes production of flowers by embryos (Yoshida *et al.*, 2001).

2.1.8. Chemically-inducible vector allows temporal control on gene expression

Inducible vectors have been widely used to achieve temporal control on gene expression, either for over-expression or knockdown assays. This has been possible by using a transactivating system. In general, an 'activator' line carrying the heterologous transcriptional activator is crossed with an 'effector' line that carries the promoter with binding sites for the heterologous transcriptional activator. The progeny will express the gene of interest (Moore *et al.,* 2006). Without the presence of the activator, the effector is non-functional (Moore *et al.,* 1998).

One of the most widely studied transactivation system is the pOp/LhG4 system, which has two components: pOp and LhG4. pOp contains a minimal CaMV 35S promoter with two copies of an optimized *lac* operator located upstream, whereas LhG4 is a fusion between $lacI^{his}$ repressor and VP16 activation domain. The pOp/LhG4 system has been reported to be stable and was still active in at least three successive plant generations (Moore *et al.,* 1998). To improve the versatility of this system, a glucocorticoid receptor was added to the activator sequence, which allows temporal control on gene expression by the use of a chemical. The ligand binding domain of rat

glucocorticoid was used, which was activated by the presence of dexamethasone (DEX). Without DEX, the transcriptional factor is trapped in an inactive complex with HSP90, a heat shock protein in the cytoplasm. The DEX treatment mediates the disassociation of the transcriptional factor from HSP90, allowing it to activate the promoter, in this case pOp promoter. The efficiency of pOp promoter was also improved by including six copies of ideal *lac* operator (Craft *et al.,* 2005; Samalova *et al.,* 2005).

By using the existing pOp6/LhGR system, Wielopolska *et al.* (2005) have modified the inducible system so that both the activator and effector are on the same vector, hence reducing the time and effort required for plants expressing the gene of interest in an inducible manner. The authors also used a bidirectional pOp6 promoter that drives a GUS reporter gene and gene of interest, which in this case, was a hairpin. This inducible RNAi vector, pOpOff2, showed rapid silencing as early as 6 hours post-induction, with maximum activity at 24 hours post-induction. Silencing of endogenous genes were also reversible, thus making this system very attractive for temporal control for gene expression.

Other transactivation systems also exists, such as *alc* system (alcohol-inducible) and Cre/*loxP* system (ß-oestradiol-inducible). However, one of the main disadvantage of these systems are full induction is toxic to plants and silencing is limited to sectors, in those respective inducible system. On the other hand, the DEX system allows full induction without toxicity to plants and efficient knockdown throughout the plant (Moore *et al.,* 2006).

2.1.9. Specific aims

The increase in number of down-regulated genes in the giant cells indicate that there is a global control on gene expression. In plants, PRC2 has been associated to play this role. PRC2 is also important during developmental stage transitions. There are several Polycomb complexes, however, each complex has a core FIE protein. To objective of this part of the study is to identify the role of *FIE* gene during the induction stage of nematode infestation. This was done by removing FIE using a DEX-inducible system. The specific aims of this experiment are:

- To achieve functional knockdown of *FIE* in tomato hairy root cultures.
- To set up a compatible assay system for inducible system and nematode infestations.
- To study nematode infestation during knockdown of *FIE* gene.

3. A ROBUST SYSTEM FOR QUANTITATIVE ANALYSIS OF ROOT-KNOT NEMATODE INFESTATION

3.1. Background

3.1.1. *Lotus japonicus* as a host for root-knot nematodes

Previous molecular studies on root-knot nematode have primarily taken advantage of *A. thaliana* as a model host (Sijmons *et al*., 1991) due to the extensive genetic and molecular resources available. This has provided much information on molecular responses to feeding site development and requirement for core cell biological processes, including those associated with regulation of the cell cycle (de Almeida Engler *et al*., 1999; de Almeida-Engler *et al*., 2012); the actin cytoskeleton (Favery *et al*., 2004; Caillaud *et al*., 2008b); plant defense (Jammes *et al*., 2005; Barcala *et al.*, 2010); nutrient transport (Hammes *et al*., 2005; Hammes *et al*., 2006); and vascular tissue formation (Hoth *et al*., 2008; Absmanner *et al*., 2013). Although much has been learned about the biology of the root-knot nematode feeding sites, key molecular determinants for the induction of feeding cells in the host remain elusive and related genetic mutants that can provide insight on this are yet to be identified despite over 20 years of molecular work with *Arabidopsis*. Alternative systems are clearly necessary and recent studies have thus begun to utilise other model host plants as the biological resources for these species rapidly expand, such as tomato (Bar-Or & Kapulnik, 2005; Fosu-Nyarko *et al*., 2009; Portillo *et al*., 2013) and rice (Kyndt *et al*., 2012; Nahar *et al*., 2013) that differ from *Arabidopsis* in that they are also natural hosts for root-knot nematodes.

One favourable host for root-knot nematode studies is *Lotus japonicus*, which is a small legume plant with a short generation time and is used as a model to study the genetic and molecular biology of legume plants and symbiosis (Handberg & Stougaard, 1992). Genetic mutations affecting development and biotic interactions are readily available and a dedicated biological resource centre also exists (Hashiguchi *et al*., 2012). *L. japonicus* has been previously reported to be a potentially powerful model for root-knot nematode infestation studies (Lohar & Bird, 2003), although a limiting factor

to its wider use has been the difficulty in identifying suitable culture conditions and readily-accessible protocols for the quantitative analysis of root-knot nematode infestation.

3.1.2. Hairy root as a system for root-knot nematode infestation assays

Stable transgenic plant lines and transgenic hairy root cultures of *L. japonicus* are able to be established using *A. radiobacter* and *R. rhizogenes*, respectively (Handberg & Stougaard, 1992; Stiller *et al*., 1997). Plant hairy root cultures have rapid growth, are genetically and biochemically stable, and can be maintained in a hormone-free media; features that have led to them being used in studies on plant metabolism (Shanks & Morgan, 1999; Christey & Braun, 2005). Due to the relatively short time period required to generate hairy root cultures (approximately 3 weeks), they also provide a rapid turnover for testing transgenic constructs and the influence of gene expression perturbation in biotic interactions prior to establishing stable transgenic plants lines (approximately 4 to 6 months).

L. japonicus hairy roots are widely used in studies on symbiotic interactions such as nodulation (Grønlund *et al*., 2005; Heckmann *et al*., 2006; Okamoto *et al*.. 2009; Soyano *et al*., 2013), however, a high transformation efficiency has proven difficult to achieve and depending on the protocol used, only 50% to 75% of the generated roots are transgenic (Stiller *et al*., 1997; Martirani *et al*., 1999; Díaz *et al*., 2005). This problem has been circumvented by including a constitutively expressed GFP gene in the transformation vector to distinguish transgenic roots based on GFP fluorescence and not including the non-transgenic roots in the assay scoring (Kumagai & Kouchi, 2003; Grønlund *et al*., 2005; Suzaki *et al*., 2012; Soyano *et al*., 2013). In the case of root-knot nematodes, comparisons against equivalent transgenic material containing either control constructs or the empty vector are preferred over the use of non-transgenic roots as the former are expected to provide greater reliability.

The use of GFP and similar reporter genes to select transgenic roots for biotic interaction studies does require a compromise as it restricts their use in common applications such as non-invasive monitoring of protein localisation or transcriptional responses associated with changes in signaling pathways. An improvement in current

transformation efficiencies is required that allows distinguishing transgenic roots from the remaining non-transgenic roots, that also supports root-knot nematode parasitic life-cycle.

3.1.3. Specific aims

Understanding the root-knot nematode parasitic process will allow the development of effective control strategies. However, lack of a standardized conventional method for quantitative measurement of host parasitism by root-knot nematodes, particularly one that enables efficient downstream analyses, is a limiting factor. The objective of this study is to set up a robust system for effective quantitative analysis of root-knot nematode infestation using *L. japonicus.* The specific aims of this experiment are:

- To establish a reproducible system to obtain pure nematode inoculum.
- To optimize *L. japonicus* hairy root transformation protocol.
- To study the compatibility of this optimized hairy root protocol with nematode infestation assays.

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FIGURES

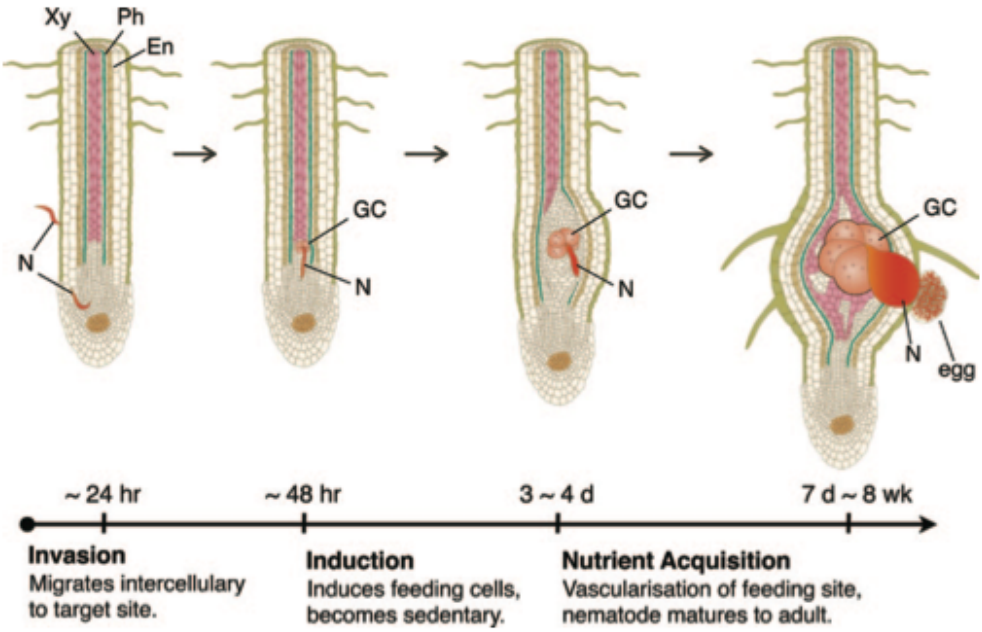

Figure 1.1:
Root-knot nematode infestation mechanism is divided into invasion, induction and nutrient acquisition stages. The induction of host cell to become large, multi-nucleated cells is a key step in the infestation process and remains unknown. The root-knot nematode (N), xylem (Xy), phloem (Ph), endodermis (En), giant cell (GC) and egg are shown (from Bartlem *et al.,* 2013).

TABLES

Table 2.1:
PcG protein components of PRC1 and PRC2 in *Drosophila* and their homologs in plants and mammalian cells (from Lund & van Lohuizen, 2004; Guitton & Berger, 2005; Hennig & Derkacheva, 2009; Sawarkar & Paro, 2010; Molitor & Shen, 2013).

Polycomb complex	In *Drosophila*	In plants	In mammals
PRC1	Pc	LHP1	CBX2 CBX4 CBX6 CBX7 CBX8
	dRING	RING1A RING1B	RING1A RING1B
	Ph		PHC1 PHC2 PHC3
	Psc	BMI1a BMI1b BMI1c	Bmi1 PCGF1-6 Mel18
PRC2	Su(Z)12	EMF2 VRN2 FIS2	SUZ12
	E(Z)	CLF SWN FIE	EZH1 EZH2
	ESC	FIE	EED
	p55	MSI1	Rbap46 Rbap48

Table 2.2:
List of primers used for amplifying genes to be used in generating plant transformation vectors. The expected amplicon size (in base pairs) and target material are also shown.

Gene	Forward primer	Reverse primer	Size	Source
YFP	5'- CACCAGCAAGGGCGA GGAGCTGT -3'	5'- GCTCGATGCGGTTCAC CAG -3'	370	pDBG12
GUS	5'- CACCATGTTACGTCCTG TAGAAACCCCA -3'	5'- TCATTGTTTGCCTCCCT GCT -3'	1812	pMDC164