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COMPARING AND CONTRASTING TWO PLANT PATHOGENS PROVIDE A UNIQUE WINDOW INTO THE DIFFERENCES IN THE INNATE IMMUNE RESPONSES BETWEEN DICOTS AND MONOCOTS

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B.S., Beijing University, 2003

Advisor: David G. Lynn, PhD.

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ABSTRACT

COMPARING AND CONTRASTING TWO PLANT PATHOGENS PROVIDE A UNIQUE WINDOW INTO THE DIFFERENCES IN THE INNATE IMMUNE RESPONSES BETWEEN DICOTS AND MONOCOTS

By Yue Liu

The eukaryotic parasitic plant *Striga asiatica* and the prokaryotic *Agrobacterium tumefaciens* are both plant pathogens that cue on host-derived phenylpropanoids for host recognition. The onset of virulence in both pathogens is regulated by a wounding event, but one manifested in slightly different ways. *S. asiatica* mimics a partial wounding response by producing reactive oxygen species on host contact, whereas *A. tumefaciens* depends on the wounding response of host plants to generate susceptible plant cells for transformation. Despite the similarity in their chemical strategy for host recognition, *A. tumefaciens* and *S. asiatica* pathogenize non-overlapping host ranges; monocotyledonous grasses are resistant to *A. tumefaciens*, but susceptible to *S. asiatica*. These two plant pathogens then provide a unique window into the differences in the innate immunity of dicots and monocots. The engineering potential of *A. tumefaciens* further provides an opportunity to map the signaling landscape at the host-pathogen interface. This study represents the first example of mapping systemically the virulence inducing signal landscape *in vivo* and in real time. I quantified not only the concentration of wound-induced phenol and sugar signals separately, but the duration of exudation. These results demonstrated that the fundamental difference in the wounding response between dicots and grasses is communicated via their wound-induced phenol exudation. *A. tumefaciens* recognizes dicots by the high level and extended exudation of wound-induced phenols, whereas grasses remain invisible to *A. tumefaciens* by not inducing phenol exudation. The modular nature of the subterranean development of *S. asiatica* further provides a valuable opportunity to study genetic regulation of development via differential expression libraries. The immune response genes and phenylpropanoid biosynthetic genes found in the differential libraries highlighted again the critical roles that the wounding response and phenol production play in plant pathogenesis. Our study on the phenylpropanoid biosynthetic genes further indicated that phenolpropanoids play a most critical role in regulating plant development beyond lignin biosynthesis. Lastly, the interaction between quinolic haustorial inducers and auxin/cytokinin signaling pathways suggest a possible evolutionary route for the parasitic plants in *Scrophulariaceae* and *Orobanchaceae* to have emerged.

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I want to take this opportunity to thank my advisor Dave for what he has shown me during the years I spent in graduate school. Dave is the first person that I talked to when I came to Emory for interview. He is very personable and this is part of the reason that I joined the lab. The Lynn lab has an exceedingly wide spectrum of research interests ranging from organic chemistry, biochemistry, microbiology to plant biology. Coming from a purely chemistry background I wanted to learn biology but was not sure whether I should take the risk of venturing into a brand new field at the beginning of graduate school. I brought up this idea to Dave and heard him telling me that "if you choose something you already know, then you won't learn anything new in the end". I therefore jumped right into the Striga project, and this journey did turn out to be rewarding to me. Dave has faith in all his students that we all will grow and become a great scientist. At his birthday last year, many of his former students came to celebrate as a lab reunion, including his first graduate student who graduated from Lynn lab 28 years ago. I found that everyone thanked him for the same reason that he truly imparted a confidence into them to not be afraid of being challenged to learn new things, and to be independent researchers able to solve problems. I thank Dave for giving me the opportunity to learn this priceless lesson early in graduate school. Above everything else, I thank Dave for showing me what science is. To him science is not merely a career, but it is about who he is. Such a person can truly communicate what science is to others. I also thank Dr. Edmondson and Dr. Conticello for their unfailing support over these years. The biochemistry courses I took from them is the foundation that I started from. They helped

me make the transition from chemistry to biology. Their annual inputs on my research have been a bright light to show me where I am and where I need to go.

At the time when I joined the lab Dr. Lizhi Liang and Dr. Yingzhen Kong had pioneered in employing the differential library approach to study genetic regulation of haustorium development, and Dr. Andrew Palmer was concluding his work on Semagenesis. They are the three giants on whose shoulder I have stood. I followed Lizhi in using the library approach to map the genetic regulation of plant development, and she taught me basic molecular biology skills. I would express my great gratitude to all the three forerunners, esp. Lizhi. I thank Dr. Yi-Han Lin for being an awesome colleague and friend. Her discovery of the Y293F mutation in VirA made my Agrobacterium project possible, and she is a great molecular biologist that helped me prepare the mutant strains of Agrobacterium. Her great scientific insights and friendship has been a highlight towards the end of my journey in graduate school. I thank Phoebe Young for being an enlightening and encouraging colleague and friend of mine. Phoebe joined the lab as a freshman and started off by working with me. At the very beginning she convinced me that I am studying the most awesome phenomenon on the earth, plants. Ever since then I was impressed by Phoebe in many aspects which has been a source of motivation to keep me go on until this day. I thank Phoebe for coming to Emory. I thank Dr. Anil Mehta and Dr. Jay Goodwin for taking the responsibility in various aspects in the lab which makes it easier for the rest of us to focus on research. They cared for all my needs during my thesis writing period to make my life easier. I also thank the rest of the lab including Tolu, Dr. Dibyendu Das, Chenrui, Dr. Junjun Tan, Savannah, Lisa and Li for being great

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   http://www.aphis.usda.gov/publications/plant_health/content/printable_version/fs_phwitchweed.pdf

Chapter 1 – Introduction

All living organisms exist within a complex web of dynamic material and energy exchange. Specific and interdependent associations are fundamental to their growth and reproduction. Some of the best known and most intricate examples are found within the plant kingdom, including the fixation of atmospheric carbon into the food chain through the sequestering of photosynthetic plastids by eukaryotic cells, the reduction of atmospheric nitrogen through the symbiosis of legumes and nitrogen fixing rhizobia, and the intimate dependence of most flowering plants on insects for sexual reproduction. Although sessile by definition, plants have never been passive in their interactions with other life forms. A single gram of fertile soil can contain 10^9 bacteria, 10^6 actinomycetes, 10^5 fungi, and millimeters of plant roots (Estabrook and Yoder, 1998). These rich microbial communities existing within the rhizosphere appear to be actively regulated by phenolic secondary metabolites released from plant roots. Such primary signaling molecules have been found to mediate the establishment of symbiotic, mutualistic or pathogenic associations between plants and other organisms and cue developmental and physiological events critical in the interaction (Baker et al., 1997). It has been argued that more than 8000 different phenols are produced by plants to communicate constantly with a multitude of diverse organisms (Estabrook and Yoder, 1998), and this need for communication may underlie the approximately 120 kg/ha of plant-derived phenols being added into grassland soil annually (Siqueira et al., 1991).

The phenylpropanoid pathway accounts for the largest group of plant secondary metabolites and is the source of most phenols (Dixon and Paiva, 1995). Apparently different biotic interactions, including pathogenic and symbiotic microbes, can elicit similar initial responses from plants as physical wounding, including the regulation of enzymes of the phenylpropanoid pathway, peroxidases, chitinases, proline-rich plant cell wall proteins, and those regulating ion fluxes (Baron and Zambryski, 1995). Many of the molecules known to be recognized by parasitic plants for host identification are structurally similar to phenylpropanoid phytotoxins produced in plant allelopathic interactions (Tomilov et al., 2006b), supporting Peter Atsatt's initial suggestion over thirty years ago that parasitic plants, like specialist insect herbivores, may cue on plant defense molecules for host recognition (Atsatt, 1977). In this thesis, I have focused on what role the interplay between phenylpropanoid production and wounding responses play in plant pathogenesis. Defining the chemical strategies that have allowed for the emergence of such complex interdependence is critical to both our understanding of evolutionary pathways and to the synthetic control of both destructive and beneficial associations. A particularly useful comparison exists with the parasitic angiosperms as represented by *Striga asiatica*, an immobile organism that survives by identifying and attaching to an equally sessile host, and *Agrobacterium tumefaciens,* a soil microbe that identifies host plants and engages in the only known example of inter-Kingdom gene transfer.

A. tumefaciens is a gram negative α-proteobacteria with an extremely broad host range and is able to induce tumors on infected plants ranging from dicotyledonous

Angiosperms to Gymnosperms (De Cleene and De Ley, 1976). Tumor induction involves transferring Agrobacterium-derived DNA to genetically transform its host (Schell, 1977; Barton et al., 1983; Herrera-Estrella et al., 1983). This capability has been extended technologically to an even wider spectrum of plants including some monocots, fungi, and even some human cells (Gelvin, 2005). Virulent strains recognize three kinds of chemically distinct host molecules, phenylpropanoids, sugars, and acidic pH, and activate virulence pathways only when the appropriate combinations of signals are perceived. Moreover, there is unusual chemical diversity, particularly in the phenylpropanoids, that appear to be critical to maintaining the broad host range (Melchers et al., 1989; Ankenbauer and Nester, 1990; Cangelosi et al., 1990; Duban et al., 1993a).

Our understanding of Agrobacterium pathogenesis revolves around a wounding event, but how this wound impacts the behavior of the bacteria at the host-pathogen interface is not understood. What is known is that all the signaling appears to funnel through the two-component signaling module known as VirA/VirG. In this thesis, the downstream virulence gene expression events have been coupled with green florescent protein (gfp) reporter constructs to allow for real time monitoring of the signal input *in vivo* at the host-pathogen interface. In addition, a series of specific mutant stains of *A. tumefaciens* have been constructed to allow for signal detection with higher sensitivity. These elements allow us to map the wound induced vir-inducing signal landscape of a plant *in vivo* and in real time. This systematic mapping has allowed us to understand what role the interplay of wounding and phenylpropanoid exudation play in determining the host range of Agrobacterium.

The other plant pathogen we studied is a parasitic plant, *S. asiatica*. Striga spp., also known as witchweeds because of their morphological impact on their hosts, have a greater impact on humans worldwide than any other parasitic plan, because their hosts are subsistence crops grown widely in Africa and Asia, including maize, sorghum, millet, rice, sugar cane and legume crops such as cowpea and ground nut (Kuijt, 1969b; Parker, 2009). Two major species alone, *Striga asiatica* and *Striga hermonthica,* result in over $1 billion in lost annual agricultural yields, in addition to the devastating impact on subsistence farmers of these regions (Nickrent and Musselman, 2004; Parker, 2009). Striga is an obligate hemiparasite, that is, they require a host for survival, but contains chlorophyll when mature (hence are photosynthetic) and obtain water, with its dissolved nutrients, by connecting to the host xylem via a haustorium. This modified root forms a morphological and physiological link between the parasite and the host and is "the organ that embodies the very idea of parasitism" (Kuijt, 1969b). Ever since the discovery of *Striga asiatica* in the United States during the 1950s (Appendix 1) a great deal of research has focused on *S. asiatica* parasitism. Host-derived signals have been identified that mediate the critical developmental steps during host recognition including both seed germination and haustorium induction (Chang and Lynn, 1986; Chang et al., 1986; Boone et al., 1995). The process of haustorial induction signifies the commitment to parasitism and the signaling mechanism is now known as Semagenesis, an active chemical strategy for host identification(Keyes et al., 2007). Semagenesis, as the name implies, involves a signal generation loop where reactive oxygen species (ROS) generated at the root tip of *S. asiatica* oxidize phenolic compounds at the host root

surface when the root tip of *S. asiatica* grows into close proximity of the host. The oxidation products of the host-derived phenols, benzoquinones, feedback to *S. asiatica* to induce haustorial development (Chang and Lynn, 1986; Keyes et al., 2007). The strategy of cueing on host-derived signals ensures that the parasite is in close proximity to its host when it commits the extremely limited nutrient store in the seed for germination and haustorial development.

Germination in obligate root parasites is usually cryptocotylar, that is, the cotyledons remain within the seed coat when the delicate radicle emerges (Kuijt, 1969b). The onset of shoot development under natural conditions occurs after host attachment, likely to be induced by another host-derived signal transferred from the host into the parasite through the haustorium. Under laboratory conditions, we have found shoot initiation depends on haustorial initiation and the phytohormone cytokinins are sufficient to induce shoot apical development. After host attachment and shoot initiation, the Striga seedling is established and exerts a powerful impact on the host by altering hormone balances and stimulating root production (Kuijt, 1969b; Graves, 1995). This highly specialized subterranean part of the life cycle of *S. asiatica* is then followed by emergence from the soil, chlorophyll biosynthesis, flowering, and seed development to complete the life cycle.

Since the subterranean developmental stages of *S. asiatica*: germination, haustorium development and shoot initiation, are all separate and require host-derived signals to proceed, we recognize that it should be possible to identify genes involved in the regulation of each step via differential expression cDNA libraries. In this thesis, I have

exploited these host-derived signaling events to create differential expression cDNA libraries for identifying these genetic elements. An understanding of the genetic mechanisms underlying haustorium development in *S. asiatica* provided initial insight into the origins of the haustorium and the evoluction of parasitism in *Scrophulariaceae* and *Orobanchaceae* families.

The eukaryotic parasitic angiosperm *S. asiatica* and the procaryotic *A. tumefaciens* are both plant pathogens that cue on host-derived phenylpropanoids for host recognition. The onset of virulence in both pathogens is regulated by a wounding event. *S. asiatica* exploits the molecular mechanisms of the wounding response by producing ROS at its root tip to actively probe for a host, but in a tightly regulated manner so as not to damage the parasite or alarm the host. When the root tip grows into close proximity to the host roots, the controlled wounding response of *S. asiatica* actively generates virulence inducers. In contrast, *A. tumefaicens* exploits a wound, typically along the soil surface in the host plant, and hence the name crown gall tumors. We show that the wound-induced phenols targeted by *A. tumefaciens* allow it to identify susceptible host cells for transformation. Despite the similarity in their chemical strategy for host recognition, Agrobacterium and Striga exploit contrasting host ranges, most notably in regard to members of the grass family of monocotyledonous plants, which are resistant to Agrobacterium, but susceptible to Striga. Monocotyledons (monocots) and dicotyledons (dicots) are the two branches of flowering plants that are morphologically distinct from each other including the number of cotyledons (seed leaves), pollen furrows, and flower pedals, the arrangement of leaf veins, stem vascular bundles and root systems. Most

monocots are small herbaceous plants in lack of secondary growth and dies down every year. In contrast, most trees and shrubs and many garden annuals and perennials are dicots, and there are many more species of dicots than there are monocots.With regard to the host range of Agrobacterium, monocots are considered to be resistant to Agrobacterium due to the different phenolic content when compared to dicots. However, it is unclear what host recognition mechanisms enables Striga, which also cues on host-derived phenols, to target monocots. Now the engineering potential of *A. tumefaciens* and the opportunity to study genetic pathways underlying virulence induction of *S. asiatica* via differential expression libraries provides a valuable window into what determines the host range of plant pathogenesis, and what molecular mechanisms evoled in both plant pathogens for host recognition.

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Chapter 2 - Dissecting the genetic regulation of haustorium development and shoot initiation of *Striga asiatica* by differential expression libraries

2.1 Introduction

Striga spp generally are obligate hemiparasites, that is, they require a host for survival but contain chlorophyll when mature (hence are photosynthetic). These parasites obtain water with dissolved nutrients by connecting to the host xylem via a specialized organ known as the haustorium. Ever since the discovery of *Striga asiatica* in the United States during the 1950s (Appendix 1), a great deal of research has focused on *S. asiatica* parasitism. Host-derived signals have been identified that mediate the critical developmental steps during host recognition including both seed germination and haustorium induction (Chang and Lynn, 1986; Chang et al., 1986; Boone et al., 1995). The requirement of a host-released germination signal ensures close proximity between *S. asiatica* and the host at germination (Worsham, 1987; Lynn and Chang, 1990; Boone et al., 1995). Following germination, *S. asiatica* depends on a second host-derived signal to switch from vegetative growth of a delicate radical to development of the parasitic organ haustorium. Prior studies have built a signaling model now known as Semagenesis for *S. asiatica* to idenfity hosts. Firstly, Chang showed that 1) host cell wall fragments removed from sorghum root surface were able to induce haustoria in *S. asiatica*; 2) Syringic acid (SA),

used as a model cell wall phenolic precursor, was oxidized to 2,6-dimethoxybenzoquinone (DMBQ) by *S.asiatica* seedlings and induced haustoria only when the concentration of DMBQ reached micromolar levels; 3) removal of the phenolic precursor prior to conversion to the quinones arrested haustorial development; 4) addition of DMBQ was necessary and sufficient for its resumption (Chang and Lynn, 1986). These results depicted how a haustorial inducer is generated: *S. asiatica* releases an oxidant that oxidizes cell wall-bound phenolic compounds on the surface of the host root cell wall, thereby producing a benzoquinone compound such as DMBQ to feedback to *S. asiatica* and induce haustorial development. Later, Kim and co-workers found that although two apoplastic peroxidases were isolated from *S. asiatica*, it is the host peroxidases that catalyzed the oxidation of phenolics to quinones (Kim et al., 1998a). Then, Keyes and co-workers revealed that the reactive oxygen species (ROS) were produced by the surface cells in the root tip of *S. asiatica* (Keyes et al., 2007), and named this signal generating process Semagenesis. Smith and co-workers presented good evidence that haustorial inducing activity of the structural analogs of DMBQ depends on their redox potential, and suggested an one-electron reduction reaction occurs when quinone signals bind to the receptors in *S. asiatica* (Smith et al., 1996). Recently, two quinone oxidoreductases (QRs) were isolated from Triphysaria, one of which was shown to be involved in the perception of the haustorial inducer and was proposed to be a DMBQ receptor that initiates haustorial induction. (Bandaranayake et al., 2010). The strategy of cueing on host-derived signals ensures that the parasite is in close proximity to its host when it commits the extremely limited nutrient store in the seed for germination and haustorial development.

Germination in obligate root parasites, including *S. asiatica,* is usually cryptocotylar, that is, the cotyledons remain within the seed coat when the delicate radicle emerges (Kuijt, 1969b). The onset of shoot development under natural conditions occurs after host attachment, likely to be induced by another host-derived signal transferred from the host into the parasite through the haustorium. Under laboratory conditions, we have found shoot initiation depends on haustorial initiation and the phytohormone cytokinins are sufficient to induce shoot apical development. Since the subterranean developmental stages of *S. asiatica*: germination, haustorium development and shoot initiation, are all separate and require host-derived signals to proceed, we recognize that it should be possible to identify genes involved in the regulation of each step via differential expression cDNA libraries.

Striga spp. are root parasites that are grouped in the angiosperm families *Scrophulariaceae* or *Orobanchaceae.* Although parasitism emerged multiple times in the evolutionary history of angiosperms (Kuijt 1969, Nickrent 1998) , a phylogenetic analysis of chloroplast gene sequences across all parasitic members of the *Scrophulariaceae* and *Orobanchaceae* families placed them into a single clade, indicating that the genetic element for haustorium development might have originated once in the evolutionary history of these two families [Young 1999]. In this chapter, I have exploited these host-derived signaling events to create differential expression cDNA libraries for identifying genetic elements regulating haustorial development and shoot initiation. By comparing and constrasting a parasitic and a vegetative developmental

stage, an understanding of the genetic mechanisms underlying the parasitism and pathogenesis of *S. asiatica* provides initial insight into the origins of the haustorium and the evoluction of parasitism in *Scrophulariaceae* and *Orobanchaceae* families.

2.2 Results

Cytokinins are sufficient to induce haustorium induction and shoot initiation

Cytokinin treatment has been reported to induce haustoria development in Cuscuta (Ramsubramanian et al., 1988). As shown in Figure 2-1A, we found trans-zeatin (a cytokinin) among other cytokinins induced haustoria with the same potency with DMBQ (Fig. 2-1A, Table 2-1). The time required for half-maximal commitment in the population to haustorium development ($t_{1/2)}$ for either 10 μM DMBQ or 10 μM trans-zeatin is 4.5 hrs. Furthermore, if left treated for extended times, the seedlings treated with cytokinins exhibited substantial shedding of the seed coat as a result of the swelling of the cotyledons (Fig. 2-1B), and release from dormancy the shoot apical meristem (SAM). In contrast, DMBQ does not induce shoot development even with exposure for up to eight days, the approximate life span of a *S. asiatica* seedling without host attachment (Fig. 2-1D, Table 2-1). The $t_{1/2}$ required for inducing shoot initiation by trans-zeatin is 7.5 hrs (Fig. 2-1C).

Table 2-1 *Comparison of DMBQ with three other cytokinins on their inducing activity of haustorium development or shoot initiation.* a: Haustorium development induced by 10 μM exposure to the indicated compound and evaluated after 24 hours. b: $t_{1/2}$ for haustorium development determined by timed exposure assays at 10 μM of the indicated compound. c: seed coat shedding evaluated after 24 h exposures at 10 μM of the indicate compound. All results are the average of 30 seedlings per experiment, conducted in triplicate, with error expressed as +/- one standard deviation.

Compound	**% Haustoria[a]**	**Haustorium $t_{1/2}$ (hr)[b]**	**% seed coat shed[c]**
DMBQ	91+/- 6	4	<1
Trans-zeatin	92+/- 5	4	87 +/- 7
Kinetin	74 +/- 11	11	58 +/- 9
6-BA	86 +/- 9	7	78 +/- 6

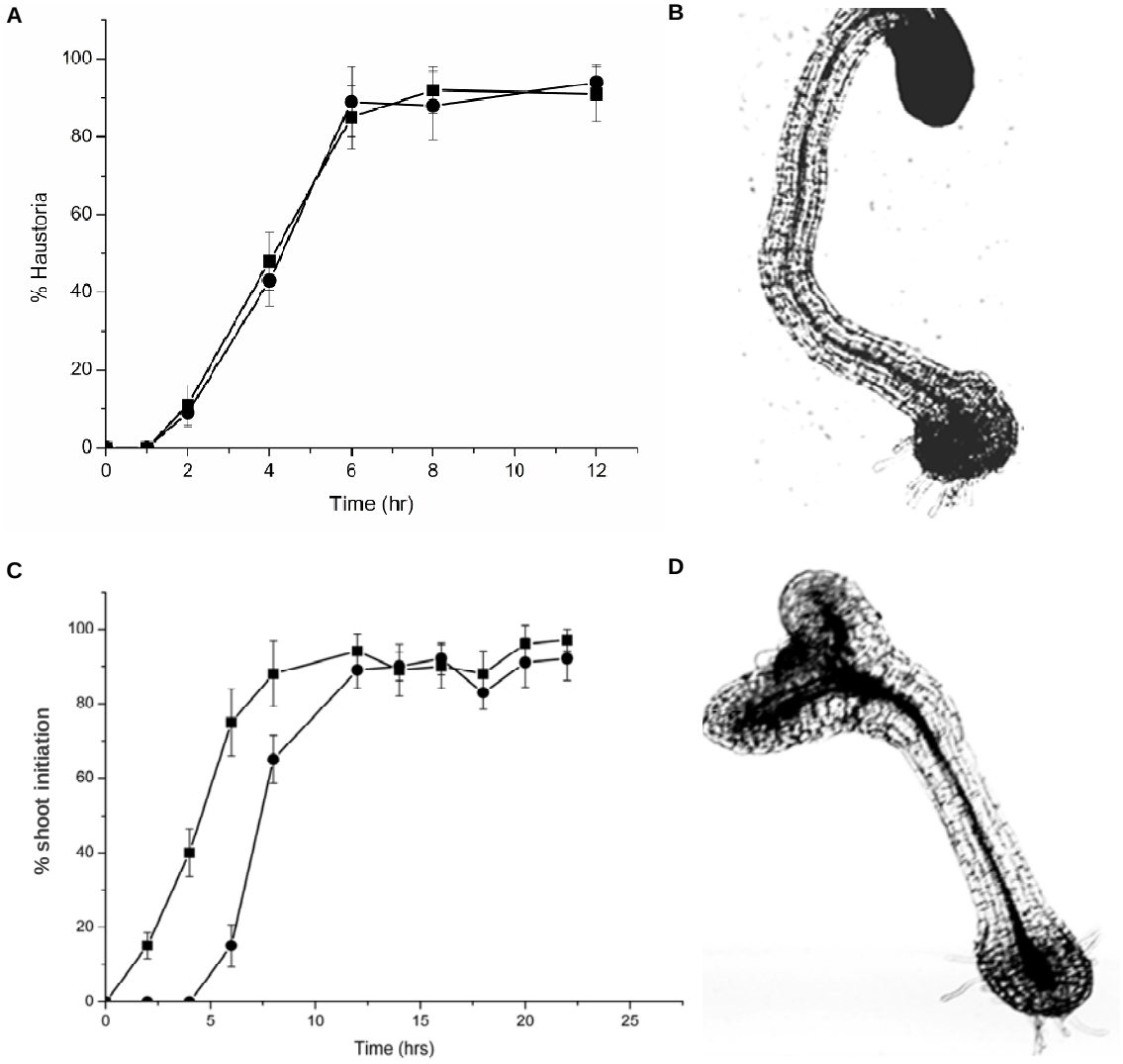

Figure 2-1 *Trans-zeatin is sufficient to induce haustorial development or shoot initiation in S. asiatica.* (A) One-day-old seedlings of *S. asiatica* are treated with either 10 µM of trans-zeatin (●) or DMBQ (■). At the indicated times a fraction of the seedlings are washed in triplicate and, placed in 0.1mM KCl. After 24 hours the samples are scored for: haustorium development (See methods). Results are the average of 30 seedlings per experiment, conducted in triplicate, with error expressed as +/- one standard deviation. (B) A 10-day-old *S. asiatica* seedling treated with DMBQ for nine days in 0.1 mM KCl solution. (C) Effects of pre-treatment with haustorial inducing quinones on seed coat shedding. Day old seedlings of *S. asiatica* are placed in 5 ml wells with either 10 µM of 2,6-Dimethoxy-benzoquinone, a known haustorial inducer (■), or just 0.1mM KCl (●). After 24 hours seedlings were then treated with 10 µM of trans-zeatin. At two hour intervals seedlings were washed then scored for seed coat shedding after 24 hours. Results are the average of 30 seedlings per experiment, conducted in triplicate, with error expressed as +/- one standard deviation. (D) A 10-day-old *S. asiatica* seedling treated with trans-zeatin for eight days in 0.1 mM KCl solution.

Constructing differential expression libraries specific to haustorium development or shoot initiation

The DMBQ induced haustorium library was made by subtractive hybridization between transcripts from seedlings treated with DMBQ for 4.5 hrs ($t_{1/2}$) and from untreated seedlings. Fifty unique sequences were obtained and compared with gene sequences in the NCBI databases using BLAST algorithms, and labeled as HDx (**H**austorium library induced by **D**MBQ number **x**) in table 2-2. The trans-zeatin induced haustorium library was prepared by subtractive hybridization between transcripts from seedlings treated with trans-zeatin for 4.5 hrs and untreated seedlings. Twenty five unique sequences were obtained and compared with sequences in NCBI database using BLAST algorithms, and labeled as HTx (**H**austorium library induced by **T**rans-zeatin number **x**) in table 2-3. The shoot initiation library was made by subtractive hybridization between transcripts from seedlings treated with trans-zeatin for 7.5 hours ($t_{1/2}$ for shoot initation) and seedlings treated with DMBQ for 4.5 hours ($t_{1/2}$ for haustorium development). Twenty one unique sequences were obtained, labeled as SIx (**S**hoot **I**nitiation library number **x**) in table 2-4.

Table 2-2 *Differential expression library for haustorial development induced by DMBQ (HD library).* Genes highlighted in bold are the ones that are found in at least one of the other two libraries.

Category	Gene ID.	Genbank Accession No.	Repeats	Homologous gene found by BLAST search				DMBQ regulated expression					
				Function	E-value	Organism	Accession No.	0hr	0.5hr	2hr	6hr	16hr	24hr
Cell division	**HD1**	**DQ445120**	**4**	**Alpha-tubulin**	**5.0E-103**	***Ceratopteris richardii***	**AY231146**	**1**	**1.1**	**0.8**	**0.4**	**0.5**	**0.5**
	HD2	**DQ442392**	**1**	**Elongation factor 1 alpha**	**9.0E-102**	***Malus x domestica***	**AJ223969**	**1**	**0.8**	**0.8**	**0.1**	**0.1**	**0.1**
	HD3	DQ445133	1	S phase specific protein	2.0E-94	*Catharanthus roseus*	D26058	1	1.1	0.3	0.4		
Cell expansion	HD4	DQ442400	1	Expansin	1.0E-115	*Nicotiana tabacum*	AF049352	0	0.0	1.0	0.9	1.0	1.2
	HD5	DQ442401	1	Expansin	1.0E-93	*Lycopersicon esculentum*	AF184233	1	3.1	3.2	3.0	3.0	3.2
	HD6	**AF291658**	**3**	**Expansin**	**2.0E-85**	***Pyrus communis***	**AB093031**	**1**	**2.0**	**2.1**	**2.5**	**2.6**	**2.5**
	HD7	**AF291657**	**1**	**Expansin**	**2.0E-87**	***Gossypium hirsutum***	**AF512542**	**1**	**1.8**	**1.9**	**1.8**	**1.9**	**1.7**
	HD8	DQ442402	2	Expansin	2.0E-110	*Mirabilis jalapa*	AY079206	1	1.0	1.2	1.3	2.5	2.4
	HD9	**DQ442395**	**3**	**Xyloglucan endotransglycosylase**	**6.0E-119**	***Malus x domestica***	**AY144594**	**0**	**1.0**	**1.4**	**2.3**	**2.4**	**3.1**
Vascular tissue differentiation	HD10	DQ431680	1	Chorismate mutase	4.0E-39	*Oryza sativa*	XM_482629	1	3.7	2.6	2.4	2.5	2.2
	HD11	**DQ431681**	**1**	**Cinnamyl alcohol dehydrogenase**	**1.0E-145**	***Ocimum basilicum***	**AY879285**	**1**	**1.8**	**2.0**	**1.8**	**2.0**	**2.0**
	HD12	DQ445123	2	Homeobox leucine-zipper protein	5.0E-64	*Zinnia elegans*	AB084381	1	1.6	2.6	2.7	2.7	2.6
	HD13	DQ442385	3	Phenylcoumaran benzylic ether reductase	1.0E-67	*Forsythia x intermedia*	AF242491	1	1.0	0.1	0.1	0.1	0.1
	HD14	**DQ442382**	**1**	**Phenylcoumaran benzylic ether reductase**	**4.0E-141**	***Forsythia x intermedia***	**AF242491**	**0**	**1.0**	**1.4**	**1.4**	**2.5**	**2.1**
Defense response	**HD15**	**DQ442383**	**1**	**Calmodulin**	**6.0E-68**	***Nicotiana tabacum***	**AB050849**	**1**	**1.1**	**1.2**	**5.7**	**5.4**	**5.5**
	HD16	DQ442394	1	Calmodulin binding protein	2.0E-27	*Arabidopsis thaliana*	NM_180054	1	2.6	2.6	2.7	2.8	2.5
	HD17	DQ442380	1	Diphenol oxidase laccase	2.0E-74	*Glycine max*	AF527604	1	2.2	2.3	2.4		
	HD18	DQ445131	1	Drug transporter	2.0E-27	*Arabidopsis thaliana*	NM_129389	1	4.5	4.5	6.4	5.9	5.9
	HD19	DQ442387	4	GDSL lipase	2.0E-62	*Oryza sativa*	ABA93963	1	1.5	1.1	0.5	0.5	0.4
	HD20	DQ442378	1	Glutathione transferase	7.0E-39	*Hyoscyamus muticus*	X78203	1	0.9	2.2	2.2	2.3	2.2
	HD21	**DQ431679**	**1**	**NADPH oxidase**	**5.0E-11**	***Zea mays***	**EU807966**	**1**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**
	HD22	**DQ442396**	**3**	**Ascorbate Peroxidase**	**2.0E-71**	***Ipomoea batatas***	**AY206407**	**1**	**0.5**	**0.1**	**0.1**	**0.2**	**0.2**
	HD23	**DQ445121**	**1**	**Peroxidase**	**1.0E-114**	***Arabidopsis thaliana***	**NM_123583**	**1**	**0.2**	**0.2**	**0.1**	**0.2**	**0.2**
	HD24	DQ442405	4	Quinone-oxidoreductase QR2	1.0E-84	*Triphysaria versicolor*	AF304462	1	7.3	7.5	8.6	8.8	8.6
Hormonal pathways	HD25	DQ442403	1	Auxin-regulated protein	4.0E-100	*Zinnia elegans*	AY090553	1	1.1	1.0	3.0	3.1	3.1
	HD26	DQ442404	1	Auxin-regulated protein	2.0E-86	*Populus tremula*	AF373100	1	3.2	3.5	3.6	3.4	3.5
	HD27	DQ442398	1	EIN3-binding F-box Protein 2	2.0E-46	*Lycopersicon esculentum*	DQ307489	1	1.2	2.3	2.4	2.5	2.5
	HD28	**DQ431687**	**1**	**Type-A RR**	**7.0E-54**	***Atharanthus roseus***	**AF534888**	**1**	**2.4**	**2.4**			
	HD29	**DQ431686**	**1**	**Type-A RR**	**6.0E-59**	***Arabidopsis thaliana***	**NM_115563**	**1**	**4.9**	**5.1**			
Metabolism and nutrient transport	**HD30**	**DQ442393**	**1**	**Aquaporin**	**6.0E-61**	***Helianthus annuus***	**X95952**	**1**	**1.8**	**2.5**	**2.5**	**2.5**	**2.7**
	HD31	DQ442399	1	Beta-glucosidase	6.0E-40	*Olea europaea*	AY083162	1	0.5	0.4	0.4	0.4	0.4
	HD32	DQ445132	1	Carbonic anhydrase	5.0E-73	*Lycopersicon esculentum*	AJ849375	1	2.5	2.7	2.5	2.5	2.7
	HD33	DQ442390	1	Carboxypeptidase	1.0E-32	*Arabidopsis thaliana*	AK176585	1	1.6	4.5	4.6	5.0	5.2
	HD34	DQ442381	1	Hydrolase	1.0E-92	*Arabidopsis thaliana*	NM_119911	1	1.0	0.2	0.2	0.2	0.3
	HD35	DQ445129	1	Desacetoxyvindoline 4-hydroxylase	3.0E-18	*Vitis vinifera*	AY226830	1	3.1	3.4	3.3	3.4	3.4
	HD36	DQ442397	1	Glycine hydroxymethyltransferase	4.0E-20	*Arabidopsis thaliana*	NM_119404	0	0.0	1.0	1.1	1.1	1.2
	HD37	DQ445126	2	Hydroxymethyltransferase	2.0E-73	*Arabidopsis thaliana*	AC021199	0	0.0	1.0	3.9	3.9	4.1
	HD38	DQ445137	1	Sorbitol dehydrogenase	1.0E-96	*Lycopersicon esculentum*	AB183015	1	1.0	2.3	2.4	2.6	2.4
	HD39	DQ442379	1	Sugar transporter	1.0E-62	*Lycopersicon esculentum*	AJ278765	1	1.8	1.9	2.0	1.9	1.9
Protein synthesis and degradation	HD40	DQ442391	1	Binding/translation initiation factor	7.0E-87	*Arabidopsis thaliana*	NM_0010368	1	2.6	2.7	2.4	2.7	2.7
	HD41	DQ445130	1	DNA binding protein	1.0E-10	*Arabidopsis thaliana*	NM_203121	1	3.9	4.0	3.8	3.8	3.9
	HD42	**DQ442384**	**1**	**DnaJ protein**	**1.0E-21**	***Camellia sinensis***	**DQ345453**	**1**	**1.0**	**0.3**	**0.3**	**0.3**	**0.4**
	HD43	DQ445136	1	DREPP4 protein	5.0E-34	*Nicotiana tabacum*	AJ277899	1	3.5	3.7	3.5	3.6	3.5
	HD44	DQ445135	1	Fimbriata-associated protein	2.0E-32	*Antirrhinum majus*	Y14857	1	1.7	1.6	1.7	1.6	1.7
	HD45	DQ445122	2	Transcription factor	1.5E-02	*Arabidopsis thaliana*	NM_100886	1	1.1	1.0	2.3	2.3	2.3
Others	**HD46**	**DQ442389**	**5**	**Epicotyl specific protein**	**5.0E-37**	***Cicer arietinum***	**X97455**	**1**	**1.0**	**1.1**	**0.6**	**0.5**	**0.5**
	HD47	DQ445128	1	Major intrinsic protein 1	5.0E-81	*Solanum tuberosum*	DQ228330	1	1.1	0.4	0.4	0.3	0.3
	HD48	DQ445124	1	Tobamovirus multiplication 3	2.0E-76	*Nicotiana tabacum*	AB193040	1	0.9	0.2	0.2	0.3	0.3
	HD49	DQ445134	1	Unknown protein	5.0E-68	*Arabidopsis thaliana*	NM_116339	1	1.0	0.2	0.1	0.2	0.1
	HD50	DQ442388	4	Unknown protein	5.0E-41	*Arabidopsis thaliana*	NM_120702	1	1.1	0.0	0.0	0.0	0.0

Table 2-3 *Differential expression library of haustorium development induced by trans-zeatin (HT library).* Genes highlighted in bold are the ones that are found in at least one of the other two libraries.

Category	Gene ID.	Repeats	Homologous gene found by BLAST search			
			Function	E-value	Organism	Accession ID
Cell division	**HT1**	**1**	**Calcium/calmodulin-dependent protein kinase CaMK3**	**6.0E-69**	***Nicotiana tabacum***	**AF435452.1**
	HT2	**3**	**Elongation factor 1 alpha**	**0.0E+00**	***Striga asiatica***	**DQ442392.1**
	HT3	**3**	**Elongation factor 1 alpha**	**4.0E-114**	***Tomato***	**X14449.1**
	HT4	**1**	**Microtubule-Associated Proteins**	**4.0E-06**	***Zea mays***	**EU971391.1**
Cell expansion	**HT5**	**9**	**Xyloglucan Endotransglycosylase**	**3.0E-28**	***Sesamum indicum***	**AY365421.1**
Vascular differentiation	**HT6**	**2**	**Alcohol dehydrodenase**	**7.0E-11**	***Arachis hypogaea***	**AY725189.1**
	HT7	**1**	**Cinnamyl-alcohol dehydrogenase**	**1.0E-80**	***Mesembryanthemum crystallinum***	**U79770.1**
	HT8	**1**	**Phenylcoumaran benzylic ether**	**3.0E-59**	***Striga asiatica***	**DQ442382.1**
	HT9	2	Trans caffeoyl CoA -3-o methyl transferase	4.0E-04	*Arachis hypogaea*	AY725194.1
Defense response	HT10	1	EST from mild drought-stressed leaves	1.0E-05	*Populus*	CU231893.1
	HT11	**1**	**FAD-linked oxidoreductase 2**	**1.0E-15**	***Glycine max***	**DQ318817.1**
	HT12	2	HTC in tomato leaves treated with	6.0E-44	*Solanum lycopersicum*	AK324519.1
	HT13	1	Metallothionin 2a	6.0E-51	*Salvia miltiorrhiza*	EF666996.1
Nutrition metabolism and transport	**HT14**	**1**	**Aquaporin**	**1.0E-51**	***Atriplex canescens***	**U18403.1**
	HT15	1	Aspartate transferase 3	2.0E-36	*Arabidopsis thaliana*	NM_121190.2
	HT16	1	Chloroplast cysteine synthase 1	6.0E-135	*Nicotiana tabacum*	AM087457.1
	HT17	1	Cytosolic invertase	5.0E-35	*Arabidopsis thaliana*	NM_202796.2
	HT18	2	Lysine/histidine transporter	1.0E-59	*Populus trichocarpa*	XM_002298561.1
	HT19	1	Methionine synthase	0.0E+00	*Phelipanche ramosa*	DQ849630.1
	HT20	1	Sodium proton antiporter	6.0E-03	*Ipomoea nil*	AB194066.1
Transcriptional protein synthesis	HT21	2	ADP-ribosylation factor	2.0E-114	*Glycine max*	AF114796.1
	HT22	2	ADP-ribosylation factor	5.0E-12	*Vitis vinifera*	XM_002281011.1
	HT23	**1**	**DnaJ protein**	**0.0E+00**	***Striga asiatica***	**DQ442384.1**
Others	HT24	1	Proline-rich protein	1.0E-25	*Phaseolus vulgaris*	AM158278.1
	HT25	**1**	**Epicotyl specific protein**	**0.0E+00**	***Striga asiatica***	**DQ442389.1**

Table 2-4 *Differential expression library of shoot initiation induced by trans-zeatin (SI library).* Genes highlighted in bold are the ones found in at least one of the other two libraries.

Category	Gene ID.	Repeats	NCBI BLAST search			
			Function	E-value	Organism	Accession No.
Cell division	**SI1**	**1**	**Elongation factor 1 alpha**	**4.0E-92**	***Anihot esculenta***	**AF041463.1**
Cell expansion	SI2	5	Alpha galactosidase	2.0E-81	*Coffea arabica*	AJ877911.1
	SI3	2	Myo-inositol oxygenase 2	6.0E-40	*Arabidopsis thaliana*	NM_127538.3
Vascular differentiation	SI4	**4**	**Alcohol dehydrogenase 2**	**2.0E-34**	***Petunia x hybrida***	**AY231365.1**
	SI5	**8**	**Cinnamyl alcohol dehydrogenase 1**	**6.0E-48**	***Nicotiana tabacum***	**AY911854.1**
	SI6	**3**	**Phenylcoumaran benzylic ether reductase**	**0.0E+00**	***Striga Asiatica***	**DQ442382.1**
Defense response	**SI7**	**1**	**Peroxidase**	**0.0E+00**	***Striga asiatica***	**AF403235.1**
	SI8	1	Superoxide dismutase	3.0E-06	*Belgica antarctica*	DQ507288.2
Hormonal pathway	**SI9**	**8**	**Response regulator 200**	**0.0E+00**	***Striga asiatica***	**DQ431688.1**
Metabolism	SI10	5	GAMYB-binding protein (GBP)	4.0E-29	*Glycine max*	DQ112540.1
	SI11	1	Glucose-6-phosphate-1-dehydrogenase	3.0E-04	*Nidula niveotomentos*	AM497810.1
	SI12	1	Lysyl hydroxylase 1	5.0E-30	*Takifugu rubripes*	AB259759.1
Cell signaling	**SI13**	2	GTP-binding protein (Ran-A1)	3.0E-109	*Nicotiana tabacum*	L16767.1
	SI14	1	RAS-like GTP-binding nuclear protein	7.0E-21	*Pyrus communis*	AJ581788.1
Protein synthesis and degradation	**SI15**	1	Heat shock protein Hsp23.9	9.0E-17	*Glycine max*	U21722.1
	SI16	2	Initiation factor 2 subunit 1	2.0E-51	*Arabidopsis thaliana*	NM_129587.2
	SI17	1	Poly(A)-binding protein	9.0E-104	*Nicotiana tabacum*	AF190655.1
	SI18	1	Small heat shock protein	7.0E-03	*Babesia bigemina*	AF332566.1
	SI19	1	Ubiquitin conjugating enzyme E2	3.0E-38	*Adiantum capillus-veneris*	EF591301.1
	SI20	3	Ubiquitin-protein ligase	4.0E-57	*Arabidopsis thaliana*	AY085670.1
Others	**SI21**	1	Transposase mRNA, partial cds	2.0E-04	*Siniperca chuatsi*	DQ251457.1

Constructing a temporal map of the genetic regulation of haustorial development and evaluating the differential expression cDNA libraries

We measured the transcription level of each gene in the HD library as a function of exposure time to DMBQ: 0 hr, 0.5 hrs, 2 hrs, 6 hrs, 16 hrs, and 24 hrs, by reverse transcription PCR (RT-RCR) with the house keeping gene actin as an internal control (Wolf and Timko, 1994) (Table 2-2). This temporal map provides temporal correlation between gene activation and onset of a phenotype. For example, five expansin genes were found, but only one of them remained uninduced until exposed to DMBQ for longer than 6 hrs. A prior study indicated that it takes more than 6 hrs of exposure to DMBQ for initiation of haustorial hairs (Smith et al., 1990a), suggesting that this expansin (**HD8**) is

possibly involved in haustorial hair growth. Meanwhile, the RT-PCR results demonstrated that the construction of differential expression cDNA libraries was efficient and identified regulated genes.

Since the development of root and shoot are separate in *S. asiatica* and the haustorium library was made with transcripts from roots only, we expect to find root specific genes in the haustorium libraries. We sampled 12 genes in the HD library to measure their transcription level in root, leaf+stem, and flower by RT-PCR, respectively. Indeed, over half of these genes are either root specific or expressed higher in the root, while the rest were expressed in all organs (Table 2-5). We then cloned the promoters of the four root specific genes, (**HD21, HD23, HD49 and HD50**), fused with beta-glucuronidase (GUS) and transformed the GUS reporter constructs into *A. thaliana* to refine the resolution of the spatial localization (Fig. 2-2). The unknown gene HD50 and the peroxidase HD23 were distinctly expressed in lateral roots only, but not in lateral root tips (Fig. 2-2A, B). The GUS staining of HD23/GUS expression is darker in the secondary lateral branches than in the primary lateral roots (Fig. 2-2B). The gene encoding a superoxide producing enzyme NADPH oxidase (HD21) exhibited a noted expression restricted to only the root tips (Fig. 2D), most consistent with the localization of ROS production during semagenesis. The rapid down-regulation of HD21 by a factor of five upon DMBQ treatment is again consistent with the rapid down regulation of ROS upon DMBQ exposure (Keyes et al., 2007). These observations suggest this enzyme might be the source of ROS for semagenesis in *S. asiatica* during semagenesis. The other unknown gene HD49 was expressed in some of the lateral roots, but not in root tips (Fig. 2-2C).

These two unknown genes HD49 and HD50 were the most down-regulated genes in the HD library with HD50 expression completely suppressed by 2 hrs of DMBQ treatment, and HD49 expression dropped to a tenth after 2 hrs of DMBQ treatment (Table 2-2). These results indicate the library was valuable in finding differentially expressed genes critical for haustorial development, and yielded root-specific genes as well.

We then evaluated the transcriptional response of these 12 genes to kinetin (a cytokinin haustorium inducer), t-butyl benzoquinone (tBuBQ) (a benzoquinone haustorium inducer), and Tetrafluorobenzo-1,4-benzoquinone (TFBQ) (a benzoquinone non-inducer) (Keyes, 2000). As shown in table 2-5, these 12 genes responded to kinetin and tBuBQ in the same manner as to DMBQ, but not to the non-inducer TFBQ, indicating the library did find genes specifically tied to haustorial development.

Table 2-5 *Localization and regulation of twelve genes sampled from the DMBQ induced haustorium library.* Genes highlighted in bold are the ones found in at least one of the other two libraries.

Gene ID	Function	Localization	DMBQ	Kinetin	tBuBQ	TFBQ
HD21	**NADPH oxidase**	Root only	Decrease	Decrease	Decrease	No change
HD23	**Peroxidase**	Root only	Decrease	Decrease	Decrease	No change
HD49	Unknown	Root only	Decrease	Decrease	Decrease	No change
HD50	Unknown	Root only	Decrease	Decrease	Decrease	No change
HD34	Hydrolase	Higher in root	Decrease	Decrease	Decrease	No change
HD24	Quinone-oxidoreductase QR2	Higher in root	Increase	Increase	Increase	No change
HD15	**Calmodulin**	Higher in root	Increase	Increase	Increase	No change
HD12	Homeobox leucine-zipper protein	All organs	Increase	Increase	Increase	No change
HD14	**Phenylcoumaran benzylic ether**	All organs	Increase	Increase	Increase	No change
HD9	**Xyloglucan endotransglycosylase**	All organs	Increase	Increase	Increase	No change
HD7	**Expansin**	All organs	Increase	Increase	Increase	No change
HD6	**Expansin**	All organs	Increase	Increase	Increase	No change

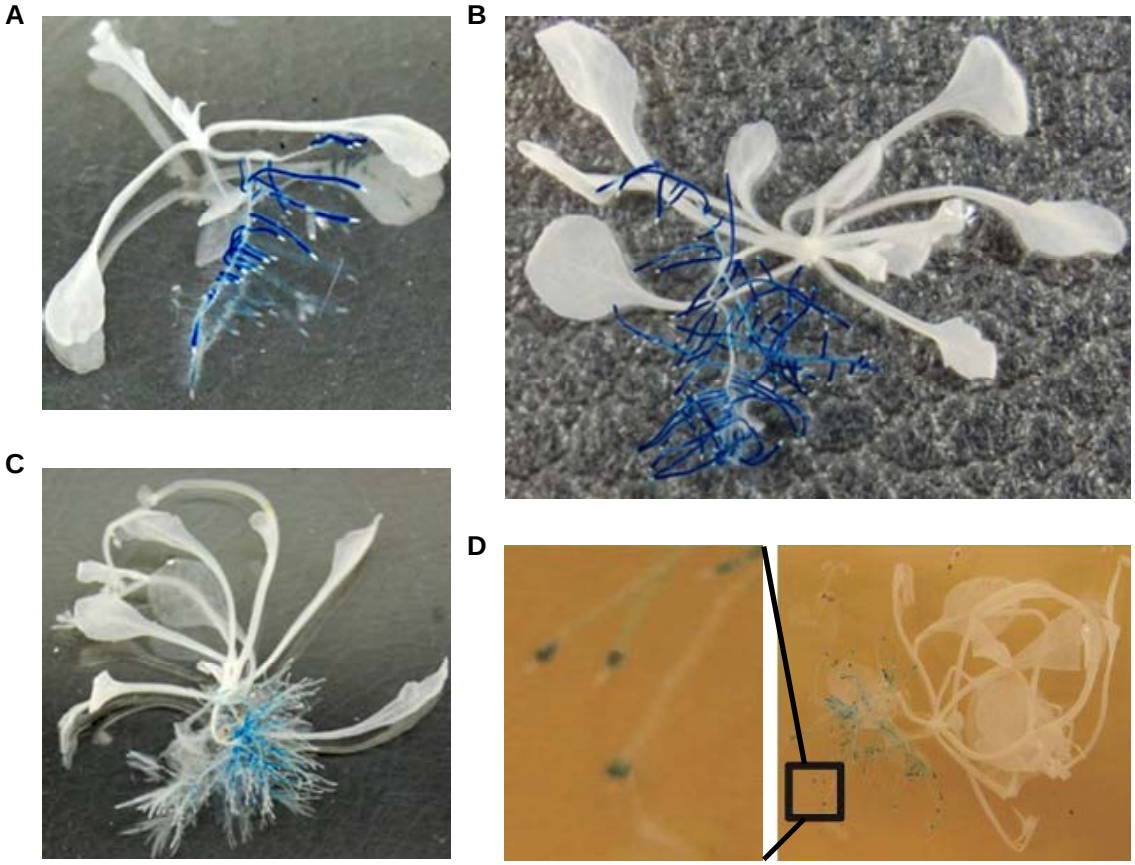

Figure 2-2 *Spatial localization by expressing GUS constructs in A. thaliana.* (A) HD50/GUS, (B) HD23/GUS, (C) HD49/GUS, and (D) HD21/GUS.

Cell cycle genes

Genes regulating or correlated with cell division are expected to be found in the haustorium libraries because the cell cycles in RAM cells was arrested immediately when seedlings of *S. asiatica* were exposed to DMBQ as measured by incorporation of ^{3}H-thymidine (Keyes et al., 2000). Notably, homologues of elongation factor 1 alpha subunit was found in all three libraries (**HD2**, **HT2**, **HT3**, **SI1**). This protein was found to interact with CDK1/cyclin B, the universal regulator of M phase and plays a role in regulating the cell cycle (Boulben et al., 2003). The expression level of **HD2** was reduced 10 folds after 2 hrs of DMBQ treatment, and a S phase specific protein (**HD3**) was decreased by 70%

after 2 hrs exposure to DMBQ, consistent with the rapid arrest of cell cydle in *S. asiatica*. In addition, alpha-tubulin (**HD1**) and microtubule-associated proteins (**HT4**) were frequently reported to mediate cell division by controling cell morphologies (Toda et al., 1984; Stotz and Long, 1999; Ambrose et al., 2007). **HD1** was down-regulated by 50% after 6 h exposure, a much slower rate than that of the elongation factor 1 or the S phase specific protein. Lastly, calmodulin-dependent protein kinases were reported to be associated with cell proliferation by phosphorylating elongation factor 2 (Bagaglio and Hait, 1994), suggesting that the calmodulin-dependent protein kinase 3 (**HT1**) found in the library might play a role in cell proliferation. In addtion to cell cycle genes, several cytokinin response regulators, the last component in the cytokinin signaling cascade, were found in both the SI library (**SI9**) and the HD library (**HD28** and **HD29**). Two auxin-induced genes (**HD25**, **HD26**) were also found in the HD library as well. The further investigation on how DMBQ interacts with the cytokinin/auxin signaling pathways is presented in Chapter 4.

Cell expansion and growth

An important phenotypic marker of haustorial development is redirection of cell expansion from longitudinal to radial dimensions, and growth of haustorial hairs centrifugal to the swelling root tip (Keyes et al., 2000). Indeed, the largest category of genes in each library is related to cell expansion. Expansins are cell wall bound proteins that induce slippage of cellulose microfibrils, and consequently, loosens the cell walls for morphological remodeling (Sampedro and Cosgrove, 2005). Three expansins have been isolated from *S. asiatica* in our prior studies (O'Malley and Lynn, 2000b), SaExpl,

SaExp2, and SaExp3, and two of them SaExp1 (**HD7**) and SaExp2 (**HD6**) were found among five expansins in the HD library (**HD4, HD5, HD6, HD7, HD8**) (Table 2-2). With the expansin superfamily comprising four families of a great number of expansin proteins, one can speculate that each expansin protein can function in a specific context. A phylogenetic analysis of all the 26 α-expansin gene sequences from *A. thaliana*, two from cucumber, one from tobacco, one from rice, two from maize, and three from *S. asiatica* reveals five subgroups A-E (Fig. 2-3), among which subgroup-A has been shown to be involved in cell expansion during xylogenesis (Gray-Mitsumune et al., 2004; Gray-Mitsumune et al., 2008). A sequence alignment of *S. asiatica* expansins with other subgroup-A expansins (AtExp3/4/6/9 and PttExp1), and OsExpa1 from subgroup-D confirmed that SaExp1 belongs to subgroup-A by finding the "RIPGV" motif immediately after the signal peptide sequence and the "KNFRV" motif at the C-terminus (Fig. 2-4). We chose SaExp1 for further study because cell expansion during xylogenesis should be an active cellular event during haustorial development.

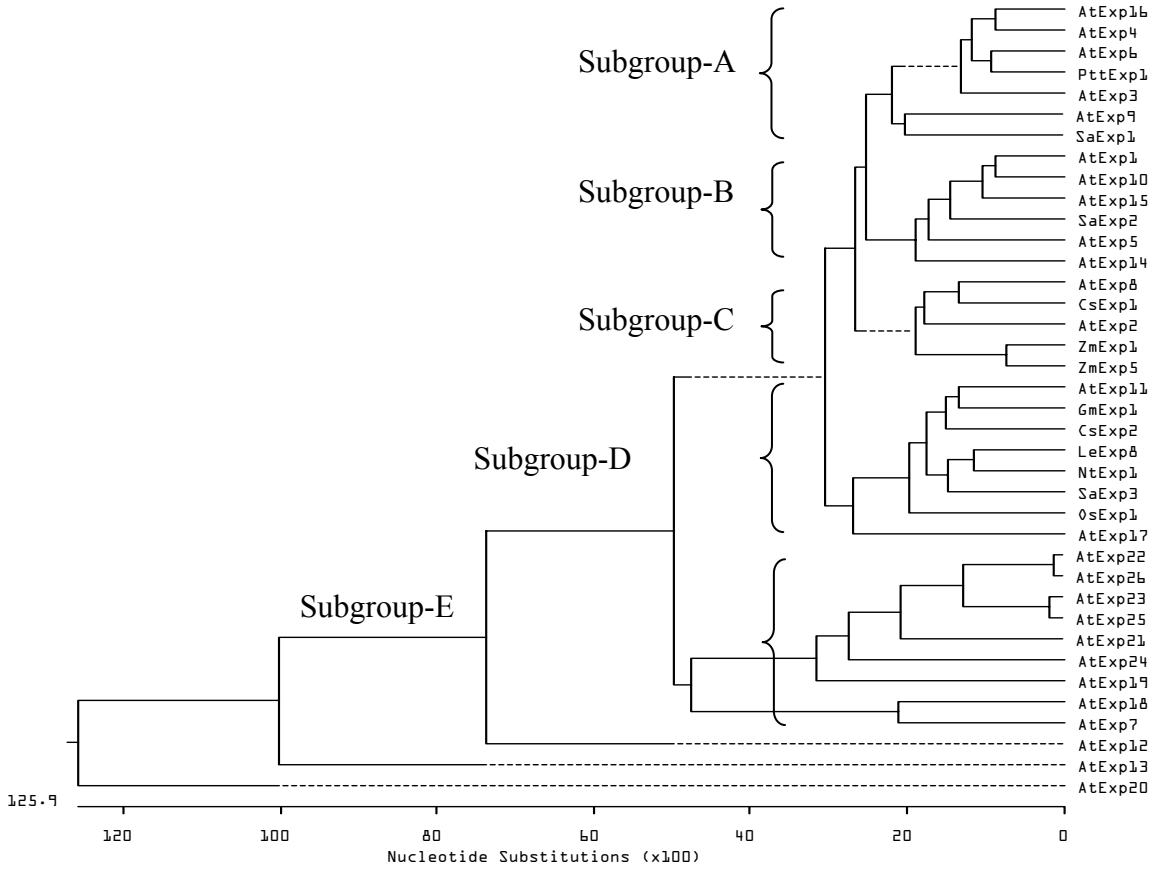

Figure 2-3 *Phylogenetic analysis of α-expansin genes.* GenBank accession numbers are AtEXP1 (AAG60095), AtEXP2 (BAB09972), AtEXP3 (AAC23634), AtEXP4 (AAB97125), AtEXP5 (BAA95756), AtEXP6 (AAC33223), AtEXP7 (AAF79645), AtEXP8 (AAB87577), AtEXP9 (CAB85531), AtEXP10 (AAF61712), AtEXP11 (AAF79895), AtEXP12 (AAF35403), AtEXP13 (AAF26104), AtEXP14(BAB11259), AtEXP15 (AAC32927), AtEXP17 (AAC72858), AtEXP18 (AAF75810), AtEXP19 (AP001309), AtEXP20(CAB37561), AtEXP21 (BAB09381), AtEXP22 (BAB09382), AtEXP23 (BAB09383), AtEXP24 (BAB09386), AtEXP25(BAB09385), AtEXP26 (BAB09384), CsEXP1 (U30382), CsEXP2 (U30460), NtEXP1 (AF049350), OsEXP1 (Y07782), ZmEXP1 (AF332169), ZmEXP5 (AF332173).

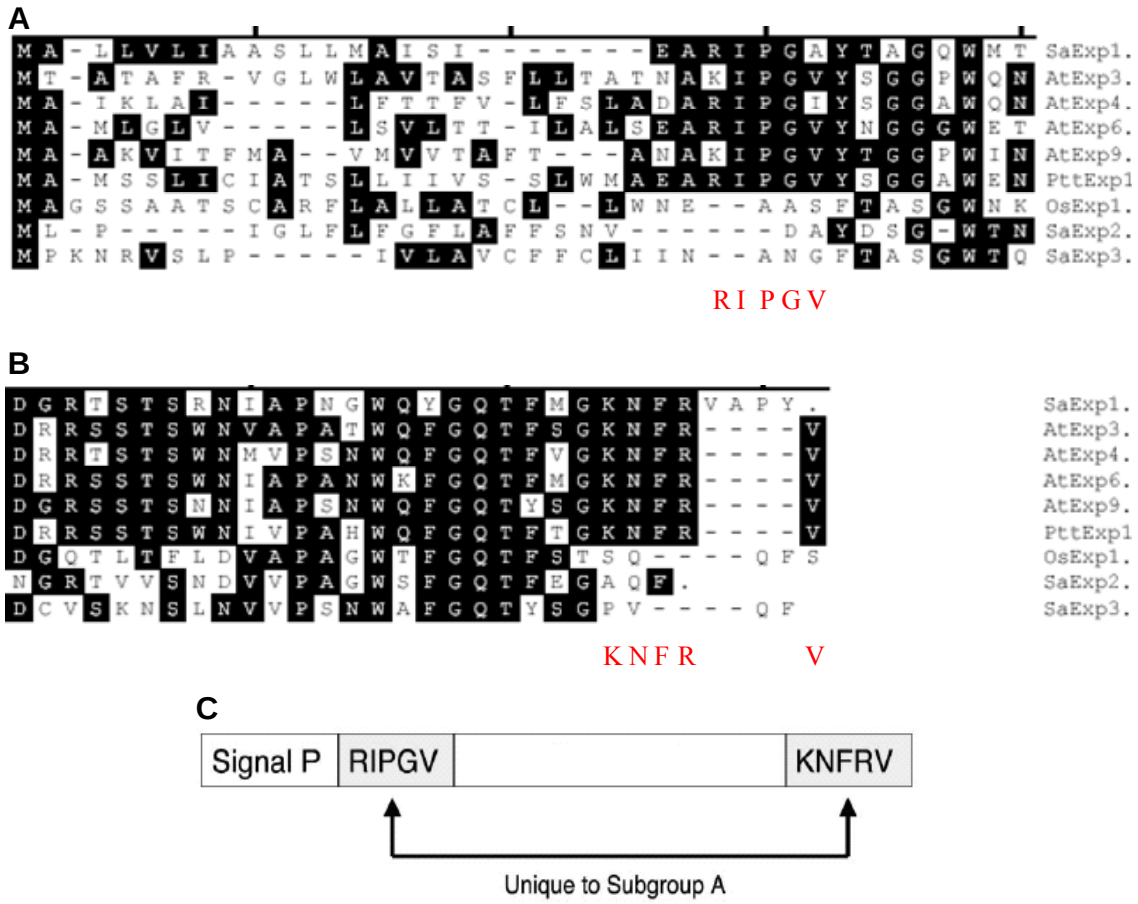

Figure 2-4 *Sequence alignment of SaExp1 with other α-expansins.* (A) Alignment of N-terminal amino acid sequences. (B) Alignment of C-terminal amino acid sequences. (C) Sequence motifs of subgroup-A α-expansins.

The promoter of SaExp1 was expressed in *A. thaliana* as a GUS reporter construct, and its expression pattern was examined in both young seedlings and mature plants of *A. thaliana*. In the seedlings, *SaExp1/GUS* was mainly expressed in the vascular tissues of stems and leaves (Fig. 2-5D), primary roots, and the base of root hairs (Fig. 2-5C). In mature plants, *SaExp1/GUS* was restricted to the base of trichomes in leaves (Fig. 2-5A). After flowering, it was expressed at the ends of the stalk connecting the flower to the inflorescence stem (Fig. 2-5B). All results are consistent with SaExp1 playing a role in xylogenesis in young seedlings. In the roots and in mature plants, SaExp1 are expressed

at the junction of different structures, suggesting that during morphological change SaExp1 may the play the role of loosening cell walls to facilitate the emergence of new structures.

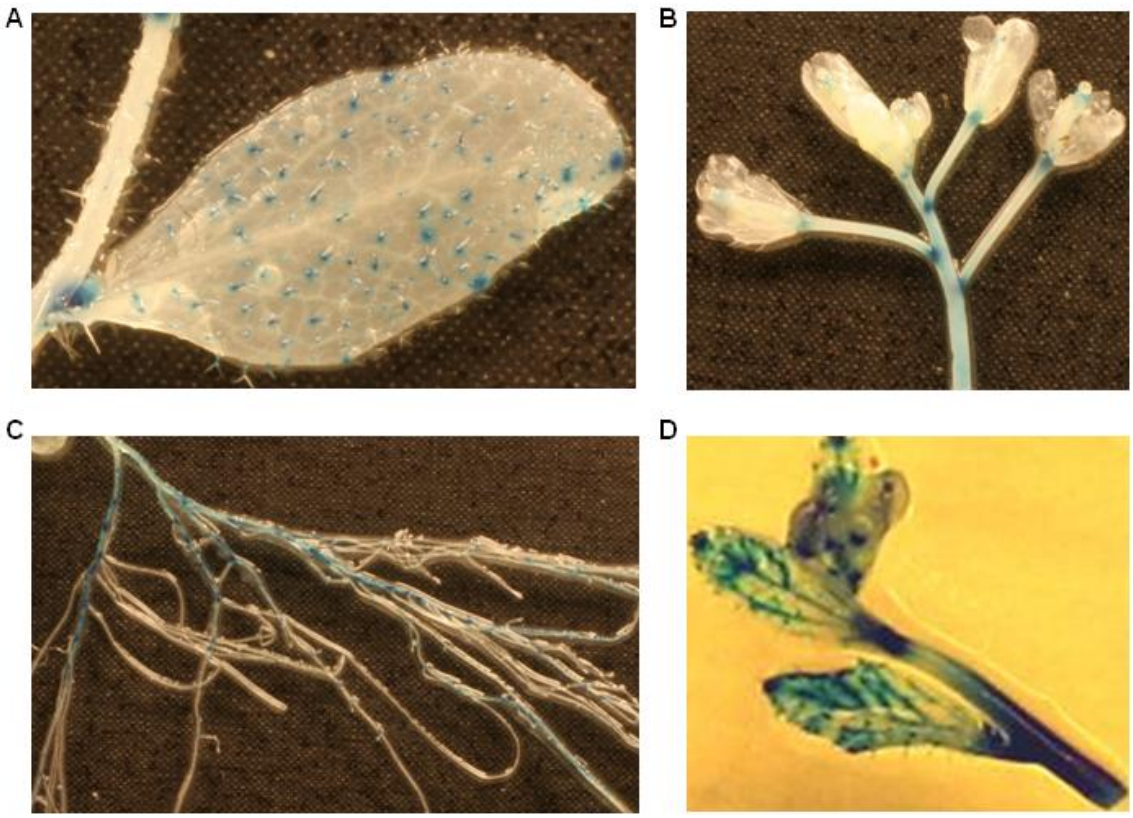

Figure 2-5 *Expression of SaExp1/GUS in A. thaliana.* (A) the leaf of a mature plant, (B) the inflorescence of a mature plant, (C), the roots of a seedling, and (D) the stem and leaves of a seedling.

Secondly, Xyloglucan endotransglycosylases (XETs) were found in both haustorium libraries, **HD9** with 3 copies and **HT5** with 9 copies, with nine being the highest repeat number found across all three libraries. XETs catalyze cleavage of a xyloglucan chain and subsequent religation to another chain. During cell expansion and elongation, the cell wall continually undergoes temporary loosening followed by rapid reinforcement, and XETs are unique enzymes in plants capable of both of these functions, modulating

xyloglucan structure as a major component of the hemicellulosic and pectic polysaccharide matrix (Eckardt, 2004), consistent with XETs playing a role in regulating cell expansion during haustorial development.

A α-galatosidase was identified in the SI library with 5 copies (**SI2**). Studies from barley and Arabidopsis showed that α-galactosidase functions in leaf morphogenesis by catalyzing cell wall loosening and cell wall expansion (Chrost et al., 2007). This finding is consistent with the observed cell expansion events during shoot initiation and may well mediate cotyledon swelling and seed coat shedding (Fig. 2-1D).

In addition to cell wall loosening genes, a myo-inositol oxygenase 2 was scored in the SI library with 2 copies (**SI3**). Myo-inositol oxygenases represent a small gene family containing four members. Studies in *A. thaliana* illustrated that this gene family is involved in the biosynthesis of nucleotide sugar precursors for cell-wall matrix polysaccharides (Kanter et al., 2005). Labeling studies with ^{3}H-myoinositol clearly showed its incorporation into pectic polymers and hemicelluloses of cell walls (Seitz et al., 2000). These results are consistent with a myo-inositol oxygenase synthesizing cell wall components as shoot development initiates.

Defense response genes and phenylpropanoid production genes

Since *S. asiaitica* produces ROS in root tip epidermal cells during semagenesis, the ROS regulating pathways is expected to be found in the libraries. Notably, a group of genes related to ROS production, processing and scavenging were found in all three libraries,

and ROS generators were found in both haustorium libraries, **HD21 and HT11.** HD21 is a NADPH oxidase (NOX) discussed in table 2-2 and Fig. 2-2, while **HT11** is a FAD-linked oxidoreductase (FOX) suggested to be defense enzymes that produce H_2O_2 to inhibit microbial growth (Carter and Thornburg, 2004; Moy et al., 2004). Meanwhile, ROS detoxifying enzymes such as superoxide dismutase (**SI8**) and peroxidases (**HD22, HD23, SI7**) were also frequently found in the libraries. Peroxidase HD23 and SI7 are the same sequence that has been found in our prior study to be an apoplastic peroxidase in *S. asiatica* (Kim et al., 1998a). The observation that both peroxidases (HD22 and HD23**)** were down-regulated within 0.5 hrs after DMBQ exposure is consistent with a role in ROS detoxification because ROS production is immediately reduced upon DMBQ exposure (Keyes et al., 2007). In contrast, the expression of the quinone oxidoreductase 2, QR2 (**HD24**) was significantly up-regulated (> 7-fold) (Table 2-2). A QR2 was cloned from another root parasitic plant *Triphysaria versicolor*, and was proposed to conduct a two electron reduction of DMBQ to protect the parasite from quinone toxicity (Bandaranayake et al., 2010), consistent with the up-regulation of QR2 in *S. asiatica* after DMBQ is generated by semagenesis.

Phenol exudation as a defense response is widely seen. Meanwhile, a phenotypic marker of haustorial development is lignin deposition along the longitudinal center in the seedling extending into the center of the haustorium (Fig. 2-1B, D). Hence, the library should find lignin biosynthetic genes in the phenylpropanoid pathway. Indeed, genes in the phenylpropanoid pathway is a recurring theme throughout all three libraries, including chorismate mutase (**HD10**), alcohol dehydrodenase (**HT6, SI4**), cinnamyl

alcohol dehydrogenase (**HD11, HT7, SI5**), and caffeoyl CoA *O*-methyl transferase (**HT9**). A further study of the phenylpropanoid pathway genes is presented in Chapter 3.

Metabolism and transport genes

The function of a haustorium is to extract nutrients from the host through a merged vascular connection. Indeed, I found genes for metabolizing and transporting nutrients in both HD and HT libraries with an abundance of metabolic genes including carbonic anhydrase (**HD32**) which catalyzes the interconversion of CO_2 and H_2O to bicarbonate and protons, carboxypeptidase (**HD33**) that mebolizes proteins, β-glucosidase (**HD31**) that breaks down starch, two amino acid metabolic genes (**HD36**, **HD37**), Sorbitol dehydrogenase (**HD38**) that converts sorbitol, a translocatable photosynthate, to fructose, and desacetoxyvindoline 4-hydroxylase (**HD35**) which is involved in the biosynthesis of vindoline, an alkaloid with unknown function in plants but used to treat acute leukemia and Hodgkin's disease (van Der Heijden et al., 2004). Transport genes found in the HD library include aquaporin (**HD30**) (Knepper et al., 2001) and a sugar transporter (**HD39**). Similarly, nutrient metabolism and transport genes were present in the HT library including aquaporin (**HT14**), Asp (**HT15**) and Lys/His (**HT18**) transporters, cysteine synthase (**HT15**) and a methionine synthase (**HT17**). In contrast, no nutrient metabolism and transport genes were found in the SI library, as probably expected for shoot initiation, highlighting the functional utility of the library approach.

2.3 Discussion

This chapter outlines my attempt to exploite the well defined modular responses of *S. asiatica* seedlings to developmental signals as an approach to identify the genes that are differentially regulated during haustorium formation or shoot initiation. The expression levels of all the genes in HD library evaluated by RT-PCR confirmed the differential library approach, and more importantly, the expression of these genes as a function of the exposure time to DMBQ were evaluated to generate a 'temporal map' to the changing expression landscape of *S. asiatica* throughout this developmental transition. I sequenced the 24 hrs period required for the haustorium to develop into three specific periods: pre-commitment (0-0.5 hrs), during terminal commitment to haustorial differentiation (2-6 hrs), and post-commitment when haustorial hairs emerge (16-24 hrs). This map is, to our knowledge, the first of its kind for the parasitic angiosperms and provides an opportunity to understand the genetic regulation of the developmental transition to parasitism.

Genes expressed during the pre-commitment phase

Of the 50 genes found in the HD library, 26 (52%) were differentially expressed as early as 0.5 hrs of DMBQ exposure. Three expansins (**HD5**, **HD6** and **HD7**) were up-regulated within 0.5 hrs, among which SaExp1 and SaExp2 (**HD7** and **HD6**) were identified and shown to be up-regulated within 0.5 hrs in our prior studies (O'Malley and Lynn, 2000a). Localization of SaExp1 expression was achieved by expressing GUS reporter constructs in *A. thaliana* (Fig. 2-5 A-C), and these results suggest that this expansin loosens cell walls at the junctions between different structures such as root/root hairs, leaf/trichomes

and flowers/stalks. Previous reports on other subgroup-A expansins homologous to SaExp1 also function in cell expansion during vascular differentiation (Gray-Mitsumune et al., 2004; Gray-Mitsumune et al., 2008). In addition, xyloglucan endotransglycosylase (**HD9**) and two lignin biosynthesis genes, chorismate mutase (**HD10**) and cinnamyl alcohol dehydrogenase (**HD11**) were up-regulated within 0.5 hours of DMBQ regulation, consistent with the remodeling of the meristem which becomes obvious by 10 hrs after induction (Fig. 3-1). And most notably, a phenylcoumaran benzylic ether reductase, which was the only sequence found in all three libraries (**HD14, HT8, SI6**) was induced as early as 0.5 hours exposure, and is the subject of further study in Chapter 3.

Several ROS related genes were differentially regulated in the earliest phase following DMBQ exposure. These genes are particularly interesting given Keyes et al demonstrated that ROS production is immediately reduced upon exposure to DMBQ (Keyes et al., 2007). Indeed, some of the most dramatic changes in expression were observed in this category with the NOX gene (**HD21**) and peroxidases **HD22** and **HD23** being significantly down regulated (5-10 folds), while expression of the quinone oxidoreductase, QR2 (**HD24**) significantly up-regulated by 7-fold, being one of the most differentially regulated genes. Peroxidases (**HD22** and **HD23**) have also been implicated in the detoxification of ROS and their down regulation is likely coupled to the reduced production of semagenic ROS by the parasite upon DMBQ exposure. Moreover, the peroxidase **HD23** is shown to be root-specific, expressed in the entire differentiation zone of all the lateral roots, but not in lateral root tips (Fig. 2-2B). The GUS staining is darker in secondary lateral roots than in primary lateral roots, and this pattern is consistent with

reports on a root-specific peroxidase involved in root differentiation (Nakamura et al., 1988). The up-regulation of QR2 supports a previous hypothesis that this enzyme plays a role in detoxifying potentially toxic xenognostic benzoquinones by two electron reduction to hydroquinones (Smith et al., 1996; Bandaranayake et al., 2010). Notably, a drug efflux transporter (**HD18**) from the multidrug and toxic compound extrusion (MATE) family of transporters was also significantly up-regulated within 0.5 hours by 6-fold. Loss of function mutants in MATE proteins show diminished resistance to pathogen challenges through a salicyclic acid mediated pathway as well as an increase in ROS production (Sun et al., 2011). Arguably, the rapid increase in the expression of this gene could once again help detoxify the seedling from exposure to xenognostic benzoquinones.

With regard to genes associated with hormone regulation, two cytokinin response regulators (**HD28** and **HD29**) and one of the two auxin regulated proteins (**HD26**) were up-regulated within 0.5 hours. The early timing of the regulation of auxin/cytokinin signaling pathways is consistent with the hypothesis that *S. asiatica* exploits pre-existing auxin/cytokinin pathways to mediate haustorial development as will be further developed in Chapter 4.

Regulated genes in the commitment phase

Of the 50 genes found in the DMBQ induced haustorium library, 23 (46%) were differentially expressed during the commitment phase, 2-6 hrs after DMBQ exposure. Expansin **HD4** was expressed after 2 hrs of DMBQ treatment, and xyloglucan

endotransglycosylase **HD9** was further up-regulated starting from 6 hrs, suggesting that these genes might play a role in growth of the haustorial hairs or later swelling events of the root tip. Elongation factor 1 (**HD2**) and α-tubulin (**HD1**) were significantly down-regulated during this period which likely reflects on the relationship between the rate of microtubule assembly and haustorium development (Ursin et al., 1991; Bao et al., 2001). The reduced expression of α-tubulin in *A. thaliana* is associated with radial expansion of the root tip, inhibited root elongation, and ectopic root hair formation, all features consistent with haustorium maturation. With regards to plant defense responses, NOX, the two peroxidases, and QR2 remain differentially regulated throughout this period as well. Down-regulation of both peroxidases is even more substantial at these later time points. Among the genes associated with hormonal regulations, the auxin-regulated protein (**HD25**), which did not appear earlier, was up-regulated at 6 hours. The other auxin-regulated protein (**HD26**) and two cytokinin response regulators (**HD28** and **HD29)** remained up-regulated throughout this phase.

Genes in the post-commitment phase

Only one gene, Expansin (**HD8**), was not regulated until after 6 hrs of DMBQ exposure, consistent with HD8 being involved in haustorial hair growth which does not begin until after 6 hrs. The reason that the HD library is enriched with genes regulated during the pre-commitment and commitment phases but not the post-commitment phase might be that the library was made by seedlings exposed to DMBQ for 4.5 hrs, the $t_{1/2}$ for DMBQ induced haustorial development.

Comparing the two haustorium libraries

The genes found in the HD library shared significant similarity with the genes in the HT library, including the cell expansion genes with a 67% overlap and vascular differentiation genes with a 75% overlap. In the defense response category, ROS generating, ROS scavenging and calmodulin related genes are found in both libraries. Nutrient metabolism/transport genes and the transcription/protein synthesis/protein degradation category show a high overlap as well. Even in the "others" category, an identical sequence was found in both libraries with unknown function but specific to epicotyl tissue in chickpea (Muñoz et al., 1997).

Comparing two haustorium libraries with the shoot initiation library

Swelling and vascular differentiation are the phenotypic markers common to both haustorium development and shoot initiation (Fig. 2-1 B, D). Indeed, cell expansion and phenylpropanoid pathway genes were found in all three libraries. However, the genes in the vascular differentiation category has a 100% overlap between SI library and HD/HT libraries, drastically contrasting the zero overlap between the cell expansion genes. In the cell expansion category, there is no overlap between the HD/HT libraries and the SI library, relative to the 67% overlap between the two haustorium libraries, suggesting that the molecular events that lead to the swelling of cotyledons during shoot initiation are different than those leading to the well defined radial swelling of the root tip during haustorial development. Meanwhile, in contrast to haustorium libraries, no nutrient transport genes were represented in the SI library, consistent with the function of the haustorium as an absorptive organ (Seitz et al., 2000; Kanter et al., 2005).

2.4 Methods

Preparation of reagents and plant material

Trans-zeatin, 2,6-dimethoxy-benzoquinone (DMBQ), Quinone assays were performed by making fresh stock solutions (1 or 10 mM) in DMSO, then diluted into wells containing 5 ml 0.1 mM KCl to the final concentration. All solutions contain 0.1 mM KCl unless specifically stated. Seeds of *Striga asiatica* were pre-treated as previously described and germinated by exposure to 10^{-9}M Strigol ((Kim et al., 1998a). Murashige and Skoog (MS) media was purchased from Caisson Labs (North Logan, UT). All other chemicals were obtained from Sigma Aldrich (St. Louis, MO). Columbia Wild type (Col-0) *Arabidopsis thaliana* seeds were purchased from Lehle Seeds (Round Rock, TX). *Striga asiatica* seeds were obtained from the U.S. Department of Agriculture (Beltsville, MD) and all experiments were conducted under quarantine conditions for noxious plants. These experiments were conducted under the auspices of the USDA quarantine license awarded to Emory University. *Striga asiatica* seeds were obtained from Drs. R.E. Eplee and Rebecca Norris (U.S. Department of Agriculture, Witchweed Methods Development Laboratory; Oxford, NC) surface sterilized and germinated as previously described using 10^{-8}M strigol (Kim et al., 1998a). All work was done under the auspices of the USDA quarantine licenses awarded to the Emory University.

Characterization of the induction of haustorial formation and/or shoot initiation

Haustoria formation in germinated seedlings of *S. asiatica* is induced by treatment with 10 μM DMBQ or trans-zeatin. To determine the number of seedlings committed at any

exposure time the seedlings are washed and scored for haustoria formation after 24 hours. Shoot initiation was induced by treatment with 10 μM trans-zeatin and is scored by the shedding of seed coats resulted from the swelling of two cotyledons.

Constructing the haustorium and the shoot initiation libraries

The DMBQ induced haustorium library is made by subtractive hybridization of cDNAs made from two populations of *S. asiatica* seedlings: untreated seedlings and seedlings treated with DMBQ for 4.5 hours. Likewise, the trans-zeatin induced haustorium library is subtraction between untreated seedlings and seedlings treated with trans-zeatin for 4.5 hours. The shoot initiation library is subtraction made between DMBQ treated seedlings for 4.5 hours and trans-zeation treated seedlings for 7.5 hours. 3. SSH library construction. The subtractive hydridization was done using the PCR Select cDNA subtraction kit (Clontech, CA, USA). Then the screening of the coarse library was done using PCR-Select Differential Screening Kit (Clontech). The gene fragments obtained as positive hits in the screening were cloned into Topo TA cloning kit (Invitrogen). The ESTs were sequenced using M13 primers and compared with sequences in NCBI database using BLAST algorithms. The full cDNA sequences of some selected genes were obtained with the SMART Race cDNA Amplification Kit (Clontech).

RNA isolation and rt-PCR expression analysis

Total RNA was extracted from *S.asiatica* seedlings with RNeasy Plant Mini Kit (Qiagen) and the integrity was analyzed by electrophoresis in 8% formaldehyde, 1.5% agarose gel. Superscript ^{TM} III transcriptase (Invitrogen) was used to catalyze the reverse transcription

at 50 °C. To analyze the expression pattern of these genes, RT-PCR method was employed with using actin as the internal standard marker. Primers for actin are: reverse: 5'-CAGGCTGTTCTCTCCCTTTAT-3', forward: 5'-TCCGATCCAGACACTGTACTT-3' , adding four primers (two for actin, two for the gene in question) together in a PCR system with *S.asiatica* cDNA from different organs or different treatments with annealing temperature at 58°C. Analyze the PCR product in 2% agarose gel.

Cloning of the gene promoters and transformation of A. thaliana for GUS staining

About 1-2Kb DNA sequence was cloned upstream a few selected genes' start codon with TAIL PCR. Put promoter sequences into PBI101 vector with appropriate restrictive enzymes to get the genes' promoter GUS constructs. Constructs were introduced into the Columbia (Col-0) ecotype with *Agrobacterium tumefaciens* GV3101 by vacuum infiltration and the transformants were selected on MS plates with Kanamycin (50 μg/ml).

GUS staining

Arabidopsis transformants are vacuum infiltrated for 5 minutes in the staining solution (50 mM sodium phosphate buffer, pH 7.0, 0.2% triton-X-100, 1 mM X-Gluc), incubated at 37°C, and fixed in 75% ethyl alcohol. The pictures were taken with a Canon digital camera.

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Chapter 3 - Phenylpropanoids and regulation of plant growth and development

3.1 Introduction

The phenylpropanoids consist of a significant group of biosynthetic building blocks in vascular plants. Complex primary and secondary wall lignols, antibiotics and other protectants against biotic and abiotic stress, as well as regulators of plant cell growth and development are all derived from this pathway. This latter category includes the plant auxin indole acetic acid (IAA) (Normanly et al., 1997), growth factors isolated from crown gall tumors that activate cell division in tobacco pith culture dehydrodiconiferyl alcohol glucosides (DCGs), the flavanoids in maize and petunia necessary for pollen viability (Coe et al., 1981; Taylor and Jorgensen, 1992), simple endogenous modulators of auxin transport (Mathesius et al., 1998; Brown et al., 2001), and monolignols that regulate pathogenesis of prokaryotic pathogens (Duban et al., 1993b; Palmer et al., 2004b).

The parasitic angiosperms present an unusual juxtaposition of monolignol biosynthesis, organogenesis, and defense response in the case of haustorium development of the obligate hemiparasite *Striga asiatica* (Schrophulariaceae). At early points in its development, lignin deposition in cell walls and the vascular tissue is limited. Yet, the

seedling is poised for rapid differentiation of the haustorium, the organ that forms the complete vascular bridge between host and parasite (Riopel and Baird, 1987; Riopel and Timko, 1995). The induction of haustorial organogenesis marks the initiation of a significant investment in the development of vascular elements in *S. asiatica*. Previous studies found that a continuous exposure of several hours to host cell wall phenylpropanoids is required for terminal commitment to haustorial development (Smith et al., 1990b).

The time-dependence of this developmental transition presented us an opportunity to study the genetic regulation of the commitment from vegetative growth to haustorial development by constructing differential expression libraries. In this chapter, I report a direct investigation of the genes in monolignol biosynthesis found in the differential expression libraries. A *Striga* asiatica chorismate mutase *SaCM1* (DQ431680), the partitioning step in the shikimate pathway that directs the carbon flux into biosynthesis of phenylalanine (Krappmann et al., 2000; Knaggs, 2003) --- the starting material of phenylpropanoid pathway (Eberhard et al., 1993; Amthor, 2003; Kloosterman et al., 2003), and a *Striga asiatica* cinnamyl alcohol dehydrogenase *SaCAD1* (DQ431681) --- a terminal step in the phenylpropanoid pathway (Wyrambik and Grisebach, 1979) were selected initially because they bracket the biosynthesis of monolignols, the building blocks of vascular differentiation as one of the phenotypic markers of haustorium development. A striga asiatica Phenylcoumaran benzylic ether reductase *SaPCBER1* (DQ442382), a downstream enzyme of the phenylpropanoid pathway which catalyzes the ring-opening reduction of diconiferyl alcohol (DCA) --- a dimer of coniferyl alcohol ---

was found in all three differential expression libraries and selected for further investigation in this chapter because of the notable discovery on DCAs. The DCA glucosides (DCGs) were discovered as phenylpropanoid derivatives isolated from Vinca rosea tumor cells and have been shown to have cell division promoting activities when added directly to tobacco pith or leaf assay systems, and in cytokinin treated dividing cells the concentration of DCGs increased by two orders of magnitude (Wood et al., 1969; Wood et al., 1972; Wood et al., 1974; Binns et al., 1987b; Teutonico et al., 1991a; Black et al., 1994; Tamagnone et al., 1998b). The model proposed was that cytokinin up-regulated DCGs to mediate cell division. Biosynthesis experiments showed that these DCG compounds are not cell wall fragments, as previously suggested, but are biosynthesized directly from coniferyl alcohol through dimerization followed glycosylation (Orr and Lynn, 1992). DCGs are a prominent example where phenylpropanoid derivatives serve the role of regulation in plant growth and development, and raise questions as to whether this developmental role generalize to other phenylpropanoid derivatives. In this study, we addressed this question not by applying phenolic compounds exogenously, but by changing the expression level of phenylpropanoid biosynthesis enzymes.

3.2 Results

The phenylpropanoid pathway mediates DMBQ induced vascular differentiation during haustorium development in Striga asiatica

1.5 day old seedlings were treated with 10 μM DMBQ and observed optically after 10 hrs. By 10 hrs a dark line has formed along the longitudinal center of the seedling, indicating increased lignin deposition (Fig. 3-1B). When excited with 254 nm UV light, this central line exhibits significant autofluorescence that is most characteristic of lignin (Fig. 3-1C).

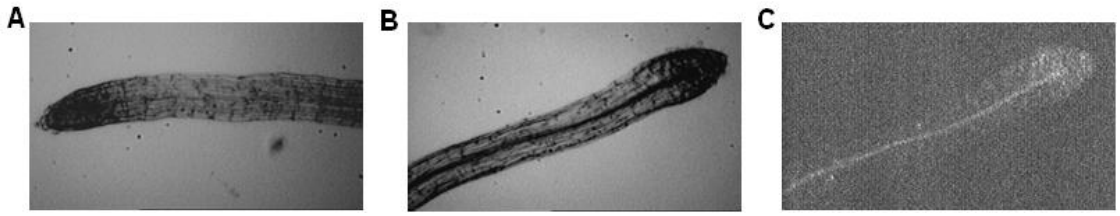

Figure 3-1 *Lignin deposition during haustorial development.* (A) No significant lignin deposition was seen in the untreated 1.5-day-old seedling. (B) Lignin deposition was detected as a dark center line in 1.5-day-old seedlings treated with 10μM of DMBQ for 10 hrs. (C) Lignin deposition was detected as a fluorescent center line when excited at 254 nm.

We then determined whether and where the selected lignin biosynthesis genes, SaCM1 and SaCAD1, are expressed. The organ-specific transcription of SaCM1 and SaCAD1 is evaluated by RT-PCR from root tissue of *S. asiatica* seedlings, leaf + stem and flower tissue from mature *S. asiatica* plants grown in culture (Fig. 3-2A). Both genes are expressed to similar level independent of the organ type. To further evaluate tissue-specific localization, the promoter of either gene was isolated and fused to β-glucuronidase (GUS) and these GUS constructs were transformed into *Arabidopsis thaliana.* As shown in Fig 3-2B, transcriptional activation of SaCM1 was seen as GUS staininig in the leaf vascular tissue in an *A. thaliana* seedling which had not bolted. In Fig. 3-2C, SaCAD1 expression was seen in the leaf vascular tissue, parts of the stem and flowers in an *A. thaliana* plant that had bolted and flowered. SaCAD1 expression did not occur throughout the stem possibly because the phenylpropanoid production is not constitutive after the plants have matured and lignin has been deposited.

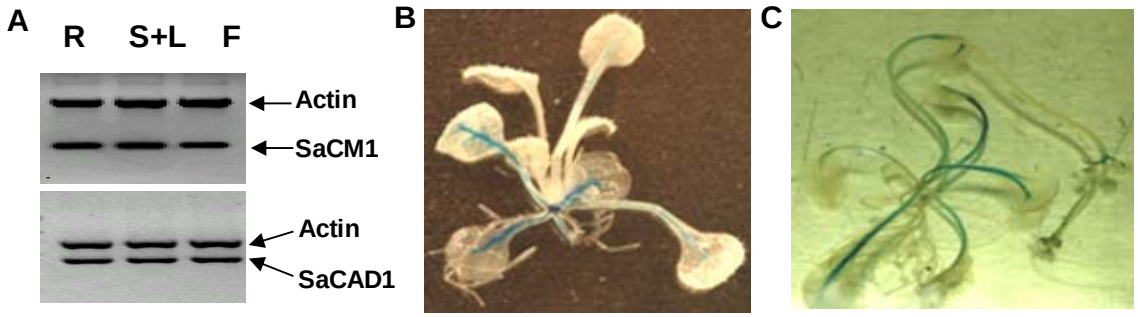

Figure 3-2 *Spacial expression patterns of SaCM1 and SaCAD1 in S. asiatica and in A. thaliana.* (A) Organ-specific expression level of SaCM1 and SaCAD1 were analyzed by RT-PCR in R: root of *S. asiatica* seedlings, S+L: stem and leaf of mature *S. asiatica* plants, F: flowers of mature *S. asiatica* plants. Actin was used as an internal standard. (B) Localization of SaCM1/GUS expressed in *A. thaliana*. (C) Localization of SaCAD1/GUS in *A. thaliana*

Now the expressions of SaCM1 and SaCAD1 have been shown to be spatially correlated with vascular differentiation, we then determined whether the regulation of phenylpropanoid pathway by haustorial inducers is temporally correlated with rapid lignin deposition during haustorial development. SaCM1 and SaCAD1 transcripts levels were evaluated at six time points over a course of 24 hrs of DMBQ treatment. As shown in Fig. 3-3, DMBQ up-regulated both genes as early as 0.5 hrs. SaCM1 transcription reached maximum at 2 hrs, remained at that level from 2-16 hrs, and returned to basal level after 16 hrs (Fig. 3-3A). SaCAD1 induction was weaker, reached a plateau between 2 and 6 hr, but remained elevated throughout the period of 24 hrs (Fig. 3-3B). This DMBQ induced temporal expression profile was consistent with the rapid lignin deposition by 10 hrs after exposure to haustorial inducers (Fig. 3-1), and remained the same with two other haustorial inducers, benzoquinone (BQ) and kinetin (a cytokinin) (Fig. 3-3C-F). The non-inducer tetrafluorobenzoquinone (TFBQ), however, did not alter gene expression over the entire 24 hrs period (Fig 3-3G, H).

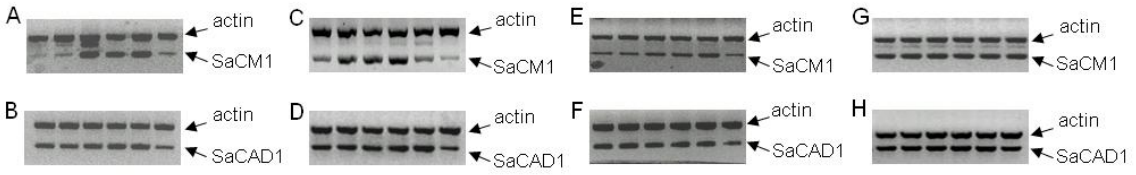

Figure 3-3 *Temporal expression patterns of SaCM1 and SaCAD1 in S. asiatica seedlings treated with different compounds analyzed by RT-PCR*. (A,E) DMBQ, (B,F) BQ, (C,G) kinetin, (D,H) TFBQ. On each gel figure the six lanes from right to left are 0 hr, 0.5 hrs, 2 hrs, 6 hrs, 16 hrs, and 24 hrs of exposure to the compound.

Do phenylpropanoid derivatives play other roles in plants rather than lignin production?

Coniferyl alcohol is one of the three monolignols synthesized by phenylpropanoid pathway. Glucoside conjugates of the dimer of coniferyl alcohol, dehydrodiconiferyl alcohol glucosides (DCGs) were found to be growth factors that mediate cytokinin induced cell division (Wood et al., 1969; Wood et al., 1972; Wood et al., 1974; Binns et al., 1987b; Teutonico et al., 1991a; Orr and Lynn, 1992; Black et al., 1994; Tamagnone et al., 1998b; Attoumbré et al., 2006). A Phenylcoumaran benzylic ether reductase (PCBER), a downstream enzyme of the phenylpropanoid pathway that catalyzes the ring-opening reduction of dehydrodiconiferyl alcohol (Mijnsbrugge et al., 2000; Vander Mijnsbrugge et al., 2000; Shoji et al., 2002; Turley, 2008) was the only sequence (DQ442382) found in all the three differential expression libraries discussed in Chapter 2. If the ring-opening reduction of DCA inactivates its developmental activity, then the observation that PCBER is up-regulated in the haustorium library (Fig. 3-4A) but down-regulated in the shoot initiation library (Fig. 3-4 B) is consistent with the DCGs' role of mediating cytokinin induced cell division. When the promoter of SaPCBER1 was transformed into *A. thaliana* as a GUS reporter construct, its expression was localized to lateral buds (Fig. 3-4C), areas of indefinite shape and size of GUS staining on leaves (Fig.

3-4C, F), maturation zone in roots excluding root tips (Fig. 3-4F, G), and flower stalks (Fig. 3-4D, E), a pattern that overlaps precisely with cytokinin functions including releasing apical dominance, delaying leaf senescence, controlling cell division in the roots, and promoting flowering. When young seedlings and mature *A. thaliana* are compared, SaPCBER/GUS was expressed at higher relative levels in the seedlings. Larger areas of GUS staining were seen in the roots and all the leaves in young seedlings (Fig. 3-4F). A gradient of blue staining increased from the elongation zone to the maturation zone, and the root tips where cell division is active are not stained in the seedlings (Fig. 3-4F, G). In contrast, smaller area of GUS staining in a fraction of leaves and weaker staining in the roots were seen in the mature *A. thaliana* (Fig. 3-4C). These results provide new perspectives on the roles that phenylpranoid derivatives play in plant growth and development beyond lignin biosynthesis.

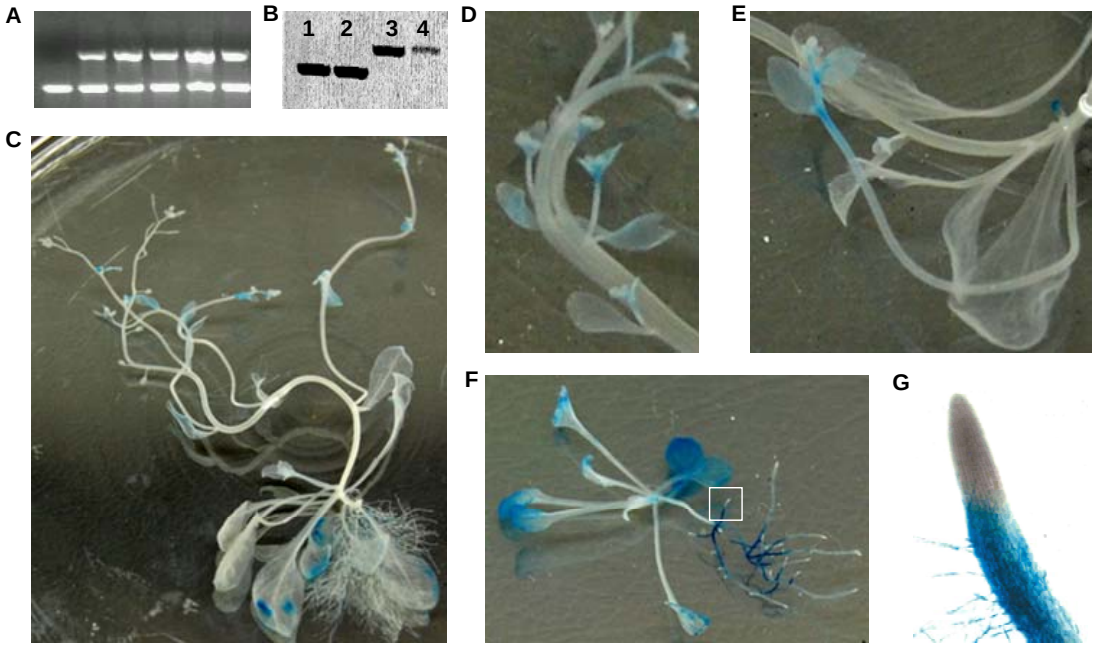

Figure 3-4 *Temporal and spatial expression pattern of SaPCBER1*. (A) Regulation of SaPCBER1 transcription when *S. asiatica* seedlings were treated with DMBQ for (left to right) 0 hr, 0.5 hr, 2 hr, 6 hr, 16 hr, 24 hrs as evaluated by RT-PCR, the bottem band is actin as a internal PCR control, (B) Regulation of SaPCBER1 transcription during shoot initiation as compared between *S. asiatica* seedlings treated with DMBQ for commitment to haustorium development (lane 3) and seedlings treated with trans-zeatin for commitment to shoot initiation (lane 4) with actin as an internal loading control with the pool of transcripts from either seedling population as the templates (lane 1, 2). (C) GUS staining in a mature *A. thaliana* plant expressing SaPCBER1/GUS, (D, E) GUS staining in flower stalks in a mature *A. thaliana* transformant, (F) GUS staining in a young *A. thaliana* seedling expressing SaPCBER/GUS, (G) Enlargement of the boxed area in (F).

Does altering the level of phenylpropanoids by overexpessing SaCM1 or SaCAD1 affect plant growth and development?

The next question we asked was whether altering the expression level of SaCM1 and SaCAD1, the two enzymes bracketing the biosynthetic pathway of phenylpropanoids, affect development by changing the endogenous level of phenylpropanoids. When over-expressed under the 35S constitutive promoter in *A. thaliana, 35S/SaCM1* transformants displayed significantly enhanced lateral growth as compared to the wt: all the lateral buds

developed into leafy branches vs. minimal lateral growth in the wt, increased sizes of leaves by 2-3 folds, and expanded girth of stem by 2-3 times than that of the wt (Fig. 3-5).

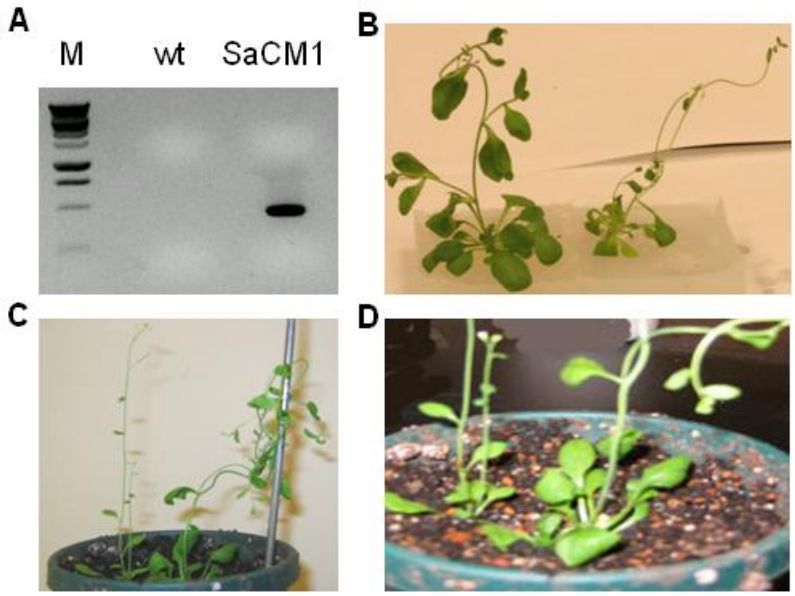

Figure 3-5 *Over-expression of SaCM1 in A. thaliana significantly increased the size of the plants.* (A) Over-expression of SaCM1 in *A. thaliana* transformants was verified by RT-PCR. (B) a wild type *A. thaliana* seedling (right) and a 35S/SaCM1 transformant seedling (left) grown on MS medium, (C) a wild type *A. thaliana* seedling (left) and a 35S/SaCM1 transformant seedling grown in soil, (D) A enlargement of the bottom third of (C).

Most notably, 35S/SaCM1 transformants showed a significant increase in the number of stem vascular bundles from 6 in the wild type (Fig. 3-6C) to 9 in the transformants (Fig. 3-6D, the pith tissue in the middle was lost during section slicing). This increase to 9 bundles is highly consistent across multiple seedlings tested relative to the 6 in wt. Increased vascular differentiation is further observed in the number of lignified cell layers in the transformants by 2-3 times compared to wt (Fig. 3-6E vs. F). When the young seedlings of *35S/SaCM1* transformants were exposed to 254 nm UV light, the seedlings exhibited a significant autofluorescence in the vascular tissue, indicating a significant increase in lignin content by a factor of 2-3 (Figure 3-6A, B). Conversely, the

overexpression of *SaCAD1* did not present any obvious phenotypic difference in the vascular differentiation or lignin content (data not shown).

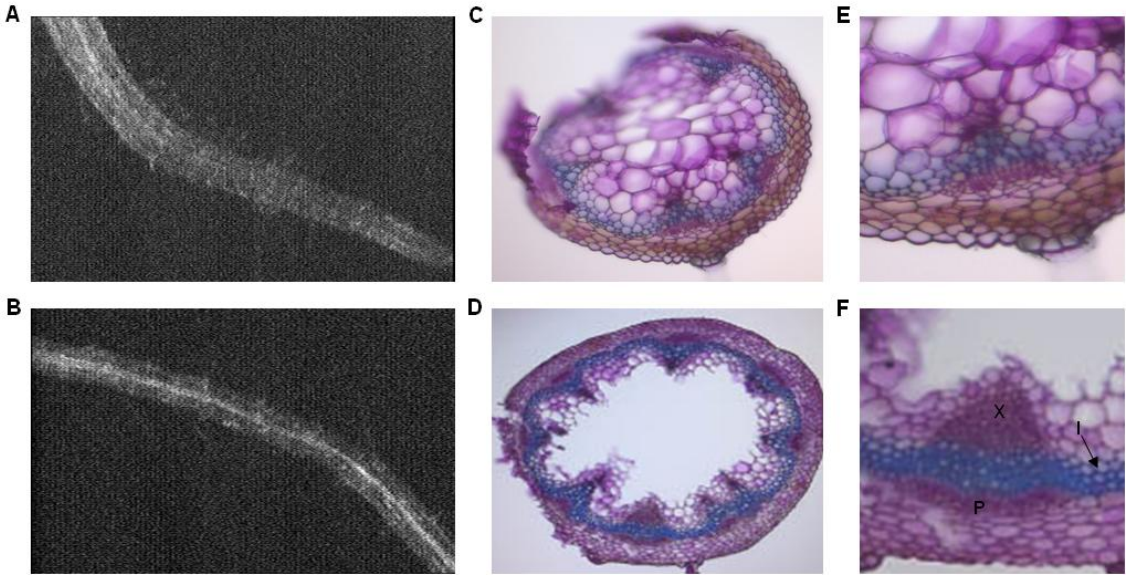

Figure 3-6 *Over-expression of SaCM1 in A. thaliana alters developmental pattern of the stem vascular bundles.* (A, C, E) Wild type *A. thaliana* seedlings, (B, D, F) SaCM1 over-expressing *A. thaliana* transformants, (A, B) fluorescence of lignin excited by 254 nm UV light, (C, D) Toluidine Blue O staining of the stem cross-section of *A. thaliana* transformant seedlings, (E) the enlargement of one vascular bundle in C, (F) the enlargement of one vascular bundle in D, X: xylem, P: Pholem, I: intervascular tissue.

3.3 Discussion

Successful parasitism among parasitic angiosperms like Striga spp. depends on proper penetration and the subsequent acquisition of host resources through haustorium, a highly vascularized organ. Using our three differential expression libraries, we found genes involved in the monolignol biosynthetic pathways and selected three for further investigation. A chorismate mutase was selected because it bifurcates carbon flux into the phenylpropanoid pathway, and a cinnamyl alchohol dehydrogenase was selected because

it is the final step of monolignol. The up-regulation of SaCM1 and SaCAD1 in response to haustorial inducers were further shown to be correlated with the deposition of lignin, as observed by increased autofluorescence excited at 254nm. Similar lignin deposition has previously been observed in the vascular tissue of other parasitic plants during haustorium formation such as Castilleja (Dobbins and Kuijt, 1973). Localization of SaCM1 and SaCAD1 to vascular tissue was confirmed by transforming the promoters for these two genes into *Arabidopsis* as GUS reporter constructs.

The temporal and spatial expression patterns of these two phenylpropanoid biosynthesis genes were correlated with lignin deposition during haustorium development. However, the critical question we aimed to address in this study was whether phenylpropanoids and their derivatives play any role in regulating growth and development in plants beyond lignin biosynthesis. This research was inspired by the observation that a DCGs metabolizing enzyme SaPCBER1 was the only gene found in all three differential expression libraries. The DCG metabolizing enzyme SaPCBER1 was up-regulated during haustorium development but down-regulated during shoot initiation. Such prediction would be consistent with the prediction that DCG disappearance mediates the halt of cell division during haustorium development and their increases promote cell division during shoot initiation. When the promoter of SaPCBER was transformed into *A. thaliana* as a GUS reporter construct, its expression was localized to lateral buds, areas of indefinite shape and size on leaves, areas of the roots where growth and differentiation occurs, but not in the root tips. This pattern is consistent with the typical cytokinin actions including release of apical dominance and activating lateral buds, delay of leaf senescence, and cell

division activities in the roots. These results suggest a more general developmental role for both DCGs and PCBER during plant growth and development?

To further test this assertion, the endogenous expression level SaCM1 or SaCAD1 was regulated in *A. thaliana* transformants. When SaCM1 was overexpressed in *A. thaliana*, not only was the stature of plants increased significantly, but most notably, the developmental pattern of vascular bundles increased by 50%, and the number of layers of lignified cells in both xylem and phloem tissues were multiplied by 2-3 folds, suggesting an abnormonal development during vascular differentiation. This phenotype of increased vascular bundles and multiplied layers of lignified cells in the vascular tissue is distinct from the chorismate mutase overexpression phenotype noted for soybean hairy root tissues (Doyle and Lambert, 2003). Chorismate mutase is secreted into plant cells by *Meloidogyne javanica*, a plant-pathogenic nematode. Transgenic expression of MjCM1 in soybean hairy roots results in a noted absence of vascular tissue, a phenotype that can be rescued by the addition of indole-3-acetic acid (IAA). These results were interpreted as MjCM1 overexpression reduceing IAA biosynthesis by partitioning the carbon flux into the phenylpropanoid pathway. In both cases of CM overexpression in *A. thaliana* and soybean, hairy roots the pattern of vascular tissue was profoundly altered, but one is a 50% increase; the other is a 100% decrease. This difference may arise from the altered phytohormone sensitivity of hairy roots generated by *Agrobacterium rhizogenes*-mediated plant transformation (Zambryski et al., 1989). Other mutations that alter monolignol biosynthesis have also been shown to alter plant cell development (Tamagnone et al., 1998a; Streatfield et al., 1999; Chabannes et al., 2001).

Overexpression of *AmMYB308*, a gene encoding a MYB transcription factor from Antirrhinum, down regulates lignin accumulation in tobacco, and these transformants show abnormal leaf palisade development and premature cell death (Tamagnone et al., 1998a). The levels of two diastereomeric DCGs were also significantly reduced in these plants, and the phenotype was rescued by supplying these lignol DCGs exogenously. Mutations within the *A. thaliana* plastid inner envelope phosphoenolpyruvate /phosphate translocator result in similar morphologies that are also correlated with monolignol synthesis. Again, the developmental changes were rescued by feeding aromatic amino acids, the biosynthetic precursors of the phenylpropanoids (Streatfield et al., 1999). Finally, overexpression of phenylalanine ammonia-lyase, the first enzyme in the phenylpropanoid pathway, down-regulated phenylpropanoid biosynthesis in tobacco and caused abnormal plant development and flower morphology (Elkind et al., 1990). Therefore, these studies suggest a model (Binns et al., 1987a; Teutonico et al., 1991b) where phenylpropanoid production/accumulation controls morphology.

3.4 Methods

Preparation of Striga asiatica Seeds

Striga asiatica seeds were obtained from the U.S. Department of Agriculture, Beltsville, MD, maintained under quarantine, and when needed, pre-treated as described and germinated using 10^{-9}M strigol (Kim et al., 1998b). All work was done under the auspices of the USDA quarantine licenses awarded to Emory University. *Striga asiatica* seeds were treated for germination using a serial regime of the following solutions: 3%

chromic acid for 3 mins, 1% tween-20 for 1 min, 7% bleach for 7 minutes, 75% ethyl alcohol for 2 mins, and then placed in water in sealed flasks for 10-14 days. Seeds were induced to germinate by 10 nM Strigol. Germinated seedlings were used for RNA extraction and fluorescence measurements at 36 hours old.

Striga asiatica Plant Cultures

Germinated sterile seedlings were transferred to Murashige and Skoog (MS) medium supplemented with different concentrations of 6-BA and IAA. Two treatments provided the best results: 6-BA 1 mg/L with IAA 0.1mg/L, or 6-BA 1 mg/L with IAA 0.5 mg/L. All plants were cultured at 23°C under a 16 hr light/8 hr dark photoperiod. After about one week, regenerated shoots were visible and the resulting *S. asiatica* plants generally flower in approximately 1-1.5 months under these conditions.

Detection of Lignin Deposition during Treatment with DMBQ

Lignin deposition was evaluated by observing fluorescence from 254nm excitation. Seedlings were placed in depression slides and the images collected on a Lycia microscope.

PCR-select cDNA Subtractive Hybridization

RNA extraction from 36 hrs old seedlings with or without treatment with 10 M DMBQ for 6 hrs was performed using the RNeasy Plant Mini Kit (Qiagen). A differential expression library was then constructed using the PCR-Select™ cDNA Subtraction Kit (clontech). Gene fragments ranging from 500bp to 1kb were cloned using the PCR-

Select™ Differential Screening Kit (clontech), while full length cDNA sequences were obtained by the SMART cDNA Amplification Kit (Clontech). All fragments were cloned by using Topo TA cloning kit (Invitrogen).

Expression Analysis by Relative RT-PCR

Total RNA was extracted from *S. asiatica* germinated seedlings with RNeasy Plant Mini Kit (Qiagen) and the integrity was analyzed by gel electrophoresis (8% formaldehyde, 1.5% agarose). Superscript™ III transcriptase (Invitrogen) was employed for reverse transcription at 50°C. The expression pattern of these genes was analyzed by virtual RT-PCR employing actin as an internal control. Primers for actin are: reverse 5'-caggctgtt ctctccctttat-3', forward 5'-tccgatccagacactgtactt-3' (Florea and Timko, 1997), primers for *SaCM1* are: reverse 5'-gcacaggccagtgccttgcttcgac-3', forward 5'-gacttgctttgtcaaagggatcatg-3', primers for *SaCAD1* are: reverse 5'-ggtggggtggggcacatggggg-3', forward 5'-gctgtagcggacgtcattcttct-3'. The four primers, two for actin and two for the individual gene, were added together in a PCR system with cDNA from different organs of *S. asiatica* or different time point during haustorium formation. The PCR Annealing temperature was set at 58°C and analyzed via a 2% agarose gel.

Promoter Cloning and GUS Assay

Approximately 2Kb of the DNA sequence upstream of the start codon (ATG) was cloned for both *SaCM1* and *SaCAD1* using TAIL PCR. The promoter was removed with XbaI and BamHI and ligated into the pBI121 vector so as to place GUS, the reporter gene,

behind the promoter. Constructs were then transformed into Agrobacterium GV3101 and introduced into *Arabidopsis* Columbia (col-0) ecotype using the vacuum transformation method (Bechtold et al., 1993). Transformants were selected on solid MS media plates with Kanamycin (50ug/ml). *Arabidopsis* transformants were vacuum infiltrated for 5 minutes in the staining solution (50mM sodium phosphate buffer PH 7.0, 0.2% triton-X-100, 1mM X-Gluc), incubated at 37°C overnight for the color to develop, and fixed in 75% ethyl alcohol. The pictures were taken with a Canon digital camera.

Gene-overexpression Constructs and Histological Analysis

The complete cDNA sequence of both genes were placed behind the 35S promoter of the pBI121 plasmid, and transformed into *Arabidopsis* Columbia (col-0) ecotype. Transformants were screened by the methods described herein. Successful transformation and expression were confirmed by time course analysis by Reverse Transcription-PCR and DNA sequencing. Both *Arabidopsis* wild type and transformants are grown in agar boxes with a 16hr light/ 8hrs darkness photoperiod. Selected mature plants were then potted in soil. Hand-cut *Arabidopsis* stem slides were stained in 0.05% toluidine blue O (TBO) for 1 min, then rinsed in ddH_2O, placed on slides with a single drop of water, and imaged under an optical microscope. Root vascular development was compared by observing lignin deposition via the aforementioned fluorescence assays where *SaCM1* over-expressing transformants readily autofluoresce because of dramatically enhanced lignin deposition, while the wile type *Arabidopsis* served as a negative control.

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Chapter 4 - Haustorium inducing benzoquinone signals interact with auxin/cytokinin signaling pathways to mediate haustorial development in *Striga asiatica*

4.1 Introduction

The generic two-component systems (TCS), also known as a Histidine-to-Aspartate (His-to-Asp) phosphorelay, are signal transduction modules involved in a variety of cellular responses to environmental stimuli (Alm et al., 2006; Laub and Goulian, 2007). The signal receptor histidine kinase (HK) and the phosphate-accepting aspartate were first discovered in *E. coli* and were considered to exist only in prokaryotes (Aiba et al., 1989a; Aiba et al., 1989b). Now 30 different TCSs we know exist in *E. coli* alone (Mizuno et al., 1996), throughout fungi (Alex et al., 1996; Schuster et al., 1996) and even in plants. In *A. thaliana*, three histidine kinases (HKs), five histidine phosphotransferases (HPTs) and numerous response regulators (RRs) have been identified as components of the Arabidopsis cytokinin signaling pathway (Hwang and Sheen, 2001; Haberer and Kieber, 2002; Muller and Sheen, 2007; To and Kieber, 2008). The model of cytokinin signaling pathway in *A. thaliana* consists of four major steps: (1) cytokinin binds to the CHASE domain of the HK receptor and causes phosphorylation of the His residue, (2) phosphorylated His-HK activates cytoplasmic HPT by transferring the phosphate to its

Asp residue, (3) activated HPT translocates into the nucleus and phosphorylates type-B response regulators (BRRs), and (4) the phosphorylated type-B RRs bind to the promoter sequence of type-A RRs to turn on transcription. The great number of HKs and RRs in the cytokinin signaling pathway may explain the great variety of developmental transitions and cellular responses that cytokinins mediate, including apical dominance, cell division, cell differentiation, root elongation, leaf senescence, and fruit or flower maturation (Mok and Mok, 2001; Haberer and Kieber, 2002; Hwang et al., 2002).

In the parasitic angiosperm *S. asiatica*, we found cytokinins also regulate haustorium development, mediating the transition from vegetative to parasitic growth. In the natural semagenesis process, haustorium development is not initiated until a host-derived quinone signal is received. As shown in Chapter 3, cytokinins induce haustorium development with the same $t_{1/2}$ as the natural inducer. Homologues of two response regulators in *A. thaliana* cytokinin signaling pathways were found in *S. asiatica* through the HD library. A fundamental evolutionary question is whether *S. asiatica* exploits pre-existing cytokinin signaling pathways to mediate the organogenesis of haustorium? We therefore asked the following three specific questions: (1) Does a typical cytokinin signaling pathway exist in *S. asiatica*? (2) If so, how does the haustorial inducer enter the cytokinin signaling pathway in *S. asiatica*? And (3) does the interaction between DMBQ and cytokinin signaling pathways exist in non-parasitic plants such as *A. thaliana*?

4.2 Results

Do components of cytokinin signaling pathways exist in S. asiatica?

Using homologous cloning with degenerate primers, we cloned three Striga histidine kinases (SHKs) and ten Striga response regulators (SRRs) (Table 4-1). According to the phylogenetic analysis of the three SHKs with other available HK sequences that were identified as signal receptors from A.thaliana and *Z. mays* (Fig. 4-1B), five distinct gene structures were illustrated in Fig. 4-1A. All three SHKs fall into the same group with *A. thaliana* and *Z. mays* cytokinin receptors and they share four conserved domains: the N-terminal CHASE domain, the Histidine Kinase A domain (HisKA), the Histidine kinase-like ATPase domain (HATPase), and the C-terminal Signal receiver domain (REC) (Fig 4-1A). The three Striga HKs (SHK278/279/300) are the only HKs that share the cytokinin binding CHASE domain and the two conserved phosphorylation sites (His in HisKA domain and Asp in REC domain) characteristic of the three Arabidopsis cytokinin receptors (AHK2/3/4) and three Maize cytokinin receptors (ZmHK1/2/3). The other four groups lack either some domains or at least one of the two phosphorylation sites. The ten response regulators cloned from *S. asiatica* were grouped as type-A or type-B RRs according to the phylogenetic analysis with all the 22 Arabidopsis response regulators (ARRs) (Fig. 4-2). In *A. thaliana*, cytokinin typically turns on the transcription of type-A RRs immediately, but has no effect on the transcript levels of type-B RRs (Sheen, 2002; Kakimoto, 2003; Mizuno, 2003). The phosphorylated type-B RRs bind to the sequence motif “AGATT” in the promoters of type-A RRs to induce transcription (Hosoda et al., 2002).

Table 4-1 *Histidine kinases and response regulators cloned from S. asiatica.* All these gene sequences have been deposited in Genbank.

	Sequence ID	Protein length/AA	Genbank ID
S. asiatica Histidine Kinases	SHK278	1003	DQ424897
	SHK279	985	DQ431691
	SHK300	975	DQ431692
S. asiatica type-A Response Regulators	SRR197	212	DQ431690
	SRR198	239	DQ431689
	SRR200	159	DQ431688
	SRR201	224	DQ431687
	SRR217	221	DQ431686
S. asiatica type-B Response Regulators	SRR380	433	DQ431685
	SRR383	456	DQ431684
	SRR384	570	DQ431683
	SRR391	542	DQ431682
	SRR392	331	DQ438147

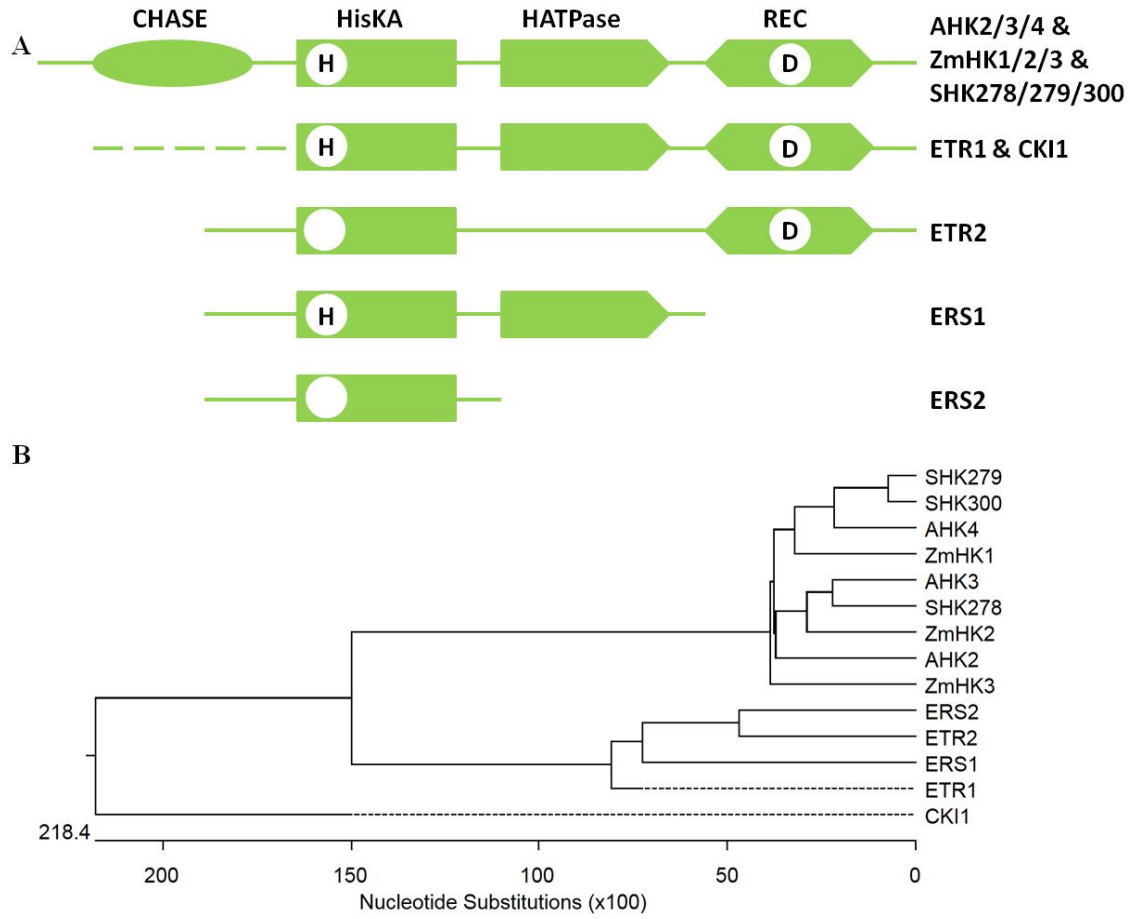

Figure 4-1 *Comparison of SHKs with other plant derived HKs on their domain structures and sequence homology by phylogenetic analysis.* (A) The full cDNA sequences of the plant HKs were aligned according to the conserved domains with the names of each domain labeled above: the extracellular ligand binding CHASE domain, the histidine kinase A domain (HisKA) where the phosphorylation site histidine residue is, the Histidine kinase-like ATPase domain (HATPase) where ATP is turned over, and the signal receiver domain (REC) which contains a phosphoacceptor, aspartic acid. (B) The Genbank accession numbers are AK175733 (AHK2), AB046879 (AHK3), AB046871 (AHK4), AB042270 (Zea mays Histidine Kinase 1) (ZmHK1), AB102956 (ZmHK2), AB121445 (ZmHK3), NM_129658 (Ethylene Response Sensor 1) (ERS1), AF047976 (ERS2), NM_105305 (Ethylene Response 1) (ETR1), AF047975 (ETR2), NM_130311 (Cytokinin Independent 1) (CKI1). Refer to table 4-1 for SHKs' Genbank IDs.

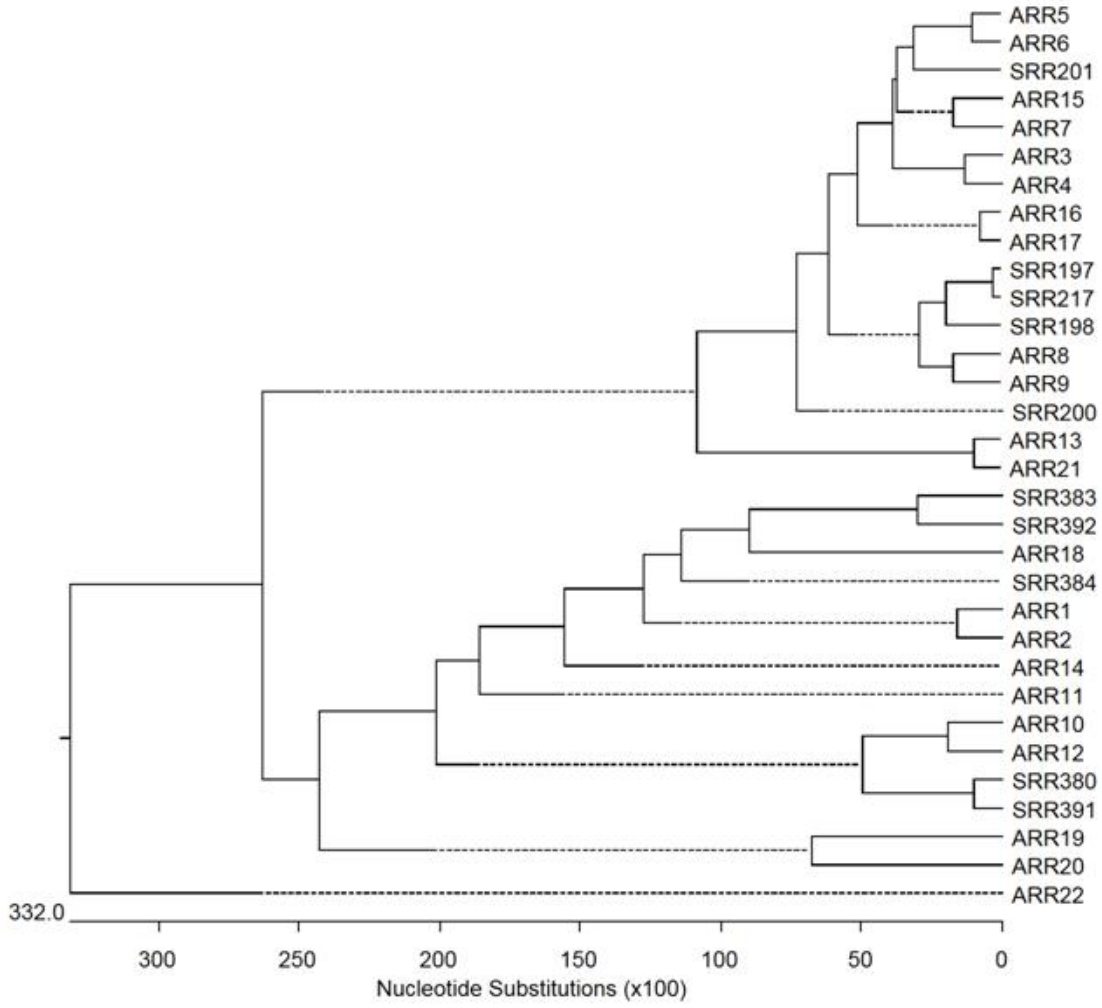

Figure 4-2 *Phylogenetic tree of ARRs 1-22 with ten SRRs.* The Genbank accession numbers of ARRs 1-22 are NM_112561, NM_117704, NM_104686, NM_100921, NM_114679, NM_125686, NM_101763, O80365, O80366, NM_119343, NM_105439, NM_128075, NM_128265, NM_126237, NM_106147, NM_129629, NM_115496, NM_125193, NM_103809, NM_116132, NM_120803, NM_180180. Refer to table 1 for SRRs' Genbank IDs.

Where are the components of cytokinin signaling pathways expressed in S. asiatica? And are they regulated by DMBQ?

With the components of the cytokinin signaling pathways cloned from *S. asiatica*, we wanted to test whether and where they are expressed, and if they are regulated and functional. Using homology cloning, we looked for the transcripts of all the three SHKs and ten SRRs in root, leaf + stem, and flower by RT-PCR with the housekeeping gene actin serving as an internal control (Wolf and Timko, 1994). As shown in Fig. 4-3, every gene was expressed to a similar level independent of the organ type, but each gene differs in expression level from each other.

Figure 4-3 *The expression level of three SHKs and ten SRRs.* (A) SHK278, (B) SHK279, (C) SHK300, (D) SRR197, (E) SRR198, (F) SRR200, (G) SRR201, (H) SRR217, (I) SRR380, (J) SRR383, (K) SRR384, (L) SRR391 and (M) SRR392 measured by RT-PCR in (1) roots, (2) leaves & stems, and (3) flowers. The housekeeping gene actin is applied as an internal control for gene expression.

When *S. asiatica* seedlings were treated with DMBQ or 6-benzylaminopurine (6-BA), the same set of SHKs and SRRs were induced at the same rate, including SHK279 and three type-A RRs (SHK200, SHK201, SHK217). The rest of the SHKs and SRRs were unchanged (Fig. 4-4). Among the three SHKs, SHK279 has the lowest basal expression level (Fig. 4-3), but is up-regulated by DMBQ treatment within 0.5 hr (Fig. 4-4A). Among the ten SRRs, the three that are regulated by DMBQ are all type-A RRs. This observation is consistent with the classification of type-A or type-B RRs in Fig. 4-2C. Cytokinins induce transcription of type-A RRs, but not type-B RRs in *A. thaliana*. It is type-B RRs that are phosphorylated first and then the activated type-B RRs bind to the

promoter of type-A RRs to induce transcription of type-A RRs. Notably, we found that all the promoter of the three regulated type-A SRRs containing at least one of these type-B binding motifs. The result that the same subset of SHKs and SRRs responded to both DMBQ and 6-BA at the same rate suggests that an expressed and functional cytokinin signaling pathway exists in *S. asiatica* and it is regulated by DMBQ.

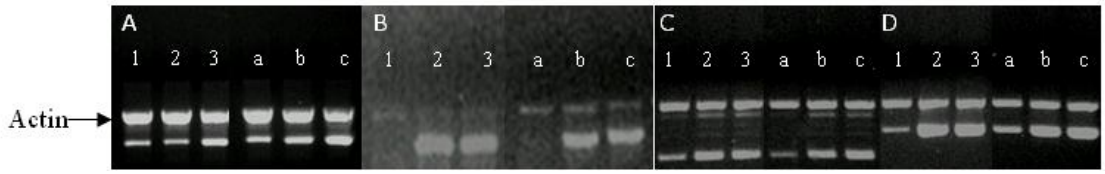

Figure 4-4 *Evaluation of how DMBQ or 6-BA treatment affects the transcription level of SHKs and SRRs in S. asiatica.* (A) SHK279, (B) SRR200, (C) SRR201, (D) SRR217. (1) untreated, (2) treated with 10 μM DMBQ for 0.5 hr, (3) treated with 10 μM DMBQ for 2hrs; (a) untreated, (b) treated with 10 μM 6-BA for 0.5 hr, (c) treated with 10 μM 6-BA for 2 hrs.

Does DMBQ interact with cytokinin receptors in S. asiatica?

Since the current evidence suggests that DMBQ and cytokinin signaling share common pathways, we wanted to determine at which point DMBQ enters the cytokinin signaling pathways in *S. asiatica*. We first explored whether DMBQ interacts with Striga cytokinin receptors using a specific cytokinin inhibitor, purine riboside. If DMBQ interacts prior to or specifically at the cytokinin receptor, then purine riboside should be able to inhibit DMBQ-induced haustorial development. As shown in Fig. 4-5, 30 uM purine riboside indeed inhibits not only cytokinin-induced haustoria as expected, but also DMBQ-induced haustoria. After six hours of preincubation with either zeatin or DMBQ, adding purione riboside did not inhibit haustoria anymore, consistent with the previous time dependent data for DMBQ induction. Therefore, DMBQ induction appears to occur upstream of the cytokinin receptors in *S. asiatica*.

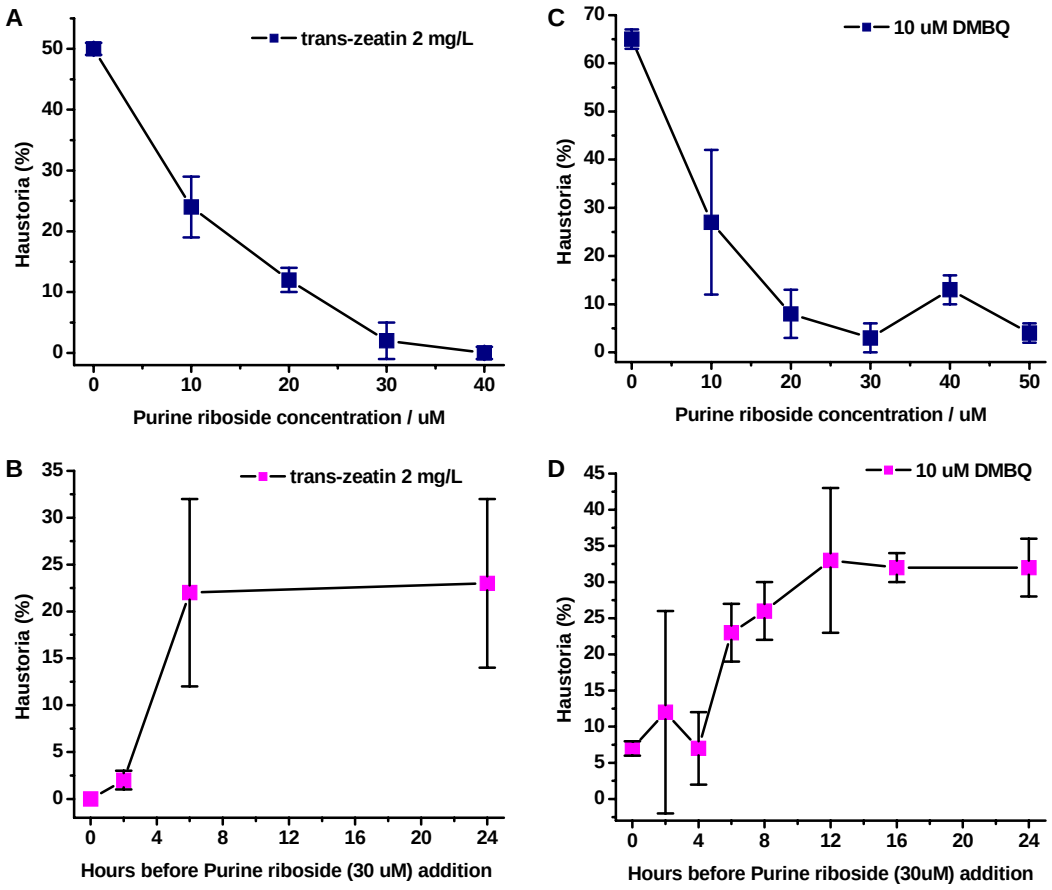

Figure 4-5 *Inhibition of haustorium development by purine riboside.* (A) Titration of purine riboside to inhibit zeatin induced haustorium, (B) Titration of purine riboside to inhibit DMBQ induced haustorium, (C) Titration of zeatin treatment duration before addition of 30 μM purine riboside to determine the exposure threshold to zeatin for commitment to haustorium development, (D) Titration of DMBQ treatment duration before addition of 30 μM purine riboside to determine the exposure threshold to DMBQ for commitment to haustorium development.

Are SHK278/279/300 cytokinin receptors?

Our possible model is that DMBQ and cytokinins use the same receptors. As shown in Fig. 4-1, SHK278/279/300 share high homology with the conserved domains and two phosphorylation sites with both *A. thaliana* and *Z. mays* cytokinin receptors, suggesting that they serve as cytokinin receptors. A yeast histidine kinase mutant system was used to examine whether cytokinins bind to SHK278/279/300 directly, in the absence of any

intermediary component provided by the genetic background of *S. asiatica*. The budding yeast gene SLN1 encodes a histidine kinase, and the *sln1Δ* null mutations are lethal when yeast cells grow on glucose media. Yeast strain TM182 carries the *sln1Δ* mutation as well as a plasmid carrying a phosphotyrosine phosphatase 2 gene (PTP2) under a GAL promoter. Therefore, TM182 can grow on galactose media because the galactose-dependent expression of PTP2 can inactivate the HOP1 MAP kinase cascade. Alternatively, introduction of a plasmid containing SLN1 into the *sln1Δ* [PGAL1-PTP2] mutants eliminates galactose dependency (Maeda et al., 1994). We designed an assay where SHKs are introduced into TM182. Successful transformants were selected by galactose media without Leucine because of the Leucine marker in the SHK-carrying plasmid. Then we grew successful transformants on glucose media containing a kind of phytohormone or DMBQ. Only when a SHK is activated can the TM182 cells survive on the glucose plate. The TM182 transformants with SHK279 and SHK300 grew on glucose medium containing one of the three cytokinins, kinetin, 6-BA or tran-zeatin, but did not survive on glucose plates supplemented with DMBQ or with other phytohormones including gibberellic acid (GA) and 1-Naphthaleneacetic acid (NAA) (Fig. 4-6A-D). SHK278, however, survived without cytokinins (Fig. 4-6E, F). These results indicate that SHK279 and SHK300 are activated by cytokinins within a yeast genetic background, most consistent with their role as *S. asiatica* cytokinin receptors. Additionally, *A. thaliana* cytokinin receptor AHK3, the one which shares the greatest sequence homology (65%) with SHK278, displayed the same constitutive phenotype as SHK278 in the yeast assay (Ueguchi et al., 2001), and there is significant evidence that cytokinins bind

directly to AHK3 in *A. thaliana* (Spichal et al., 2004; Kim et al., 2006). These results are most consistent with SHK278 serving as a *S. asiatica* cytokinin receptor.

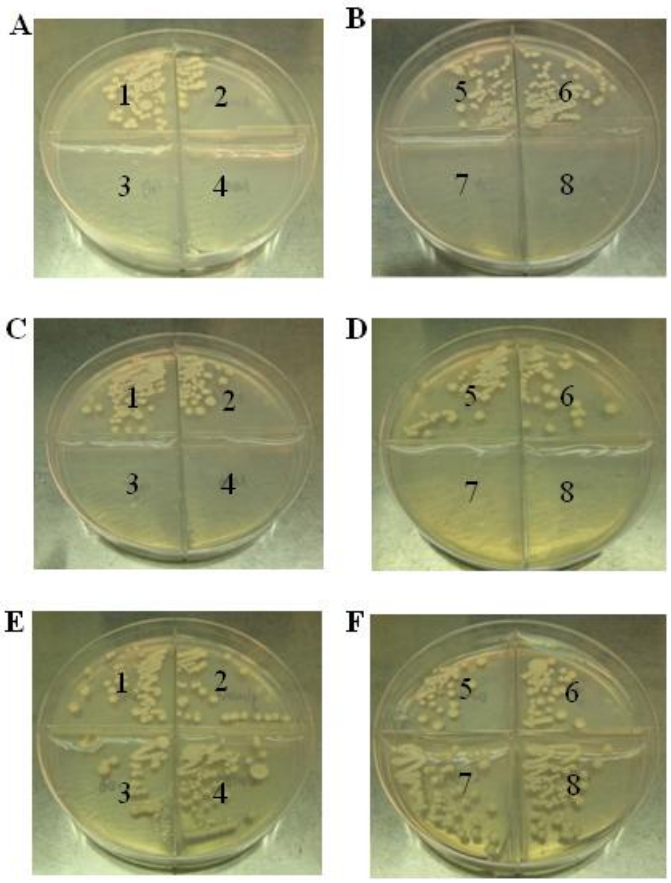

Figure 4-6 *Yeast sln1Δ lethality rescue assay.* (A,B) SHK279, (C,D) SHK300 (C,D), (E,F) SHK278. (1) Galactose medium (positive control), (2-8) glucose medium with various additive 50 µM each, (2) Kinetin, (3) GA, (4) DMBQ, (5) 6-BA, (6) trans-zeatin, (7) NAA, and (8) DMSO (negative control).

Seeing that SHKs do function as histidine kinases, we then tested whether the signal is transmitted through a His-to-Asp phosphorelay as occurs in *A. thaliana* cytokinin receptors. In addition to the conserved cytokinin binding CHASE domain, SHK278/279/300 have the same two conserved phosphorylation sites as Arabidopsis cytokinin receptors, His in HisKA domain and Asp in REC. Single and double mutants of

the His or Asp residues were expressed in TM182 (SHK278: H445Q, D904N, H445Q/D904N; SHK279: H386Q, D895N, H386Q/D895N; SHK300: H387Q, D880N, H387Q/D880N). Successful tranformants were selected by galactose media without leucine and grown on glucose media containing cytokinins to select for functional SHK mutants. None of the SHK mutants allowed the TM182 transformants to survive on glucose media (data not shown), indicating that both the His and Asp residues are required in the phosphorelay.

Does DMBQ interact with the cytokinin signaling pathways in A. thaliana?

Developmental effects characteristic of cytokinin treatment including the inhibition of root elongation and leaf senescence were evaluated in wt *A. thaliana* seedlings treated with DMBQ. Cytokinin exposure reduced root elongation (Fig. 4-7A) and leaf senescence (Fig. 4-7B), whereas DMBQ exposure exerted no effect. In order to rule out the possibility that DMBQ can interact with cytokinin signaling pathways in *S. asiatica* but not *A. thaliana* because of some unique DMBQ responsiveness of the promoter of a Striga gene, the expression of GUS reporter constructs with either an *A. thaliana* gene promoter or a *S. asiatica* gene promoter in *A. thaliana* was compared (Fig. 4-8). An unidentified *S. asiatica* gene was found to be up-regulated by both cytokinin and DMBQ in *S. asiatica* (Fig.4-8A). We chose this gene, a *S. asiatica* Cytokinin DMBQ responsive (SaCDR), to make a SaCDR/GUS construct (Fig. 4-8B) and compared its expression with that of ARR5/GUS (Fig. 4-8C) in *A. thaliana*. Both ARR5/GUS and SaCDR/GUS remained responsive to cytokinin treatment, but did not responsed to DMBQ treatment when expressed in *A. thaliana*. Therefore, the promoter alone is insufficient to convey

DMBQ responsiveness of a Striga gene, but something in the *S. asiatica* genetic background is required.

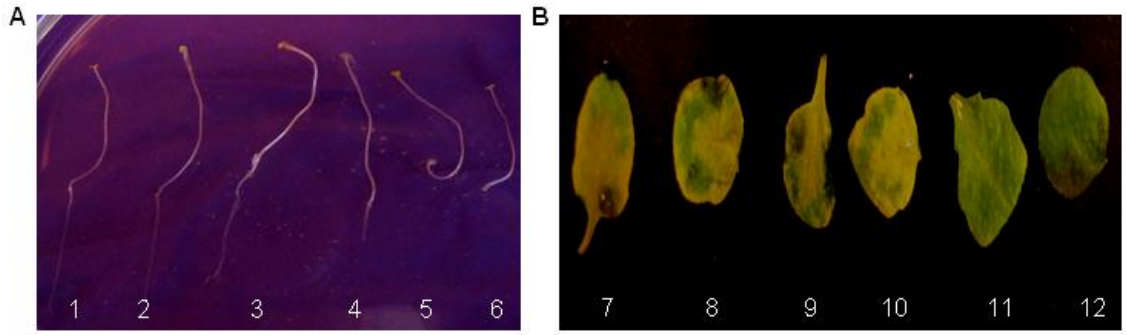

Figure 4-7 *Effects of cytokinin or DMBQ treatment on root elongation and leaf senescence in wt A. thaliana*: (1) untreated, (2) treated with 50 µM DMBQ, (3) treated with 100 µM DMBQ, (4) treated with 5 µM 6-BA, (5) treated with 10 µM 6-BA, (6) treated with 20 µM 6-BA; (7,8) untreated, (9) treated with 50 µM DMBQ, (10) treated with 100 µM DMBQ, (11) treated with 10 µM 6-BA, (12) treated with 20 µM 6-BA.

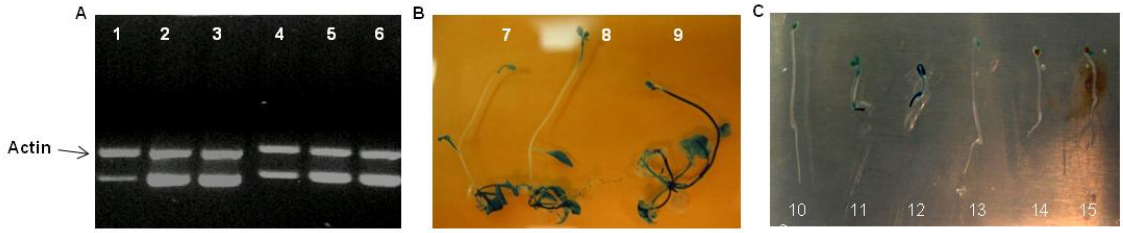

Figure 4-8 *Comparison of the effects of DMBQ treatment on a S. asiatica gene promoter with an A. thaliana gene promoter.* (A) Effects of cytokinin or DMBQ treatment on the expression level of SaCDR in *S. asiatica*, (B) SaCDR/GUS in *A. thaliana* transformants, (C) ARR/GUS in *A. thaliana* transformants. (1) untreated, (2) treated with 10 µM DMBQ for 0.5 hr, (3) treated with 10 µM DMBQ for 2hrs; (4) untreated, (5) treated with 10 µM 6-BA for 0.5 hr, (6) treated with 10 µM 6-BA for 2 hrs, (7) untreated wild type *A. thaliana*, (8) *A. thaliana* transformants expressing SaCDR/GUS treated with 50 µM DMBQ, (9) *A. thaliana* transformants expressing SaCDR/GUS treated with 10 µM 6-BA, (10) untreated, (11) treated with 5 µM 6-BA, (12) treated with 10 µM 6-BA, (13) treated with 50 µM DMBQ, (14) treated with 100 µM DMBQ, (15) treated with the DMSO, the solvent of 6-BA and DMBQ (negative control).

Does DMBQ mediate haustorial development through auxin signaling as well?

Haustorial organogenesis is characterized by the immediate halt of cell division/root elongation, accompanied by radial swelling of the root tip and centrifugal growth of haustorial hairs, and the differentiation of vascular tissue (Kuijt, 1969b; Kuijt, 1969a; O'Malley, 1996; Kim et al., 1998c; Palmer et al., 2004a). Among all the processes that occur on the cellular level, cell elongation is typically mediated by the phytohormone auxin with its ability to loosen cell walls. Indeed, two auxin-regulated proteins were found in the HD library as shown in Chapter 2, consistent with the existence of the interaction between DMBQ and auxin mediated processes. When the synthetic auxin NAA is exposed to seedlings of *S. asiatica* from 1E-6 μM to 10 μM, root elongation is suppressed very sharply when the concentration was 0.05 μM or higher, and the unsuppressed root length was quite similar to the seedlings treated with 10 μM DMBQ (Fig. 4-9A). In addition to halting root elongation, another phenotypic marker of haustorium development, root tip swelling, was induced by auxin as well, but only between 0.03-0.5 μM (Fig. 4-9B), and no root hair formation was observed. Furthermore, 2,3,5-triiodobenzoic acid (TIBA), an auxin polar transport inhibitor, suppressed haustorium induction by DMBQ with an ED_{50} of 5 μM (Fig. 4-9C). These results are consistent with DMBQ regulating the concentration of auxin to mediate the sudden halt of root elongation and the swelling of the root tip.

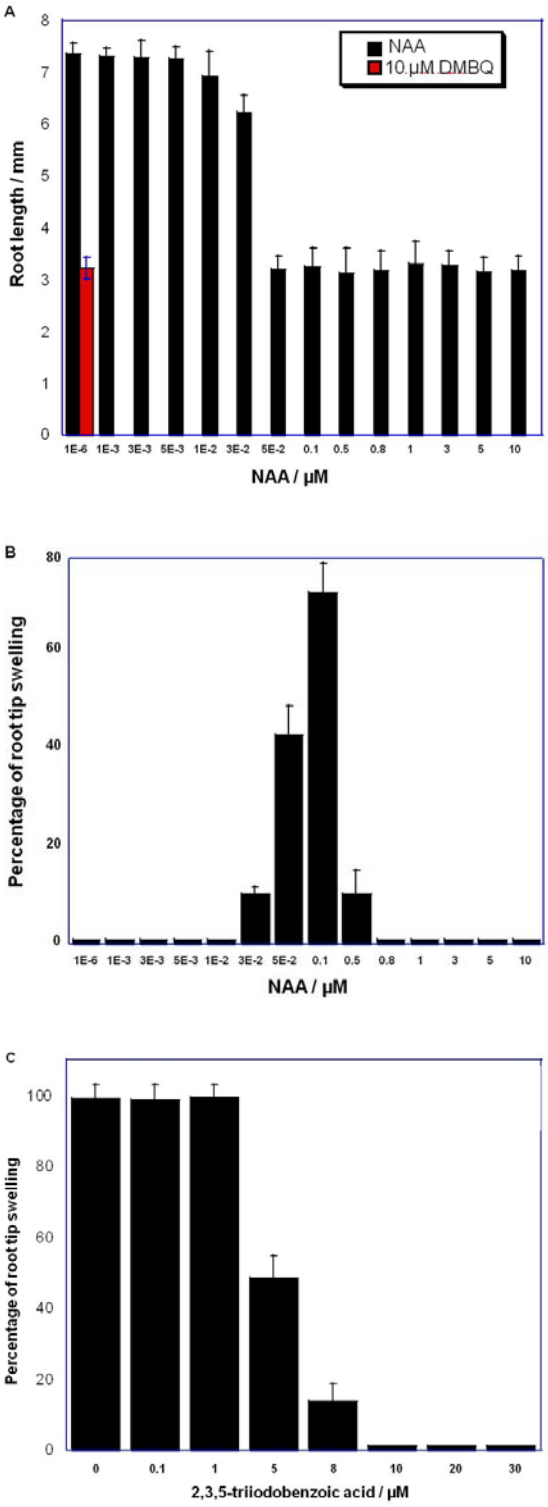

Figure 4-9 *Auxin regulates haustorial development in S. asiatica.* (A) The concentration range of auxin that can suppress root elongation as 10 μM DMBQ does, (B) the concentration window of auxin that induces root tip swelling, (C) the inhibitor of auxin polar transport inhibits haustorium induction by DMBQ.

4.3 Discussion

The differential libraries prepared in Chapter 2 have been used to further understand haustorium development. We have now cloned and characterized homologues of the cytokinin signaling pathway components from *S. asiatica*, and better understand the points where DMBQ enters the cytokinin pathways. While SHK278/279/300 may be Striga cytokinin receptors, they do not function as DMBQ receptors in the yeast hybrid screening system. The observation that purine ribose, a cytokinin binding inhibitor, blocks DMBQ induced haustorium development but that DMBQ did not bind to SHKs in yeast genetic background as do the cytokinins suggests that DMBQ interacts with Striga cytokinin receptors indirectly through a DMBQ receptor. Lastly, we have demonstrated that the interaction between DMBQ and cytokinin signaling pathway that exists in *S. asiatica* does not function in the same manner in *A. thaliana*. DMBQ does not mediate the developmental effects that are characteristic of cytokinin treatments in *A. thaliana*. These results are consistent with our basic hypothesis that the genetic background of *S. asiatica* provides an element that enables DMBQ to exploit the cytokinin signaling pathway. We rule out the possibility that this element is some unique responsiveness to DMBQ of the promoter of a *S. asiatica* gene, but rather is likely a factor either upstream or specifically at the receptor binding step that couples DMBQ perception to cytokinin signaling in *S. asiatica*. If this component is upstream to binding of cytokinins to their receptors, it could be a DMBQ receptor that interacts with the cytokinin receptors. If it is specifically at the binding step, it could be a DMBQ receptor that modulates the cytosolic

concentration of cytokinins. The interaction of DMBQ with auxin signaling in *S. asiatica* might also occur by modulating the local cytosolic concentration of auxin.

Haustorium development in parasitic plants is not the first example where host-derived phenolic compounds induce morphology change in the associated organism and this morphology change is mediated by endogenous hormones. In the establishment of nitrogen-fixing symbiosis the rhizobia cue on flavanoids exuded by host legume plants during defense response to invading bacteria. Several lines of evidence point to the involvement of endogenous hormones (Hirsch and Fang, 1994). For example, the early nodulin gene ENOD2 is induced in *Sesbiana rostrata* roots by exogenous cytokinins (Dehio and de Bruijn, 1992). Cytokinin also induces expression of ENOD2, ENOD12, and ENOD40 genes in alfalfa roots (Hirsch and Fang, 1994). A cytokinin-excreting strain of *Rhizobium meliloti* even can induce the formation of bacteria-free pseudonodules on alfalfa (Cooper and Long, 1994). Further support for a role of endogenous hormones derives from the demonstration that auxin sensitivity correlates with nodule formation in different lines of *Medicago varia* (Kondorosi et al., 1993). Finally, inhibitors of polar auxin transport induce pseudonodules and ENOD genes on alfalfa or pea roots (Hirsch et al., 1989; Scheres et al., 1992).

Numerous host-derived signals that parasitic plants recognize for host recognition have been identified and shown to be structurally similar to phenolic defense signals produced by allelopathic plants (Tomilov et al., 2006a). Among these parallels of host recognition cues/phytotoxic allelochemicals, DMBQ represents the case where the same molecule

has both biological activities. DMBQ at 10 μM mimics contact with host root and induces haustorium development, whereas at 100 μM DMBQ causes the root tip of *S. asiatica* turn brown and the seedlings die (data not shown). Our prior studies have demonstrated that haustorial inducing activity of the structural analogs of DMBQ depends on their redox potential, and suggested a redox reaction took place when DMBQ binds to its receptor (Smith et al., 1996). The observation that different haustorium-inducing benzoquinones had similar first-half volt redox potentials led to the hypothesis that it is a semiquinone intermediate that initiates haustorium development. This model was evaluated with the chemical spin trap cyclopropyl-*p*-benzoquinone. A single-electron reduction of the cyclopropyl ring of cyclopropyl-*p*-benzoquinone activates a reactive electrophilic center that irreversibly inhibits haustorium development in *Striga* in response to DMBQ (Zeng et al., 1996). This redox model posits that the first step in quinone perception is the single electron reduction of benzoquinone to semiquinone. These semiquinone radicals account for the quinone cytotoxicity as seen in the hypersensitive response of plants against microbial pathogens (HammondKosack and Jones, 1996), and treatment of cancer, bacterial or plasmodium infection. Recently, two quinone oxidoreductases were isolated from another root parasitic plant in the *Orobanchaceae* family, *Triphysaria versicolor*, TvQR1 and TvQR2. Silencing TvQR1 significantly reduced the rate of haustorium formation but silencing TvQR2 did not (Bandaranayake et al., 2010). Based on sequence homology, TvQR1 produces semiquinone radicals through one-electron reduction of benzoquinones (Rao and Zigler, 1992; Babiychuk et al., 1995; Testa, 1995; Thorn et al., 1995), whereas TvQR2 generates hydroquinones through two-electron reduction of benzoquinones. TvQR2 is highly

homologous to the QR from a wood-rotting fungus *Phanerochaeta chrysosporium*. This PcQR protects the fungus from various lignin degradation products by reducing quinines to hydroquinones in a single-step reduction that avoids semiquinone intermediates (Li et al., 1995; Brock and Gold, 1996). Spectrophotometrical studies of purified proteins of TvQR1 and TvQR2 confirmed these enzymes catalyze NAD(P)H-dependent reductions of DMBQ or other allelopathic quinones (Wrobel et al., 2002). The observation that single-electron reduction of quinones by QR1 was necessary for haustorium induction, but not the two-electron reduction by QR2, suggested that the downstream product of the QR1 route, ROS, might be involved in haustorial induction. QR2 probably plays the role of quinone detoxification, as consistent with the up-regulation of TvQR2 and SaQR2 upon DMBQ exposure, as well as other defense genes and drug exporters identified in the HD library.

The ROS signals produced by processing benzoquinones through the QR1 route may possibly be the intracellular signals that couples quinone perception to phytohormone signaling. ROS were initially thought to be toxic byproducts of aerobic metabolism, but have now been recognized as central players in the signaling networks of cells (Mittler et al., 2011). After cells acquired efficient ROS regulation mechanisms with various antioxidants and ROS scavenging/detoxifying enzymes during evolution to cope with the increased levels of atmospheric oxygen billions of years ago (Mittler, 2004; Foyer and Noctor, 2005; Halliwell, 2007), the advantages of adapting ROS as signaling molecules include: 1) ROS regulation is dynamic, extremely fast and accurate to subcellular locations as demonstrated by detection of oscillating ROS signals in root hairs

(Monshausen et al., 2007; Takeda, 2008); 2) ROS signals are auto-propagated rapidly throughout the plant as seen at a rate of up to 8.4 cm/min in *A. thaliana* (Miller, 2009); 3) different forms of ROS with different membrane permeability make ROS versatile signaling molecules (Miller, 2010); and 4) ROS signaling is highly integrated with hormonal signaling networks which allowed plants to regulate developmental processes in response to environmental stresses. Root apical meristems (RAMs) are rapidly reorganized in response to environmental triggers in the physically, chemically and biologically complex matrix, soil. This plasticity is mediated by a complex signaling module in which ROS operate in strict association with auxin, GA and cytokinin signaling pathways (De Tullio, 2010). The integration of ROS with auxin signaling networks, triggered by environmental factors, is known as the stress-induced morphogenic response. Numerous reports can be found on this phenomenon: auxin induces cell elongation via hydroxyl radical (OH•)-mediated cell wall loosening (Schweikert et al., 2000; Schopfer, 2001). Hydrogen peroxide and superoxide anion scavengers inhibit coleoptile elongation of maize (Schopfer, 2001). Hydrogen peroxide level was increased in maize root upon gravi-stimulation; inhibitors of phosphatidylinositol 3-kinase and wortmannin suppressed auxin and gravistimuli-induced ROS accumulation in maize root (Joo et al., 2005).

In summary, given these results on how DMBQ interacts with the auxin/cytokinin signaling in *S. asiatica*, we propose a model on the molecular mechanism of parasitism establishment in *S. asiatica*. It is widely observed that parasitic plants cue on host defense signals as their strategy of host recognition. *S. asiatica* probes for a host by exploiting its

own defense armory to produce a mild level of ROS (Keyes et al., 2007). When the parasite grows into close proximity to the host root, ROS oxidize the phenolic compounds in the host root cell walls (Chang and Lynn, 1986). The quinolic oxidation product feeds back to the parasite and induces a defense response, including one- and two-electron reduction of benzoquinones (Smith et al., 1996). While the two-electron reduction protects the parasite from quinone cytotoxicity, the one-electron reduction generates semiquinone radicals, which initiates the signaling pathway of haustorium induction by exploiting pre-existing auxin/cytokinin regulation systems. The observation that DMBQ interacts with cytokinin signaling pathways in *S. asiatica*, but not in *A. thaliana* is consistent with our basic hypothesis that the evolution of parasitic plants in *Scrophulariaceae* and *Orobanchaceae* families occurred through the emergence of a DMBQ receptor that is able to couple quinone perception to auxin/cytokinin signaling pathways.

4.4 Methods

Plant material and chemicals

Striga asiatica seeds were treated by 3% Chromic acid 3min, 1% tween-20 1min, 7% bleach 7min, 70% ethyl alcohol 2min, then placed in pure water for more than a week. Those seeds are treated with 10um strigol, after about one and half days, the germinate seedlings are ready for RNA extraction. The tissues of leave, stem and flower are obtained from plant culture. All chemicals are bought from Sigma Aldrich and restrictive enzymes from Promega.

Cloning of HKs, type-B and type-A RR from S. asiatica

Based on the conserved regions of *A. thaliana* HK2, 3, 4 and *Z. mays* HK 1, 2, 3, degenerate primers for HKs were designed: reverse primer-1: 5'-GCHAARTCDCAGTTYCTDGCHACYG-3'; reverse primer-2: 5'-TCHCATGARATHAGRACWCCDATGAATGG-3'; forward primer-1: 5'-GTNGCYTCAAAYCCRTCCATYTSDGGCATYTG-3'; forward primer-2: 5'-GTHGCBTGDATHACATCBGCHGTCATNGCYA-3'; Based on the conserved regions of *A. thaliana* type-A RR3, 4, 5, 6, 7, 8, 9, 15, 16, 17, degenerated primers for type-A RRs were designed: reverse primer: 5'-TNCAYGTYYTHGCHGTHGAYGAYAR-3'; forward primer-1: 5'-GWNGAYTCYTTVAYYTTYTTVARNAGHTC-3'; forward primer-2: 5'-TTCTCNGANGACAWDATYACHACHGG-3'; Based on the conserved regions of *A. thaliana* type-B RR1, 2, 10, 11, 12, 13, 14, 18, 19, 20, 21, degenerate primers for type-B RR are designed: reverse primer: 5'-ATGCCWGACATGGAYGGTTTCAARCTBCTTGA-3'; forward primer-1: 5'-TGMARYTCAWIAGWCCAAACVACDCGHKVYTTCTT-3'; forward primer-2: 5'-TTCTGIARRTGRCTDGCHACRTTYTCYC-3', Based on the conserved regions of *A. thaliana* HPt1, 2, 3, 4, 5, degenerate primers for HPt were designed: reverse primer-1: 5'-GGDHWBBTBGAYRVICARTTY-3'; reverse primer-2: 5'-RTKCATCARYTBAARGGDAGYAGY-3'; forward primer-1: 5'-RCTRCTHCCYTTVARYTGATGMAY-3'; forward primer-2: 5'-AAGCAGTGGTATCAACGCAGAGTACT(30)VN-3'. Except for HPt, the other three type of genes get predicted PCR fragments which were cloned by TOPO TA cloning kit

(Invitrogen). Theses genes' full cDNA sequences were obtained by the SMART Race cDNA Amplification Kit (Clontech).

RT-PCR method for expression analysis

Total RNA was extracted from *S.asiatica* germinate seedlings with RNeasy Plant Mini Kit (Qiagen) and the integrity was analyzed by electrophoresis in 8% formaldehyde, 1.5% agarose gel. Superscript ^{TM} III transcriptase (Invitrogen) was used to do the reverse transcription at 50°C. To analyze the expression pattern of these genes, RT-PCR method was employed with the housekeeping actin gene as the internal standard marker. Primers for actin are: reverse: 5'-CAGGCTGTTCTCTCCCTTTAT-3', forward: 5'-TCCGATCCAGACACTGTACTT-3' (Wolf and Timko, 1994), adding four primers (two for actin, two for the individual genes) together in a PCR system with *S.asiatica* cDNA from different organs or different treatments toward striga seedlings with annealing temperature 58°C. The PCR products were analyzed in 2% agarose gel.

Yeast suppression assay

Saccharomyces cerevisiae strain TM182 [MATa ura3 leu2 his3 Slin::hisG/pGP22] (Maeda et al., 1994) was used for the suppression assay. The full cDNAs of SHK278/279/300 were cut by NotI-SacII and then inserted into pCUY315 which carries CEN6, the ADH1 promoter and LEU2 marker to yield pCUY278, pCUY279 and pCUY300. The transformants were selected by growing TM182 on galactose medium without leucine because the SHK carrying plasmid has a leucine marker. The selected transformants were grown on 2% glucose plates with one of the following supplements:

kinetin, 6-BA, trans-zeatin, DMBQ, NAA, GA, or DMSO as a negative control. Transformants were also grown on 2% galactose plates as a positive control. These plates were incubated at 30 °C for CA. 3 days.

Arabidopsis root elongation and leave senescence by cytokinin and DMBQ

Sterilized *A. thaliana* seeds were spread on MS plates with different concentrations of DMBQ or cytokinin, incubated in dark for about two weeks, and root elongation was scored. The seventh Arabidopsis leaf was harvested and placed in water with different concentrations of cytokinins or DMBQ, and checked for senescence after 1 week. Pictures were taken with a Canon digital camera.

Cloning of the gene promoter and Arabidopsis transformation

About 2Kb DNA sequence was cloned upstream from the start codon using TAIL-PCR. The 2kb promoter fragment were cut by SalI and XbaI, then ligated with the PBI101 vector cut by the same restrictive enzymes to prepare the GUS constructs of the genes. The construct was introduced into the Columbia (col-0) ecotype with agrobacterium GV3101 mediated by the vacuum transformation method and the transformants were selected by the MS plate with Kanamycin (50ug/ml). F3 generation of the transformants were planted in MS medium with different additives such as cytokinin and DMBQ, then the GUS assay as described below was used to check DMBQ and cytokinin effects on the expression of these genes. As for the Arabidopsis type-ARR5 GUS transformants, the same methods mentioned above were used to check cytokinin and DMBQ effects.

GUS assay

Transformants of promoter plus GUS constructs were vacuum infiltrated for 5 minutes in the staining solution (50mM sodium phosphate buffer PH 7.0, 0.2% triton-X-100, 1mM X-Gluc), then put into 37°C overnight and fixed in 75% ethyl alcohol; the pictures were taken by Canon digital camera.

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Chapter 5 - Mapping the signaling landscape with a plant pathogen *Agrobacterium tumefaciens*

5.1 Introduction

Agrobacterium tumefaciens is a broad host range plant pathogen that induces tumors on infected plants across the dicotylendous grouping of Angiosperms, a large percent of gymnosperms (De Cleene and De Ley, 1976), and is capable of transferring DNA to genetically transform an even wider spectrum of plants including some monocots, and even fungal and human cells (Bundock et al., 1995; de Groot et al., 1998; Kunik et al., 2001). Virulent strains recognize three chemically distinct types of host molecules and activate virulence pathways maximally only when the appropriate combinations of signals are perceived. Moreover, within each class of signals there is great chemical diversity, possibly providing an important means of broadening the host range (Melchers et al., 1989; Ankenbauer and Nester, 1990; Cangelosi et al., 1990; Duban et al., 1993a). But more importantly, advancement in the molecular genetics of this pathogen should make it possible to engineer *A. tumefaciens* as an *in vivo* detector of the signaling landscape in plant tissues.

It has long been known that inoculating *A. tumefaciens* on plants rarely causes tumors unless the plant is wounded at the site of inoculation (Lippincott and Lippincott, 1975).

The requirements for wounding, which implicates targeting a hostile tissue that exudes antimicrobial chemicals as a wounding response, remain unclear. Somatic cells are typically arrested at some point in the cell cycle; cells competent for division are located in the meristem. However, the phenomenon known as cellular dedifferentiation, in which adult somatic cells change from differentiated to less differentiated states and regain cellular proliferative competence, occurs widely in multicellular organisms, including fish, amphibians, mammals, and plants (Gautheret, 1934). Compared to animals, plants have a remarkable capacity to change their cell fate, and the first examples of *in vitro* plant cell dedifferentiation (unorganized cell proliferation to form callus) were reported as early as the 1930s. Wounding induces somatic cells to dedifferentiate and re-enter the cell cycle (Butenko, 1999). The rapid proliferation after wounding may coincide with the "window of competence" initially described by Braun, who showed that transformation of wounded plants is most efficient 24 to 36 h after wounding (Braun and Mandle, 1948). Binns and Thomashow pointed out the distinct correlation in the literature between the wound-induced cell division and the competence of such cells to be transformed by *A. tumefaciens*, and proposed that the active cell cycle exposes unwound plant chromosomal DNA and enables integration of T-DNA into the host chromosomes (Binns and Tomashow, 1988). Supporting evidence has been reported in the case of both *Agrobacterium*-mediated transformation and direct transformation (Wullems et al., 1981; An, 1985; Kudirka et al., 1986; Okada et al., 1986; Valvekens et al., 1988). The idea that dividing cells are required for tumorigenesis is also supported by the reports that application of auxin prior to inoculation enhances transformation rate (Sangwan et al., 1992; Stover et al., 1997; Chateau et al., 2000), and findings that tumors were limited to

the cambium, a meristematic tissue predisposed to cell division (Sangwan et al., 1991; Ghorbel et al., 2000). Wounding therefore may provide target cells for Agrobacterium, because the cells become competent for cell division and recognizes signals for division in the wound exudates. Monitoring the virulence behavior of Agrobacterium at the host-pathogen interface, at the portals for Agrobacterium to enter into the apoplast - the continuum of intercellular space in a plant - and comparing that information with what we have learned from *S. asiatica*, a unified picture of pathogenesis may emerge.

Our understanding of Agrobacterium has now reached the point where we can engineer the pathogens as a bio-sensor of the dynamic signaling landscape at the host-pathogen interface in real time, and illustrate how wounding in various tissue types of different ages changes the signaling landscape. In that regard, the active phenols that *A. tumefaciens* recognizes are those that are methoxylated at one or two sites ortho to the phenol hydroxyl and carry a variety of substitutions para to the phenol hydroxyl and can, in fact, be two-ring derivatives, such as dehydrodiconiferyl alcohol (Melchers et al., 1989; Duban et al., 1993a; Palmer et al., 2004b) While these phenols are necessary for activation, various sugars and low pH modulate phenolic sensitivity at the detector known as VirA (Stachel et al., 1985; Stachel et al., 1986; Cangelosi et al., 1990; Shimoda et al., 1990; Banta et al., 1994; Gao and Lynn, 2005). The mechanism underlying transfer of the T-DNA from the 'tumor-inducing' (Ti) plasmid into the plant cell involve expression of 'virulence' (*vir*) genes (Brencic and Winans, 2005), (McCullen and Binns, 2006) with VirG serving as the transcriptional activator of the VirA/VirG two-component system. By placing a gfp reporter gene into the Ti plasmid under the control of a

virulence gene promoter, we sought to visualize the signaling events *in-situ* using confocal fluorescence microscopy. Further, we hoped to quantify the amount of virulence inducers in real time by flow cytometry.

Ever since the discovery of *Agrobacterium* as a vector for transforming plant cells, the pathogen has rapidly become a standard tool for plant transformation, but transformation of grain crops has been frustrated by their stubborn recalcitrance to *Agrobacterium*-mediated plant transformation (De Cleene and De Ley, 1976). Indeed, Zhang and co-workers isolated an inhibitor from the root exudate of Maize that specifically inhibited the virulence gene expression (Zhang et al., 2000; Maresh et al., 2006). Given these results, we now have an opportunity to directly monitor the virulent behavior of *Agrobacterium* at the host-pathogen interface in monocot plants. Here we report engineering *Agrobacterium* strains that de-coupled the dependence of sugar perception on phenolics and show a hypersensitive response to either phenolic or sugar signals.

Further, we exploit *S. asiatica* as a dicot which the semagenesis model predicts contains no free wall-bound phenolics. In combination, these results greatly enrich our understanding of both the host and the parasite within the dynamic interaction that is pathogenesis.

5.2 Results

Engineer A. tumefaciens strains to detect phenol and sugar signals separately

To evaluate the phenols and sugars, *in vivo*, it was important to decouple their perception. A single mutation Y293F in VirA indeed decoupled sugar sensing from phenolic signaling (Fig. 5-1). In combination with wt *A. tumefaciens* strain, it appeared possible to detect these inputs separately.

A. tumefaciens strain A348 was engineered to carry a reporter gene encoding green fluorescent protein (gfp) on the Ti plasmid under the VirE promoter (Goulian and van der Woude, 2006). On a separate plasmid, pMP7605, a gene encoding an m-cherry fluorescence protein was placed under the constitutive Tac promoter (Lagendijk et al., 2010). This strain serves as bio-sensor AB650. By inserting the Y293F VirA mutant into the locus of wt VirA in AB650, we prepared strain YHL301 as an independent sugar sensor and a more sensitive phenol sensor. A third strain, YHL324, was prepared by inserting Y293F into the locus of wt VirA in AB520 with a separate plasmid, pRG182, encoding VirB/gfp. AB520 is a strain carrying a deletion of the multiple monosaccharide transport B (ΔmmsB) (Zhao and Binns, 2011). The deletion enhances the sensitivity of Agrobacterium to sugar signals, presumably by increasing the sugar concentration in the periplasm. The dose response to AS and to glucose was measured with and without saturating concentration of the other signal (Fig. 5-1). The sensitivity of each strain, AB650, YHL301 and YHL324, is reported as the ED_{50} of each dose response curve in table 1. The pH is always buffered to 5.5.

While AB650 remained uninduced at any concentration of glucose when there is no AS in the media (Fig. 5-1D), YHL301 and YHL324 were greatly induced by 1% glucose without AS (Fig. 5-1A), confirming that YHL301 and YHL324 are sugar sensors decoupled from phenol signaling. Both the maximal induction level and the sensitivity to AS of all the three strains are enhanced at the presence of 1% glucose (Fig. 1A) as compared to without glucose (Fig. 5-1B). 1% glycerol is added (Fig. 5-1B) as an uninducing carbon source. Without AS, the sugar detection threshold of YHL324 (0.1 mM) is lower than that of YHL301 (1 mM) (Fig. 5-1D), demonstrating that AB520 has enhanced sensitivity to sugar signals than wt, which stayed true when 100 μM AS is added in the media (Fig. 5-1C). As summarized in table 5-1, all these dose response curves were fitted and the ED_{50} values were reported as the signal sensitivity of the three strains under these conditions. AB650 detects 100 μM AS (without sugar) to 8 μM AS (with saturating sugar). YHL301 exhibits enhanced sensitivity to AS by an order of magnitude than AB650 with and without glucose, and YHL324 is more sensitive to AS by up to 2 orders of magnitude than AB650. In regard to sugar signals, AB650 detects 0.6 mM glucose when phenol is saturated, but unable to detect sugar without phenol. YHL301 and YHL324 detect sugars independent of phenols, with YHL324 showing enhanced glucose sensitivity by a factor of 3 as compared to YHL301 with and without phenols (Table 5-1).

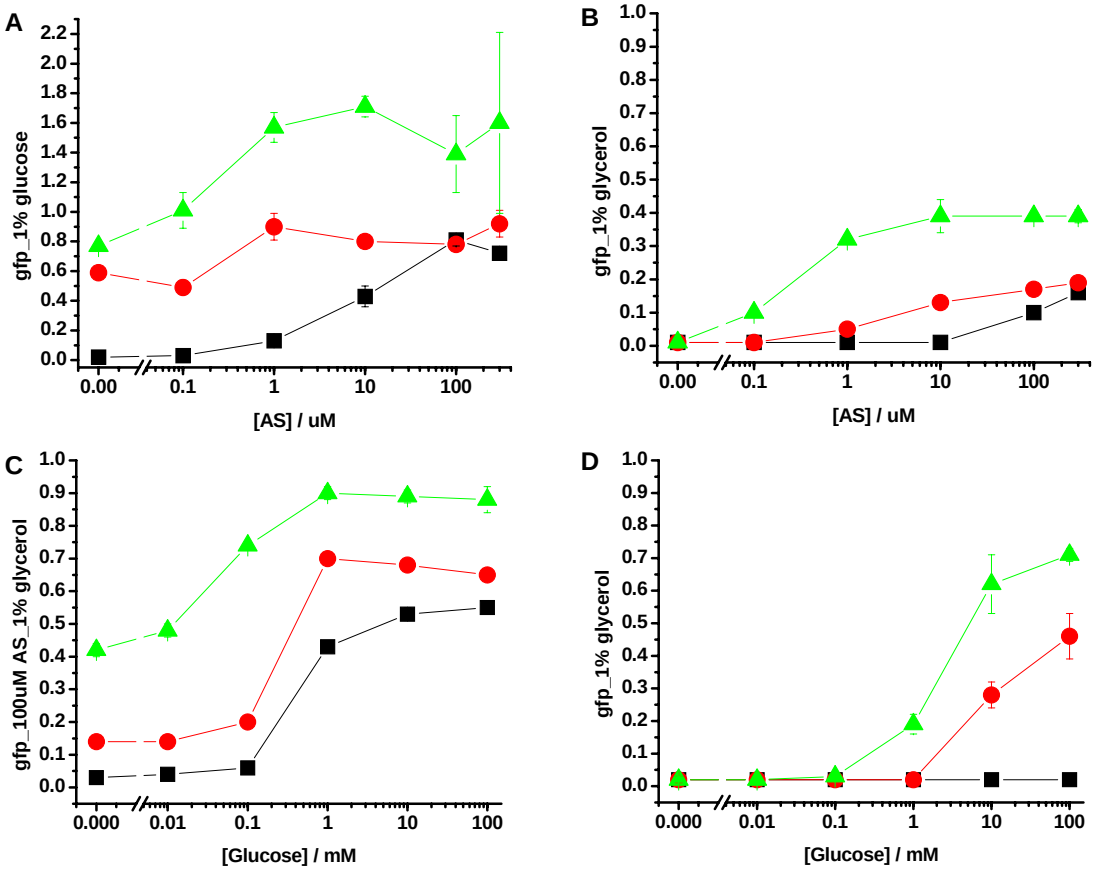


Figure 5-1 *Dose responses of the three A. tumefaciens strains to AS or glucose.* AB650 (black squares), YHL301 (red circles) and YHL324 (green triangles) to AS or glucose dose responses. (A,B) AS dose responses with 1% glucose or 1% glycerol in the growth medium, (C,D) glucose dose responses with 100 µM AS or no AS in the growth medium.

Table 5-1 *ED_{50} of the three strains of A. tumefaciens to AS or glucose.*

Strains	ED_{50} to AS / µM		ED_{50} to glucose / mM	
	w 1% glucose	w/o glucose	w 100 µM AS	w/o AS
AB650	8	100	0.6	-
YHL301	0.2	3-4	0.2	10
YHL324	0.1-0.2	0.4	0.07	3

Does wounding provide physical access for Agrobacterium to enter into the apoplastic space?

A. tumefaciens is not an obligate pathogen as it can colonize plants and form benigh bio-films on root surfaces (Matthysse and Kijne, 1998; Matthysse and McMahan, 1998). Although wounding has been considered to provide physical access to the plant tissue, it is generally expected that antimicrobial substances released at the wound prevent colonization (Broekaert et al., 1997; Orozco-Cardenas and Ryan, 1999; Berrocal-Lobo et al., 2002; Akinsulire et al., 2007; Taye et al., 2011). The way I created wounding in all the tested plants is shown in Fig. 5-2. As shown in the confocal fluorescence image in Fig. 5-3D, AB650 accumulated in the apoplast of a wounded tobacco seedling following wounding as shown by the m-cherry expression in the intercellular space between hypocotyl cells, but did not under the apoplast in the unwounded tobacco seedling (Fig. 5-3A). With regard to mature tobacco explants, m-cherry (red) or gfp expressing (green, or yellow if expressing both m-cherry and gfp) *A. tumefaciens* colonized on wound surface of the explants excessively (Fig. 5-3B, C). By taking an optical section 60-80 μm below the surface, bacterial cells were found to be able to move several cell layers into the tissue following wounding (Fig. 5-3E, F). The root of an unwounded maize seedling accumulated no bacterial cells either in the cell layers immediately below the epidermis or in the vascular bundles (Fig. 5-3G, H). The green fluorescence in Fig. 5-3C, F, G, and H are autofluorescence of lignin in the cell walls.

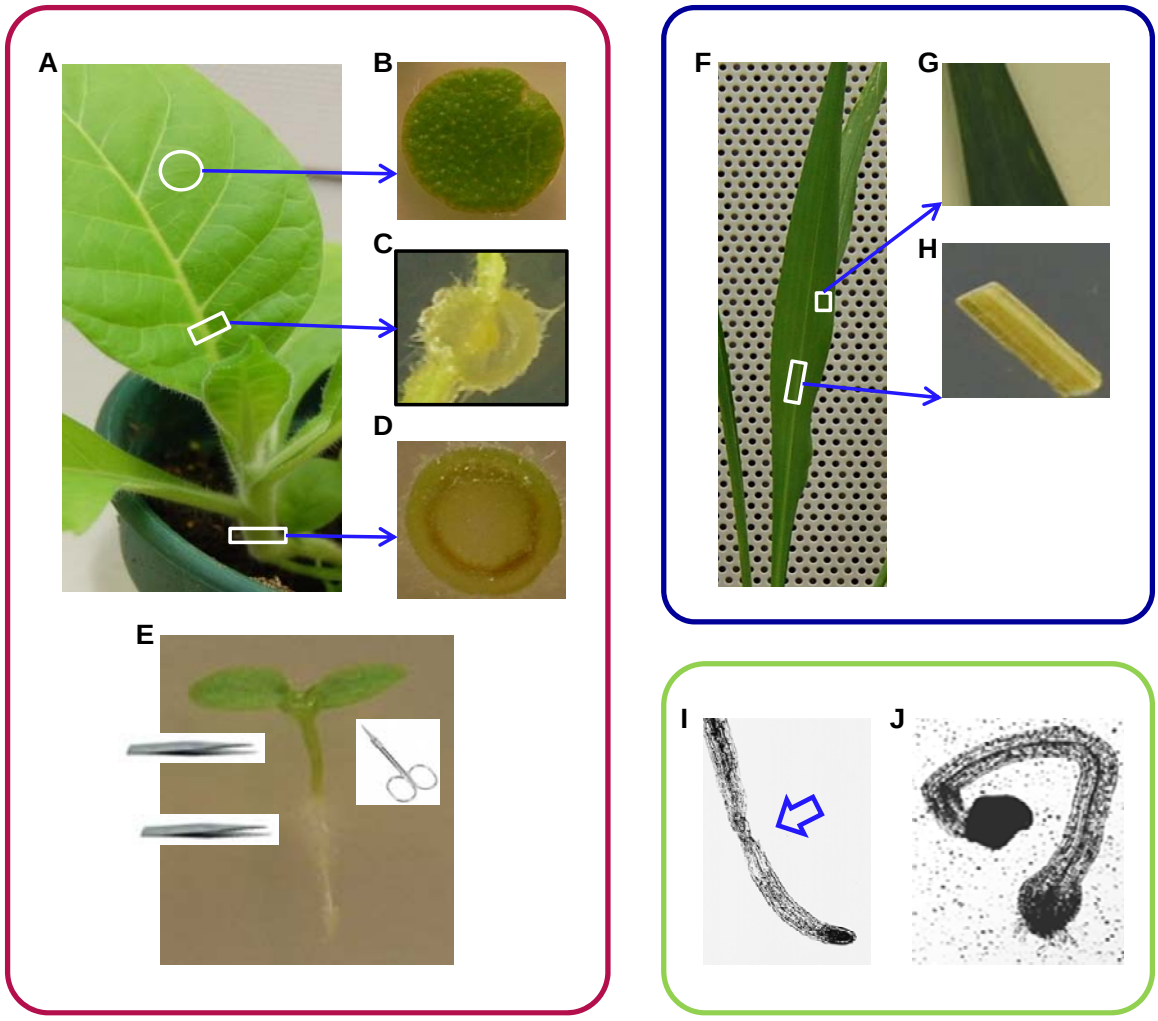


Figure 5-2 *Illustration on how plant tissues were prepared for co-cultivation.* Flowered tobacco plants (A) were cut into leaf explants (B) by cork borer, mid-vein explants (C) and stem explants (D) by razor blade. 1-2-weeks-old tobacco seedlings were wounded by cutting the cotyledons by make-up scissors, or pitching the hypocotyls and root by pointed tweezers (E). 2-months-old maize plants (F) were cut into leaf explants (G) and mid-vein explants by razor blades. Maize stem explants were not prepared because at this stage of the plant, the upright stalk was mainly composed of leaves wrapped in a concentric manner. Therefore, the stalk slices would be a mixture of mid-vein and leaf tissue. 1-day-old *S. asiatica* radicals were conditioned by pitching (I) or haustorium induction (J) before exposure to *A. tumefaciens*.

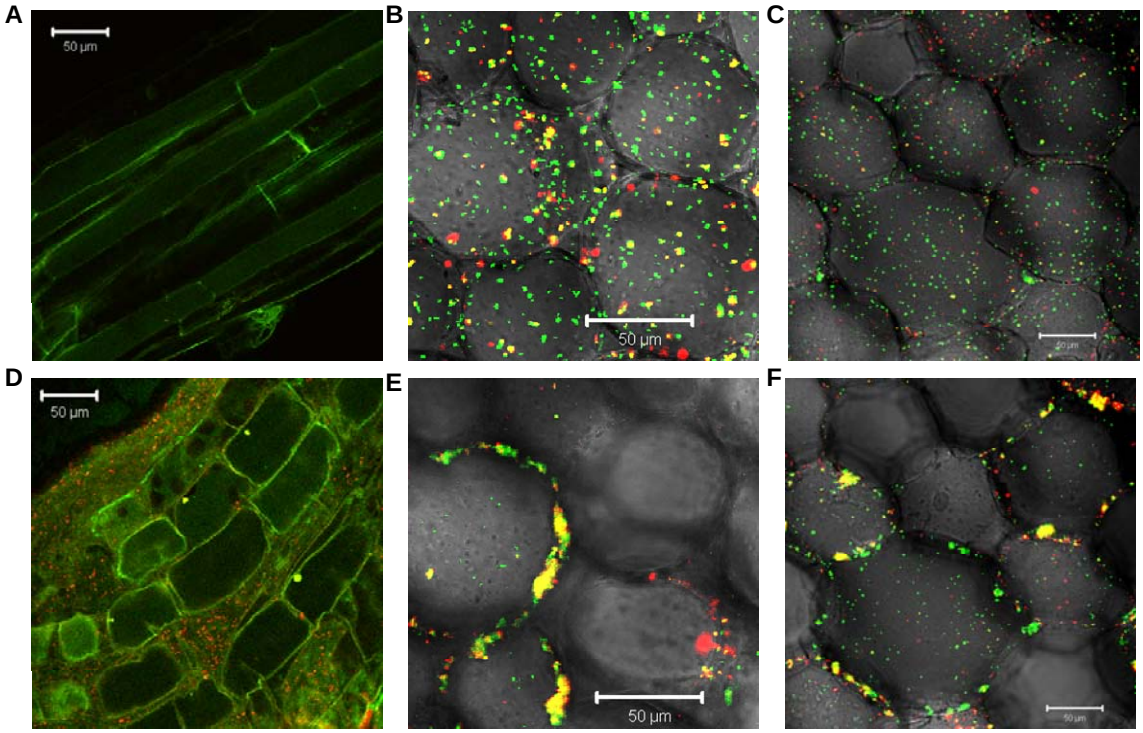


Figure 5-3 *Wound sites provide physical entry into the apoplast.* Unwounded (A) and wounded (D) tobacco seedlings co-cultivated with AB650, YHL324 colonizing the cortex tissue of tobacco stem explants on the surface (B) or 75 µm below surface (E), YHL324 colonizing the pith tissue of tobacco stem explants on the surface (C) or 60 µm below the surface (F).

Is A. tumefaciens able to colonize monocot cells as they do to dicot cells?

Attachment of *Agrobacterium* to plant cell surface is the first crucial step for tumor initiation (Lippincott and Lippincott, 1969; Lippincott and Lippincott, 1977). Several reports indicate that the attachment of *Agrobacterium* to the plant cell requires not only the activity of bacterial genes (Douglas et al., 1985; Thomashow et al., 1987) but appropriate plant cell-wall components (Neff et al., 1987; Binns, 1990), and monocot cell walls were thought to have fewer bacterial attachment sites than dicot cells (Lippincott and Lippincott, 1978). Few attachment sites could account for the lack of tumor formation in some monocots, and reports that Agrobacterium attaches to carrot in greater

number than to corn or oat protoplasts from suspension cultures (Matthysse et al., 1982) and that they do not attach to embryonic leaf fragments of maize support this contention (Lippincott and Lippincott, 1978). However, attachment studies have used cells prepared by many different techniques (Lippincott and Lippincott, 1977, 1978; Ohyama et al., 1979; Matthysse and Gurlitz, 1982; Matthysse et al., 1982; Draper et al., 1983; Douglas et al., 1985) which may have altered their physiological characteristics considerably. For example, *A. tumefaciens* cells rapidly agglutinate and attach to mechanically isolated protoplasts of *Asparagus officinalis* (Draper et al., 1983). As shown in Fig. 5-4C (AB650) and D (YHL301), both strains colonized the intercellular spaces in the mid-vein explants of maize and stem explants of tobacco (Fig. 5-4A, B). However, colony density did appear to be reduced, consistent with the hypothesis that monocots have fewer Agrobacterium binding sites than dicots (Lippincott and Lippincott, 1978). Notably, the YHL301 cells are clearly induced (green and yellow) in tobacco (Fig. 5-4B) but no obvious induction is seen in the maize explants (Fig. 5-4D). This critical difference in virulence induction is addressed in following sections.

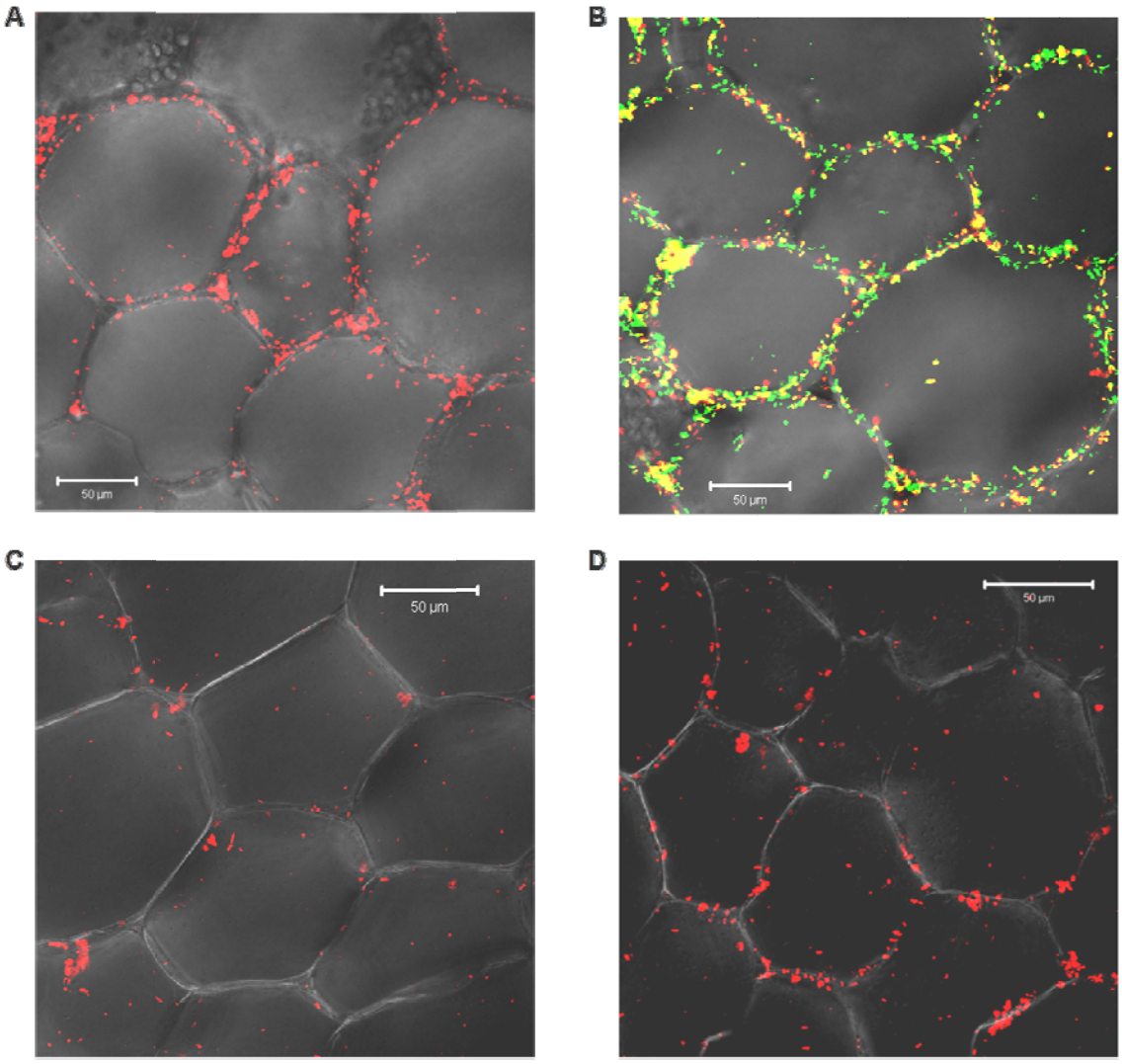


Figure 5-4 *Comparison of the colonization of A. tumefaciens in tobacco or maize explants.* The cortex tissue of tobacco stem explants were bound with AB650 (A) or YHL301 (B), and the cortex tissue of maize mid-vein explants were bound with AB650 (C) or YHL301 (D).

Does A. tumefaciens target phenolic compounds released by wounded cells or living cells at a wound site?

Chemotaxis is a crucial first step in this plant–microbe interaction (Shaw, 1991; Vande Broek and Vanderleyden, 1995; Chesnokova et al., 1997), required for colonization (Shaw, 1991) and infection (Hawes et al., 1988; Hawes and Smith, 1989; Chesnokova et

al., 1997). Motility in *A. tumefaciens* is based on clockwise-rotating flagella (Tanaka, 1985; Loake et al., 1988; Shaw, 1991), and flagellar synthesis, and assembly and switching have been characterized in great detail (Chesnokova et al., 1997; Deakin et al., 1997b; Deakin et al., 1997a; Deakin et al., 1999). Micromolar concentrations of sugars (Loake et al., 1988) and nanomolar amount of wound phenolics have been reported to attract *A. tumefaciens* to the to plant wounds. Further, chemotaxisis is reported to require phosphorylation of VirA/ VirG (Ashby et al., 1987; Ashby et al., 1988; Shaw et al., 1988; Palmer and Shaw, 1992) to activate the chemotaxis operon of *A. tumefaciens* (Wright et al., 1998). Micromolar concentrations of wound phenolics VirA/VirG will then induce virulence gene expression (Winans, 1992; Zambryski, 1992). These signals are present at a plant wound site. Phenols and sugars can be released from breached cells as pools of monomeric building blocks of plant cell walls and acidic pH is characteristic of plant cell wall and vacuoles. On the other hand, the sugar and phenol signals can be exuded from the living cells buried beneath the wound surface to regulate the wound response and *A. tumefaciens* needs to target living cells at a wound rather than dying wounded cells. To resolve this issue, we compared *A. tumefaciens* chemotaxis between vascular cells and the adjacent pith or cortex tissue in tobacco stem explants. High colonization and high induction of YHL324 was observed on the surface of the stem explants across all three tissue types (Fig. 5-5A-C), however, YHL324 ironically did not enter the vascular channels even though these wide conduits are readily accessible to the bacteria. Instead, chemotaxis down to 50-60 μm below the surface was observed in both the adjacent pith and cortex tissue within 2 days of co-cultivation (Fig. 5-5D-F). The colonization and induction on the surface of the tobacco stem explant might be due to virulence inducers

released from both living cells and breached wound cells, however, the selective chemotaxis into the apoplast of the living tissue instead of the dead cells in the vascular tissue is consistent with active exudation from living cells. Signals exuding living cells appear to dominate passive release from wound-breached cells.

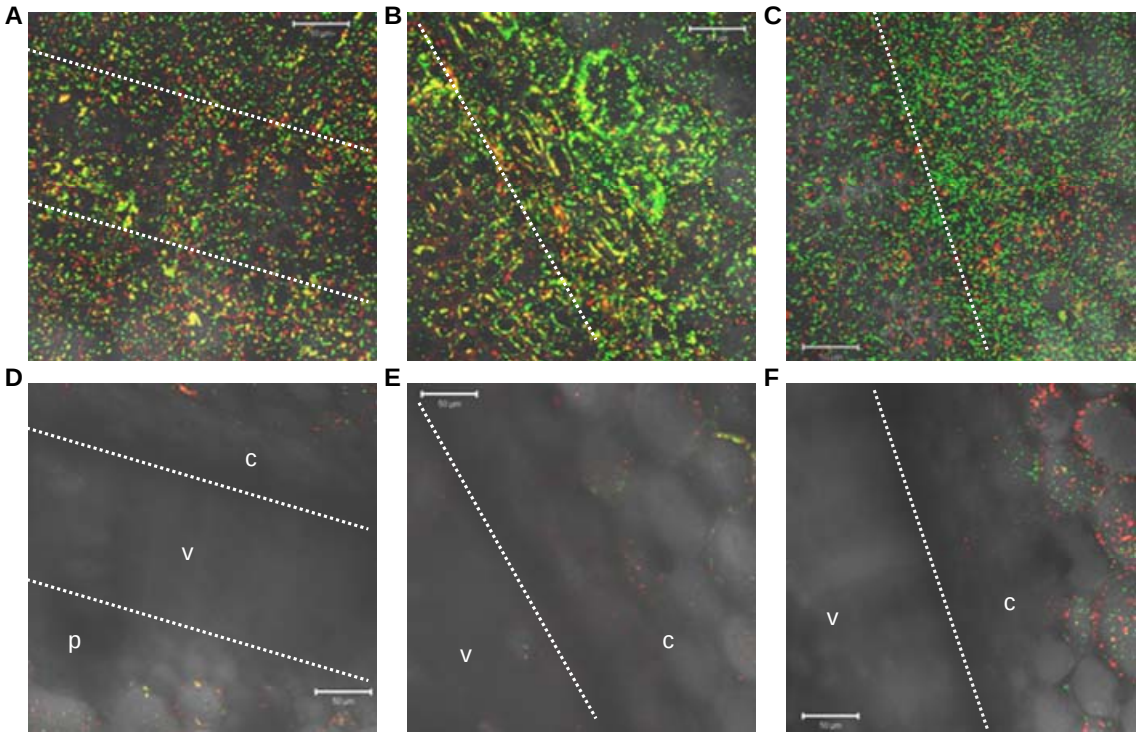
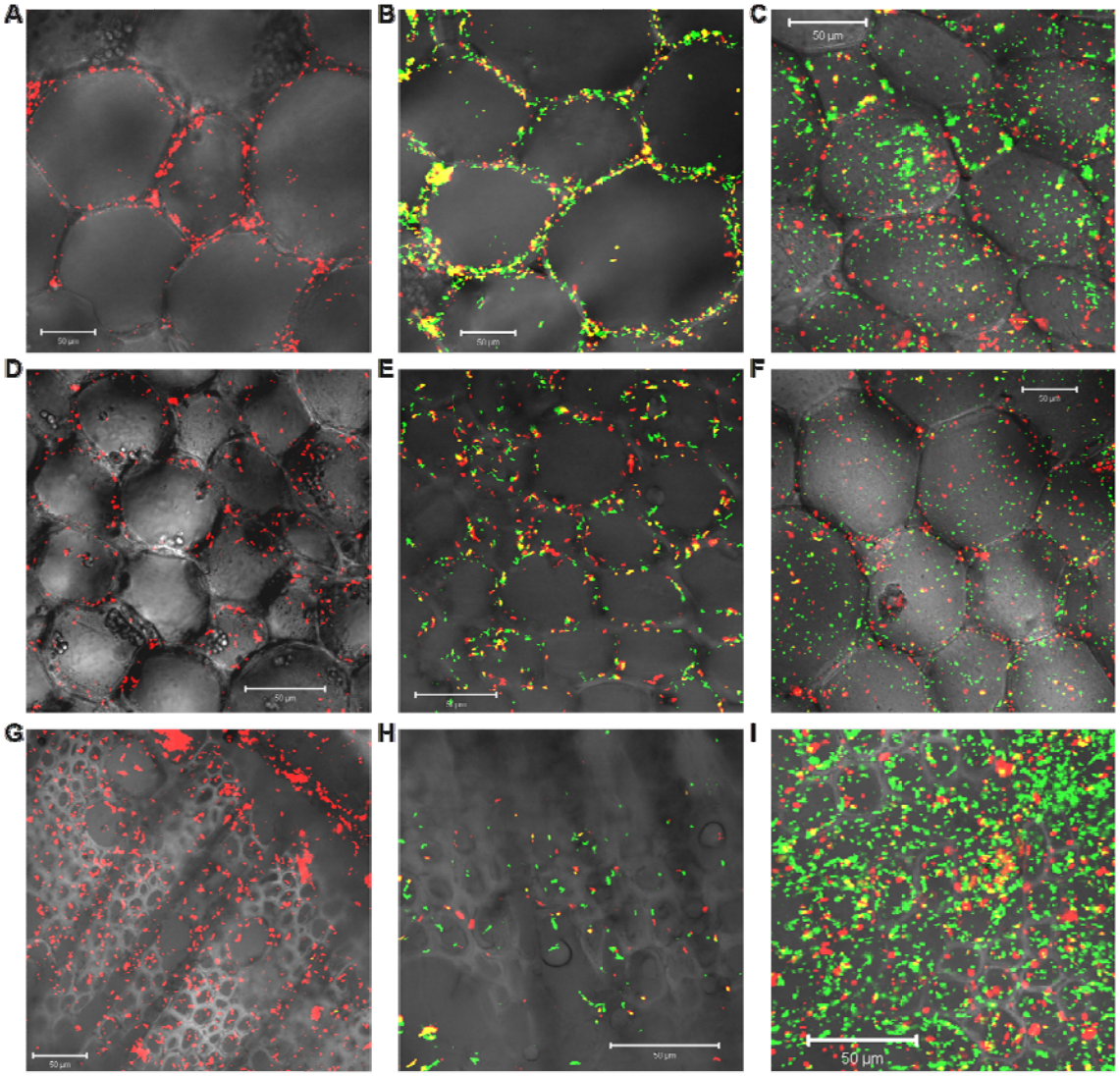


Figure 5-5 *Chemotaxis into the apoplast of tobacco explants within 2 days of co-cultivation on glycerol plates.* (A-C) on the surfaces of the stem explants; (D) an optical section 60 μm below (A); (E) an optical section 50 μm below (B); (F) an optical section 50 μm below (C). The white dashed line are the junction between vascular tissue (v) with the pith tissue (p) inside the vascular ring, or the junction between the vascular tissue (v) and the cortex tissue outside of the vascular ring.

Does wounding release virulence inducers, and is the exudation organ/tissue type dependent?

Wounding has been shown to significantly increase phenolic secretion (Stachel et al., 1985), and I hypothesized that Agrobacterium cued on the wound-induced molecules to identify competent plant cells. Consistent with this hypothesis, AS and alpha-hydroxyacetosyringone, HO-AS, were identified as vir-inducing phenols that were released in 10 times higher amounts upon wounding in tobacco root cultures (Stachel et al., 1985). These C6-C2 acetophenones appear restricted to members of the Solanaceae; phenylpropanoids such as benzaldehydes, benzoic and C6-C3 cinnamic acid derivatives were found in wound exudates of species in other plant families (Spencer and Towers, 1991). Unwounded mature plants of either tobacco or maize failed to induce virulence at all as examined by fluorescence imaging or measured by flow cytometry (data not shown). Wounded tobacco explants exhibited high chemotactic and virulence inducing capacity within 2 days of co-cultivation across the stem pith (Fig. 5-6 A-C), stem cortex (Fig. 5-6 D-F), and stem vascular tissue (Fig. 5-6 G-I), mid-vein cortex (Fig. 5-6 J-L) and mid-vein vascular tissue (Fig. 5-6 M-O), and leaf epidermis (Fig. 5-6 P-R) on glycerol plates. AB650 were not significantly induced by co-cultivating with any of the tobacco explants (left column in Fig. 5-6), whereas YHL301 was induced by all organ/tissue types of the tobacco explants (middle column in Fig. 5-6) and YHL324 was even more highly induced by all organ/tissue types of the tobacco explants (right column in Fig. 5-6). YHL324 also displayed higher colonization density as compared to AB650 or YHL301, which may be due to highly induced chemotaxis. To date, this study is the first

systematical mapping of the signal landscape *in vivo* across different organ and tissue types.



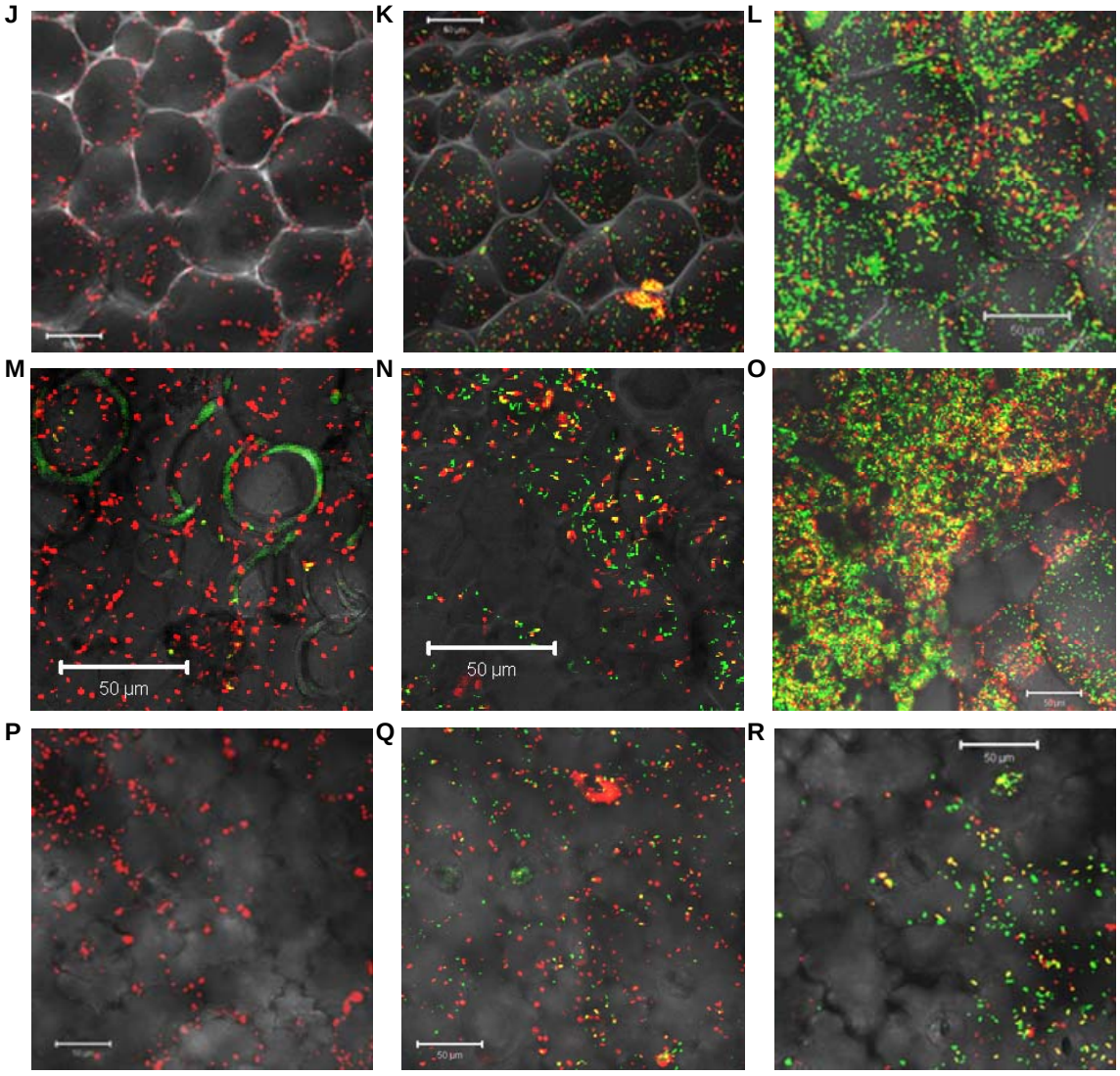


Figure 5-6 *Virulence induction events in mature tobacco explants co-cultivated on glycerol plates.* The left column (A, D, G, J, M, P) shows co-cultivation of AB650, the middle column (B, E, H, K, N, Q) shows co-cultivation of YHL301, and the right column (C, F, I, L, O, R) shows co-cultivation of YHL324 with (A-C) stem pith tissue, (D-F) stem cortex tissue, (G-I) stem vascular tissue, (J-L) mid-vein cortex tissue, (M-O) mid-vein vascular tissue, and (P-R) leaf epidermis.

Do monocots produce phenols upon wounding?

Monocots were initially considered to be resistant to *Agrobacterium* infection because the plants lack *vir*-inducing exudates on wounding (Hooykaas, 1989; Smith and Hood, 1995). The successful transformation of rice suggested that there might be a low level of vir-inducers released from rice (Raineri et al., 1990), but the rate of transformation was greatly enhanced with addition of exogenous AS (Hiei et al., 1994; Vijayachandra et al., 1995). Moreover, the phenols from monocots differ widely from those of dicots and also amongst the monocot species (Usami et al., 1988; Messens et al., 1990). Further, plant growth regulators or secondary metabolites have been reported to inhibit the process of *vir* gene induction, such as a heat labile, bacteriostatic compound from maize (Sahi et al., 1990), and 2-hydroxy-4,7-dimethoxybenzoxazin (MDIBOA) from the roots of maize seedlings (Zhang et al., 2000; Maresh et al., 2006). Given these *in vitro* results, we were poised to observe *in vivo* whether monocots exude vir-inducers sufficient to induce vir expression. As shown in Fig. 5-7, both AB650 (Fig. 5-7A, D) and YHL301 (Fig. 5-7B, E) failed to be induced by either the mid-vein or the leaf explants of maize; however YHL324 was induced by either tissue (Fig. 5-7C, F). Therefore, maize appears to show lower vir inducing capacity, due either to lower amount of inducers produced upon wounding or to the prescence of vir inhibitors.

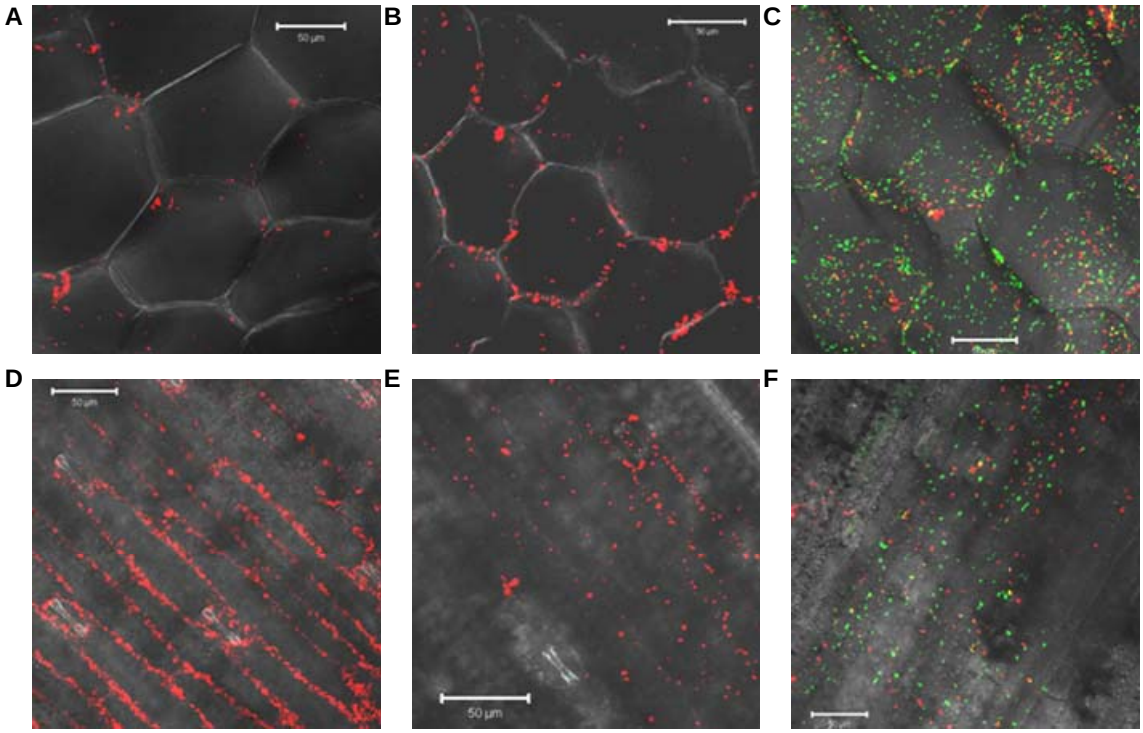


Figure 5-7 *Virulence induction events in 2-month-old maize explants co-cultivated on glycerol plates.* (A) mid-vein explants with AB650, (B) mid-vein explants with YHL301, (C) mid-vein explants with YHL324, (D) leaf explants with AB650, (E) leaf explants with YHL301, (F) leaf explants with YHL324.

How does age affect the exudation of wound induced vir-inducers?

When I inoculated two-week-old tobacco seedlings with R10, an *A. tumefaciens* strain with octopine-type Ti plasmid, pTiR10 (Sciaky et al., 1978), and co-cultivate the seedlings in petri dishes on solidified growth medium, no symptoms of infection were seen (Fig. 5-8A). However, after I transplanted these seedlings to soil, one of the transplants developed a grown gall tumor (Fig. 5-8B). This plant did not grow new leaves nor bolt afterwards, but quickly became nutrient deficient and remained the same height until death. In this case, colonization probably has taken place in the seedling, and transformation may be facilitated by wounding during transplantation or abrasions at the crown during radial expansion after being transplanted into the soil. We speculated that

young seedlings have lower tumorigenesis potential. Consistent with this hypothesis, AB650 failed to be induced in all tissue types (Fig. 5-9 H-L) regardless of wounding status; YHL324 was the only strain induced by wounded seedlings in hypocotyls (Fig.5-9 A-C), root (Fig. 5-9 D-F) and cotyledons (Fig. 5-9G), and YHL324 also colonized at significantly higher level than AB650. Wound sites recruited YHL324 (Fig. 5-9C, E) more readily than AB650 (Fig. 5-9H, I, L), and bacterial clusters, as shown in Fig. 5-9B, E, F, were frequently seen in all the treatment groups, although it was not clear whether clustering provides any advantage during pathogenesis.

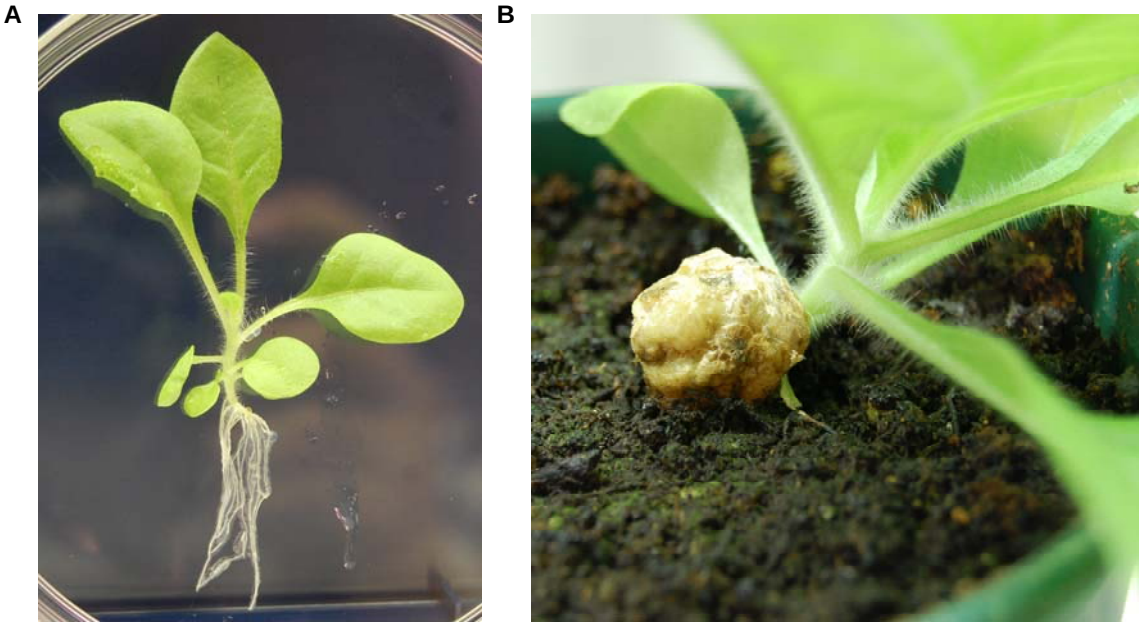


Figure 5-8 *Tumorigenesis potential as related to the age of plants.* Tobacco seedlings inoculated with *A. tumefaciens* strain R10 did not show symptoms of infection (A), but developed crown gall tumors after transplanted into soil (B).

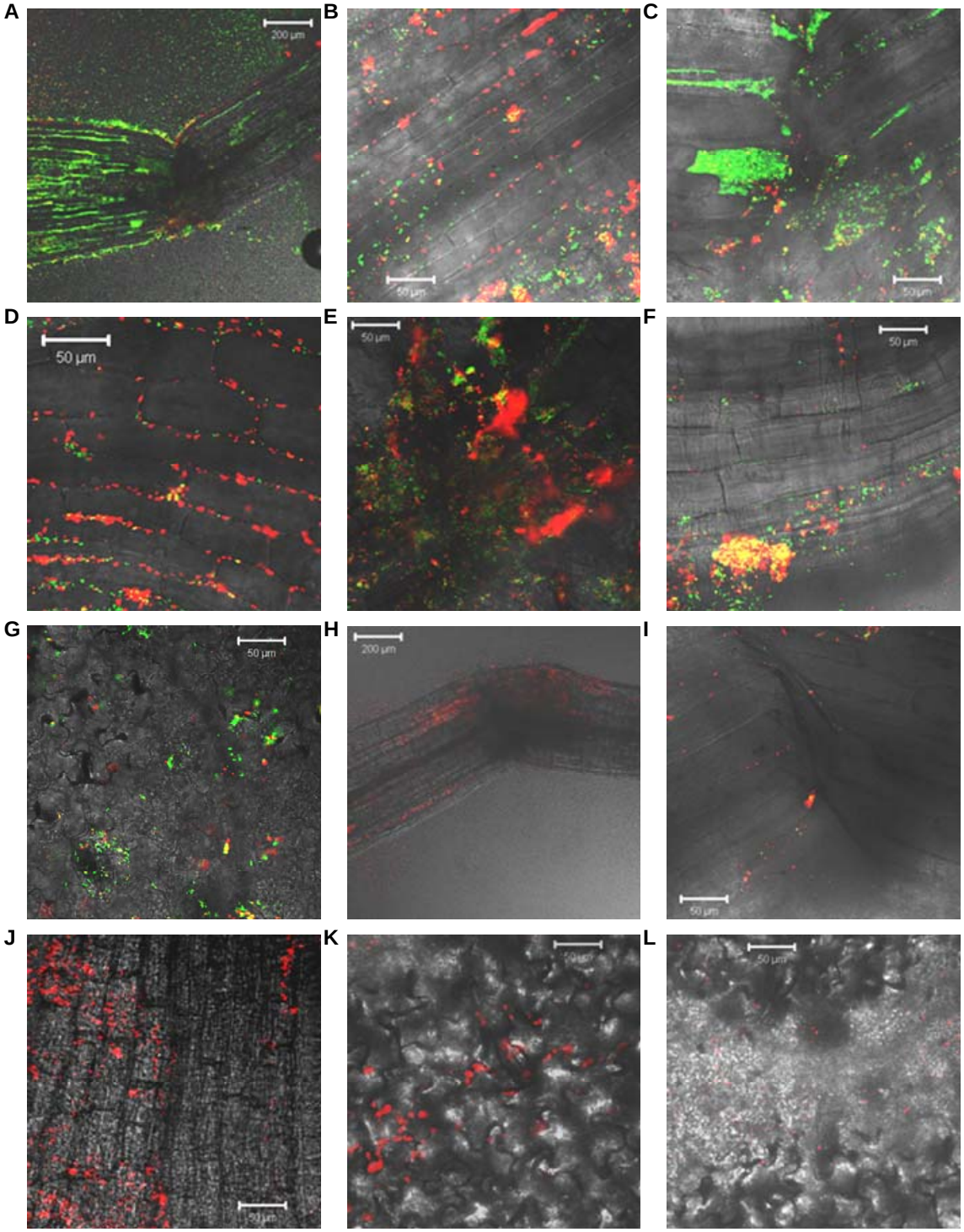


Figure 5-9 *Virulence induction events in two-week-old wounded tobacco seedlings co-cultivated on glycerol plates.* (A-G) co-cultivated with YHL324 on the suface of hypocotyls (B), at the wound site of the hypocotyls (C), at a cell layer beneath the root epidermis (D), at a wound in the root (E), on the surface of the root (F), on the epidermis of the leaf (G). (H-L) co-cultivated with AB650 on the hypocotyl (H), at a

wound in the hypocotyl (I), on the surface of the root (J), on the leaf epidermis (K), or at the cut in the leaf (L).

Quantifying the virulence inducers released at a wound

To quantify the plant released virulence inducers, the bacteria and plant tissue were co-cultivated following inoculation on MS plates at pH5.5 in all treatments. The pH is buffered to pH5.5 because we found that without buffer the plant tissues changed the pH of the growth medium and caused sporadic and large fluctuation in the virulence response under otherwise identical conditions (data not shown). In measuring other vir-inducers, the concentration of the phenols prepared as the limiting factor by supplementing MS media with saturating concentration (1% w/v) of glucose (glucose plates). Therefore, the virulence induction level is only impacted by the phenol inducers released from the plant tissue. Likewise, the concentration of the sugars is measured from MS media with saturating concentration of AS (100 μM) (AS plates). After measuring the gfp expression in co-cultivated *A. tumefaciens*, the gfp fluorescence was compared to the fluorescence level in the standard curves of AS or glucose dose response in Fig. 5-1A and C to estimate the concentration of plant released signals.

The other complication in estimating signal concentration by measuring gfp fluorescence and comparing it to the standard curves is that virulence induction differs when *A. tumefaciens* is grown by themselves in liquid growth medium as compared to co-cultivated with plant explants on the surface of solid medium. To rule out the possibility that the induction level during co-cultivation is higher than in liquid bacterial culture is

because VirA has multiple phenol binding sites so that a variety of phenols exuded by the plant explants exert synergistic effects on VirA activation, I compared phenol dose response of AB650 to either a single phenol inducer, AS, or a mixture of eight phenol inducers (Duban et al., 1993a) with each at an eighth of the concentration of AS. As shown in Fig. 5-10, the mixed phenol failed to exhibit any higher induction level than the single inducer, AS, suggesting the cooperative binding of multiple phenols to VirA does not exist. The difference between solid vs. liquid medium is possibly due to other factors such as oxygen availability. We solved this problem by using multiple strains (AB650, YHL301 and YHL324) with a gradient of sensitivity to either signal. The concentration was estimated by measuring the ratio of the responses from multiple strains and matching the responses onto a similar ratio on the dose response curves of these strains in Fig.5-1A and C.

As shown in Fig. 5-11, 5-12, 5-13, populations of *A. tumefaciens* were washed off from the tobacco or maize explants by vortexing at specified time points over the 22 days of co-cultivation. The mean gfp fluorescence per bacterium as measured by flow cytometry was plotted as a function of time to present the amplitude and kinetics of virulence induction on these tissues. Induction by tobacco seedlings was followed out to only eight days because afterwards the seedlings became significantly necrotic. Virulence induction by co-cultivation on glucose plates (Fig. 5-11), AS plates (Fig. 5-12) and on glycerol plates (Fig. 5-13) were used for quantifying concentrations of plant released AS equivalents, glucose equivalents and a combination of both signals.

On glucose plates, except for tobacco stem explants (Fig. 5-11A), YHL324 exhibited unexpected lower and slower induction than YHL301 on tobacco mid-vein (Fig. 5-11B), tobacco leaf (Fig. 5-11C), maize mid-vein (Fig. 5-12G) and maize leaf explants (Fig. 5-12H), possibly due to the loss of gfp containing plasmid. Unlike AB650 and YHL301, where gfp is Ti-encoded, the gfp reporter in YHL324 is encoded on a separate plasmid maintained by kanamycin. In liquid culture, kanamycin is readily available to every bacterium, while on solid medium every bacterium on the explants may not be exposed to the medium and could lose the gfp containing plasmid, thereby lowering the population mean of gfp fluorescence. Moreover, we found antibiotic suppresses gfp expression (data not shown), which could contribute to the lowered response of YHL324. On the tobacco stem and mid-vein explants (Fig. 5-11A, B) YHL301 responded at similar rate and level as did AB650, indicating the explants released saturating concentration of phenols according to Fig. 5-1A. On the tobacco leaf explants (Fig. 5-11C), YHL301 was induced at higher levels and more quickly than AB650, consistent with a lower concentration of phenols released from tobacco leaf explants. Most notably, the induction patterns seen on the maize explants were drastically different from tobacco explants. At the maize leaf explants (Fig. 5-11E) YHL301 was induced twice as high and fast as AB650, and the maize mid-vein explants (Fig. 5-11D) induced YHL301 four times as high (0.8 vs. 0.2) and twice as fast (8 days vs. 15 days) as AB650, indicating the maize explants released much lower concentrations of phenol signals. Intriguingly, unlike the tobacco explants which sustained a steady state of high level induction for up to more than 18 days, the maize explants released a spike of YHL301 induction that disappeared rapidly. The remaining induction level of YHL301 after the peak was probably due to the saturating

glucose signal in the medium. An AB650 culture pre-induced with 100 μM AS takes up to 8 days after removal of AS for the pre-existing gfp to be completely turned over (Fig. 5-14). The observation that the spike of induction on maize explants disappeared within 4-7 days after the peak is most consistent with previous reports that, in contrast to dicots, monocots have extremely weak and short wound responses. The baseline induction of YHL301 is around 0.2. Therefore, the duration of phenols released was estimated by measuring for how many days the YHL301 induction level was above this threshold 0.2 (Table 2).

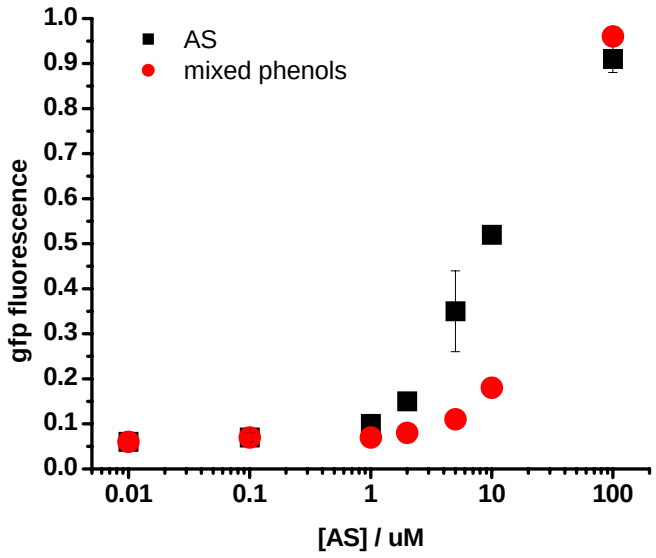


Figure 5-10 *Determination of whether phenols bind to VirA in a cooperative mode.* The dose response of AB650 to a single inducer, AS, (black squares), or to a mixture of eight vir-inducing phenolic compounds (red circles) with each one at a concentration of an eighth of the corresponding AS concentration.

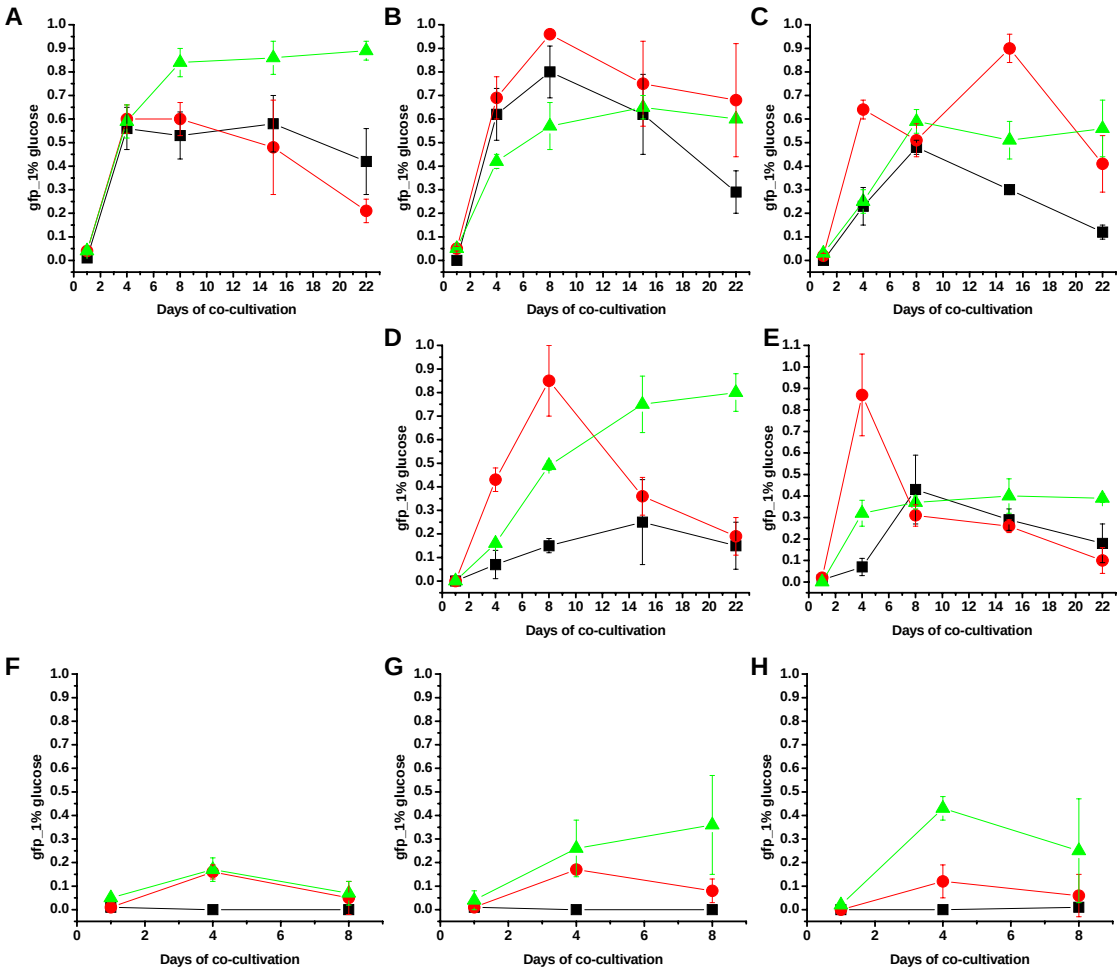


Figure 5-11 *Quantification of wound-induced phenols by co-cultivation on glucose plates.* AB650 (black squares), YHL301 (red circles), and YHL324 (green triangles) co-cultivated with tobacco stem explants (A), tobacco mid-vein explants (B) and tobacco leaf explants (C), maize mid-vein explants (D), maize leaf explants (E), hypocotyls of wounded tobacco seedlings (F), leaves of wounded tobacco seedlings (G) and roots of wounded tobacco seedlings.

YHL324 responded very differently on AS plates than on glucose plates that its induction was indeed higher than YHL301 in all cases (Fig. 5-12). It is possibly because the over-saturating concentration of glucose (56 mM) on the glucose plates affected the normal physiology of YHL324, whereas the AS plates allowed YHL324 to perform normally according to its enhanced signal sensitivity. In the absence of phenols, it only takes 10-20

mM of glucose to reach the saturated induction of YHL324 (Fig. 5-1D). Above 10 mM of glucose, the induction level of YHL324 dropped by four days of culturing in liquid media (data not shown), suggesting that the aberrantly lower and slower response of YHL324 to co-cultivation on glucose plates is due to an inhibiting effect of over saturating signals, as supported again by the observation that among all the co-cultivation groups, the maximum YHL324 induction level was seen on glycerol plates from both tobacco and maize explants. YHL324 is so sensitive that even maize explants, without any exogenous source of signals, induced YHL324 to the maximum. Further, on the AS plate, the ratio of responses among the three strains is very similar between tobacco and maize explants (first row in Fig. 5-12 vs. second row in Fig. 5-12), and amongst different organ types of tobacco (Fig. 5-12 A-C) or maize explants and maize explants (Fig. 5-12 D, E), indicating tobacco and maize releases similar amount of sugars at wound sites, suggesting that the different susceptibility to Agrobacterium infection between dicots and monocots is correlated with wound-induced phenols rather than sugars.

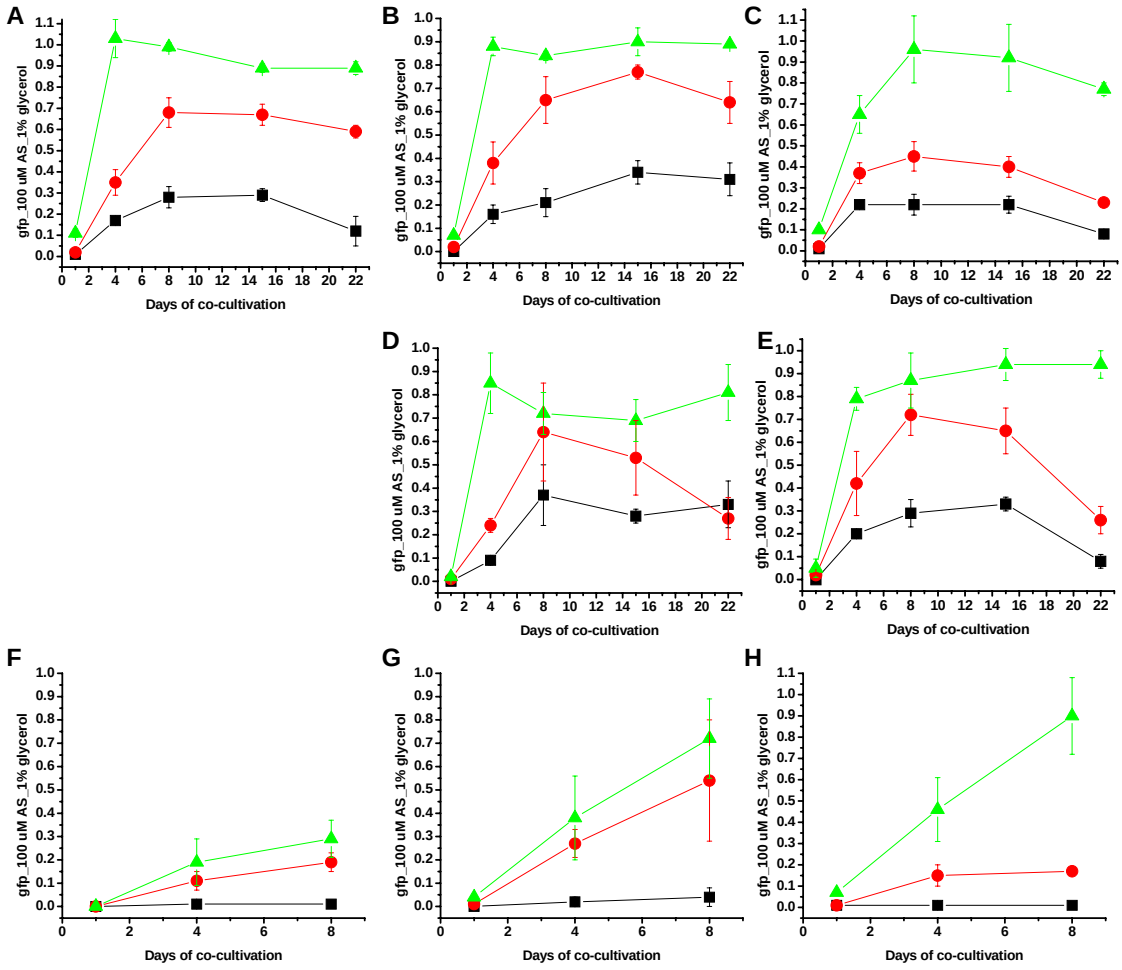


Figure 5-12 *Quantification of wound-induced sugars by co-cultivation on AS plates.* AB650 (black squares), YHL301 (red circles), and YHL324 (green triangles) co-cultivated with tobacco stem explants (A), tobacco mid-vein explants (B) and tobacco leaf explants (C), maize mid-vein explants (D), maize leaf explants (E), hypocotyls of wounded tobacco seedlings (F), leaves of wounded tobacco seedlings (G) and roots of wounded tobacco seedlings.

On glycerol plates, the tobacco explants (Fig. 5-13 A-C) displayed much higher induction potential (the combinatorial effects of both inducers) than the maize explants (Fig. 5-13D, E) as shown by the induction level of YHL301 and AB650, although YHL324 was highly induced by both plants. We quantified the induction potential of each type of explant as conditioned by wounding, organ/tissue type, and age by measuring how AB650

responded to co-cultivation on glycerol plates without exogenous inducers (Table 5-2). Maize plants showed extremely low induction potential (Fig. 5-13D, E), similar to young tobacco seedlings (Fig. 10F-H), but mature tobacco explants showed high induction (Fig. 5-13 A-C).

The concentrations of AS equivalents or glucose equivalents were estimated mainly according to the ratio of YHL301 induction to AB650 induction (Table 5-2). YHL324 response was used only as an auxiliary reference because the ratio of YHL324 response to the other two strains is not proportionally translatable between solid medium and liquid medium due to the antibiotic requirement. This phenomenon can be seen on tobacco seedlings as the root were in contact with the kanamycin containing solid medium, while the hypocotyls and half of the leaves were not. This organ-dependent incomplete exposure of the seedlings to kanamycin explains the aberrantly lower YHL324 response on the hypocotyls or leaves (Fig. 5-13F, G) relative to the roots (Fig. 5-13H), while the roots exude lower levels of phenols and sugars as shown by the YHL301 response on glucose plates (Fig. 5-13F, G vs H) and on AS plates (Fig. 5-13F, G vs. H). Therefore, the dose response measurements in Fig. 5-1 were normalized to the maximum YHL301 (instead of YHL324) fluorescence value among all the dose response measurements; the co-cultivation measurements in Fig. 5-11, 5-12, and 5-13 were normalized to the maximum YHL301 value among all the co-cultivation measurements.

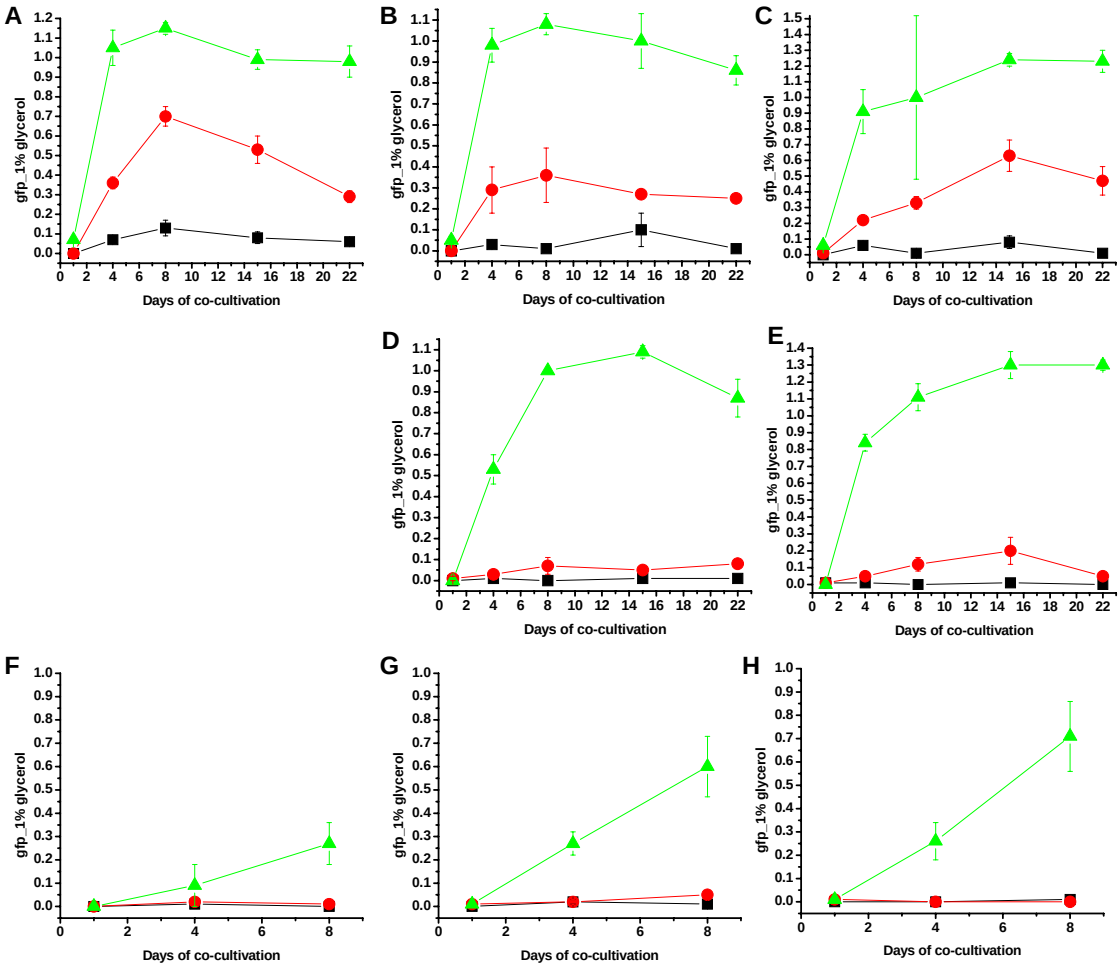


Figure 5-13 *Quantification of the combinatorial effects of both phenols and sugars by co-cultivation on glycerol plates.* AB650 (black squares), YHL301 (red circles), and YHL324 (green triangles) co-cultivated with tobacco stem explants (A), tobacco mid-vein explants (B) and tobacco leaf explants (C), maize mid-vein explants (D), maize leaf explants (E), hypocotyls of wounded tobacco seedlings (F), leaves of wounded tobacco seedlings (G) and roots of wounded tobacco seedlings.

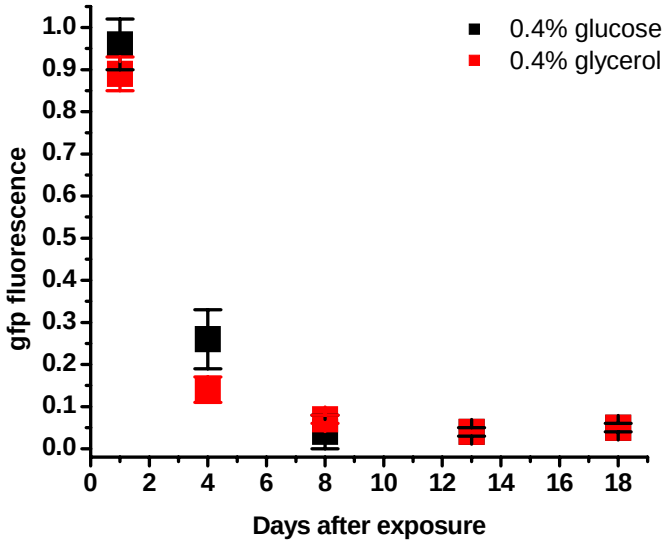


Figure 5-14 *Evaluation of how rapidly gfp proteins are disposed in A. tumefaciens.* AB650 was grown in ½ MS + 0.4% glucose medium under pre-inducing condition with 100 µM AS from day 0 to day 1. At day 1, AS was removed by pelleting the bacteria, wash the bacteria with ½ MS two times and then resuspend the bacteria in ½ MS + 0.4% glucose (black squares), or ½ MS + 0.4% glycerol (red squares). The fluorescence level of remaining gfp was monitored at indicated days

Table 5-2 *Summary of the quantified phenolic, sugar, or comibinatorial effect of both inducers, released from tobacco and maize as conditioned by wounding, organ/tissue types, or age.* The concentration and duration of phenol or sugar exudation is quantified according to Fig. 5-11 or Fig. 5-12, respectively, with Fig. 5-1 as the reference. The induction potential is presented as how AB650 with wt signal sensitivity responded to plant tissue without exogenous inducers (Fig. 5-14), normalized to the maximum induction level seen from YHL301 across all the treatment groups. The vir-inducers exuded by unwounded plant tissue or young tobacco seedlings were not detected (ND) by AB650 or YHL301.

Plant Condition	*Vir*-inducers		6-months-old tobacco			2-months-old maize		Young tobacco		
			Stem	Vein	Leaf	Vein	Leaf	Stem	Vein	Leaf
Wounded	Phenolic	Conc.	> 100 uM	> 100 uM	10-100 uM	1-5 uM	5-10 uM	0-0.1 uM	0-0.1 uM	0-0.1 uM
		Peak length	> 18 days	> 18 days	> 18 days	< 11 days	< 7 days	< 7 days	< 7 days	< 7 days
	Sugar	Conc.	0.5-1 mM	0.5-1 mM	0.1-0.5 mM	0.1-0.5 mM	0.5-1 mM	0-0.1 mM	0-0.1 mM	0.1-1 mM
		Peak length	< 18 days	< 18 days	< 18 days	< 18 days	< 18 days	> 7 days	> 7 days	> 7 days
	Induction potential		0.1	0.03	0.08	0.01	0.01	0.01	0.02	0.01
Unwounded	Phenolic			ND	ND	ND	ND	ND	ND	ND
	Sugar			ND	ND	ND	ND	ND	ND	ND

The signal lansdcape of S. asiatica

S. asiatica is a parasitic plant that produces ROS at its root tip as a chemical probe for host recognition (Chang and Lynn, 1986; Keyes et al., 2007). This semagenesis model requires that *S. asiatica* contain reduced levels of wall-bound phenols, otherwise they will be oxidized by ROS to auto-induce haustorium development. On the other hand, once haustorium is induced, ROS is down-regulated, lignin biosynthesis is up-regulated, and the lignin precursor phenylpropanoids are potent vir-inducers for Agrobacterium. We therefore inoculated *A. tumefaciens* on *S. asiatica* seedlings conditioned either by haustorium induction (Fig. 5-15C, E) or wounding (Fig. 5-15F, H). Under no circumstances was any strain of *A. tumefaciens* induced, including the double hypersensitive strain YHL324. YHL324 was only slightly induced when co-cultivated with *S. asiatica* on growth medium supplemented with saturating concentration of glucose (Fig. 5-15H). This observation supports the prediction of the semagenesis model. These are very young seedlings with a lifespan of only several days without host attachment, and the seedlings of tobacco also have much reduced phenols compared to mature tobacco. This age dependence appears to be a critical feature in the success of the novel semagenesis strategy.

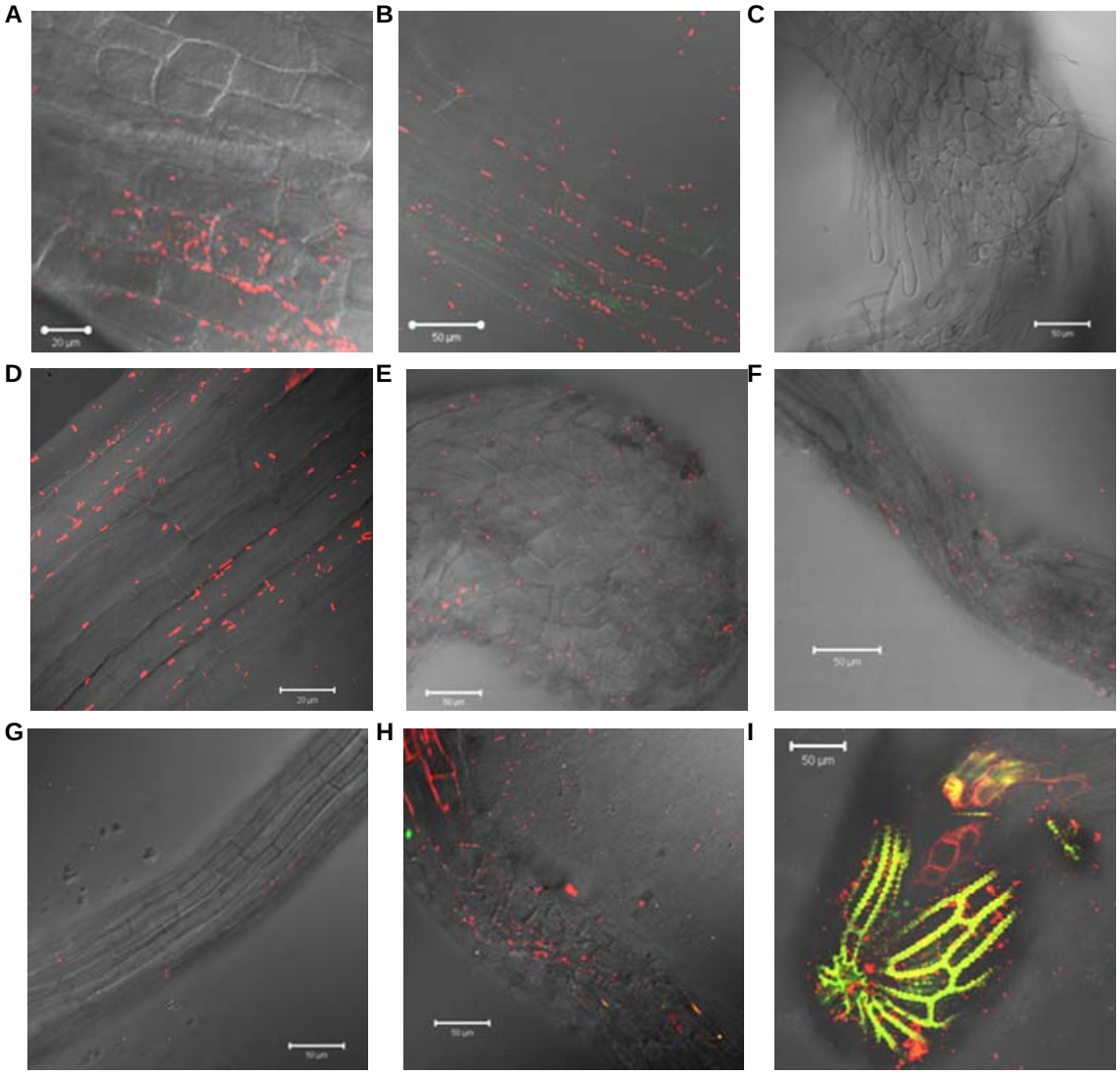


Figure 5-15 *Virulence induction in several-day-old S. asiatica seedlings.* (A-C) AB650, (D-F) YHL301, and (G-I) YHL324. (A) a longitudinal optical section through the vascular tissue in unwounded root on glycerol plates, (B) a shallow longitudinal optical section above the vascular tissue on glucose plates, (C) a wounded haustorium on glycerol plates, (D) a unwounded root on glycerol plates, (E) a unwounded haustorium on glucose plates, (F) a wounded root on glycerol plates, (G) an unwounded root on glycerol plates, (H) an wounded root on glucose plates showing the pinched tissue in the middle, (I) a germinated seed highlighting the coat tissue on glycerol plates.

Can the hypersensitive strain overcome the resistance of monocot grain crops to Agrobacterium infection?

We have shown that binding and virulence induction of *A. tumefaciens* at a maize wound is significantly lower than that in a tobacco. However, the hypersensitive strain YHL324 overcame this limitation and is highly induced by maize explants. We therefore tested whether YHL324 can induce crown gall tumors on maize explants. We inoculated maize explants with either YHL320 (YHL324 without the gfp containing plasmid) or wild type strain A348 and co-cultivated on glucose plates or glycerol plates. Inoculation of tobacco explants were run in parallel as a positive control. After two weeks of co-cultivation, tobacco explants inoculated with either strain started to show sign of tumor initiation, however, up to 51 days neither YHL320 nor A348 induced any crown gall tumor on maize explants. The tobacco stem explants co-cultivated with YHL320 displayed reduced pith tissue and failed to produce obvious tumor growth, but the tobacco stem explants co-cultivated with A348 developed prominent tumor growth (Fig. 5-16A). YHL320 may have induced a severe necrotic response. On tobacco mid-vein and leaf explants, both A348 and YHL320 induced significant tumor growth. No distinct pattern of enhancement in rate of tumorigenesis on tobacco explants was seen in YHL320 co-cultivation than A348. Interestingly, A348 inoculation exhibited higher rate of tumor formation on glucose plates than on glycerol plates. In contrast, YHL320 inoculation induced higher rate of tumor formation on glycerol plates than glucose plates (Table 5-3). This difference in response to exogenous sugar vir-inducer may be explained by the difference in the sensitivity to vir-inducers between A348 and YHL320. A348 has rather low sensitivity to the glucose compound in the medium facilitates virulence induction, while YHL320 can

be induced by the glucose in the medium alone, therefore the over saturating concentration of glucose in the medium mis-leads and prevents YHL320 from locating its host cells. Additionally, wt *A. tumefaciens* strain R10 induced tumors on tobacco explants even when the co-cultivation medium contained no exogenous carbon source (Fig. 5-16 M-P).

I also examined the localization of tumor development. As shown in Fig. 5-16 C-P, tumors were formed preferentially at the junction between two tissue types, the location of cambium cells. Cambium cells retain cell division potential and can be activated by wounding. Scarring were seen around the cut edge of the leaf disks and the tumors grew there (Fig. 5-16 I-L). When leaf explants contain veins, tumors were initiated preferentially at the cut junction between leaf and vein (Fig. 5-16 M-P) in a polar fashion (at one of the two ends) (Fig. 5-16M, N). Only when co-cultivation lasted for more than 35 days did the other cut end and other spots around the cut edge of the leaf disks initiate tumor growth (Fig. 5-16O, P). This localization pattern is consistent with the hypothesis that wounding conditions cells at a wound site and causes formation of rapidly dividing callus tissue for Agrobacterium to target.

Table 5-3 ***Tumor induction rate on tobacco explants co-cultivated with wt A348 and hypersensitive YHL320***

Strains	Plates	Stem	Mid-vein	Leaf
A348	Glucose	96% (22/23)	72% (20/32)	85% (22/26)
	Glycerol	38% (9/24)	44% (14/32)	40% (10/25)
YHL320	Glucose	0%	20% (7/35)	64% (14/22)
	Glycerol	0%	64% (21/33)	92% (23/25)

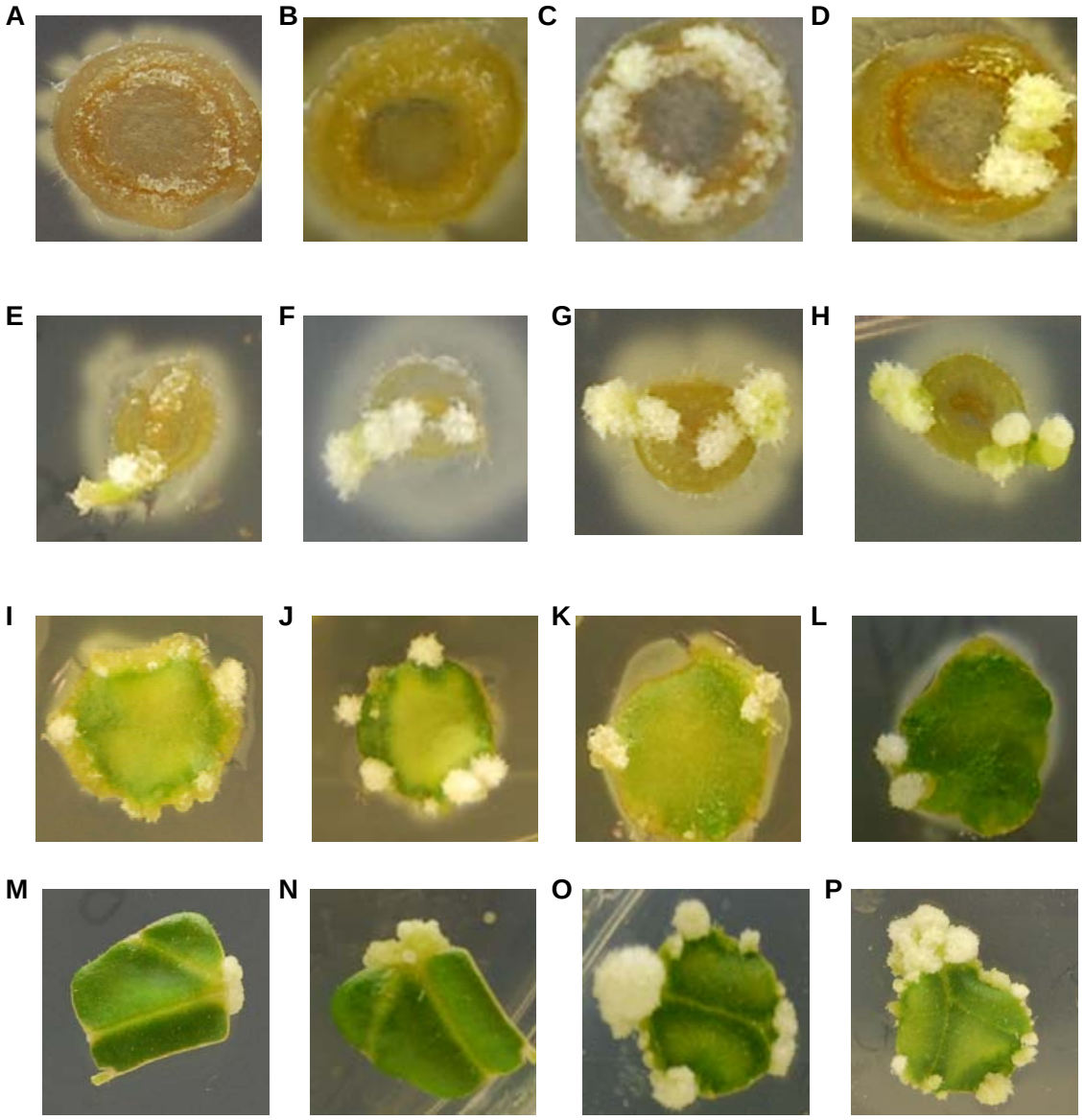


Figure 5-16 *Crown gall tumor induction on tobacco explants.* Tobacco stem explants were co-cultivated with YHL320 on glucose plates (A), and glycerol plate (B), or with A348 on glucose plates (C) and glycerol plates (D); tobacco mid-vein explants with YHL320 on glucose plates (E) and glycerol plate (F), or with A348 on glucose plates (G) and glycerol plates (H); tobacco leaf explants with YHL320 on glucose plates (I) and glycerol plate (J), or with A348 on glucose plates (K) and glycerol plates (L); tobacco leaf explants co-cultivated with R10 on ½ MS plates for 20 days (M), 34 days (N), or 78 days (O, P). (A-L) 51 days of co-cultivation.

5.3 Discussion

Our results established that Agrobacterium infection of monocots is blocked at many steps during pathogenesis. First, wound-released phenol in monocots is 1-2 orders of magnitude lower as a transient spike as compared to a high level of sustained exudation of wound-induced phenols in dicots, while the exudation of sugars were of similar level and duration. This sharp contrast in the profile of wound-released phenols provides direct evidence to previous demonstrations that extremely weak wound responses (Hiei et al., 1997) and absence or low levels of *vir*-inducing exudates hamper Agrobacterium-mediated transformation of monocots (Hooykaas, 1989). The concentrations of maize released vir inducers were derived from measuring virulence expression at the host-pathogen interface in this study, which directly reflects the entire system of vir-inducers and vir-inhibitors exuded by maize.

The cells at a wound appear to be competent for transformation by Agrobacterium, and these cells have been reported to synthesize phenols upon wounding in yew plants (Dubravina et al., 2005). *In situ* imaging allowed us to observe the chemotaxis of *A. tumefaciens* to living cells a couple cell layers below the wound surface but not to the adjacent dead vascular cells at the same depth. It is the living cells that Agrobacterium must target and it is these cells that produce and exude phenols at a wound. The dead vascular cells below the wound surface do not synthesize or exude phenols in response to wounding.

Further, I observed that binding of *A. tumefaciens* to host cell walls occurs less frequently in monocots than in dicots, consistent with the hypothesis that monocots provide fewer binding sites for Agrobacterium. This difference may be due to the differences in cell wall chemistry between dicots and monocots, especially, the members of *Poaceae* family (Carpita 1996). While the dicot cell wall is composed of β-linked glucose residues with interlocking chains of β-D-xyloglucans, the glucuronoarabinoxylans and linear chains of β-*D*-xylose comprise the interlocking polysaccharides in grasses.

Once in the tissues, both YHL301 and YHL324 were induced by dicot explants, but YHL301 failed to be induced by monocots. YHL324 is 10^3 more sensitive in signal perception and is the only strain among the three that was induced by monocots. Meanwhile, young tobacco seedlings exuded 2 or 1 orders of magnitude less phenolic or sugar signals than mature tobacco plants but YHL324 was induced by these seedlings, yet this hypersensitive strain was not able to induce tumors in either tissue. The incompatibility of Agrobacterium with monocots appears to lie in more intrinsic factors in the plants and the apparent differences in the wounding response between dicots and monocots.

Wound healing is a fascinating process and requires not only defense against pathogen but also requires cell dedifferentiation, cell proliferation and cell differentiation to repair and reconsititute the wounded tissue. Monocot cells, unlike the dicots, lose the ability to dedifferentiate at a very early stage of development (Graves et al., 1988). This explains why the breakthrough in *agrobacterium* –mediated transformation of monocot cells were

seen when embryogenic calli or immature embryos were inoculated (Chan et al., 1993; Ishida et al., 1996; Cheng et al., 1997). When wounding does occur, wounding in monocots is not always followed by extensive cell divisions. Rather the wounded monocot tissues differentiate into a lignified or sclerified ring of hardened cells that quickly seal the wound from invading *Agrobacterium* (Kahl, 1982; Mahalakshmi and Kurana, 1997). This rapid differentiation of wound sites in monocots leaves only a few cells marginally competent for either plant regeneration or transformation or both. The actual number of cells receiving the T-DNA is also critically low (Graves et al., 1988). In contrast, wounding in dicots converts potentially competent cells to actually competent ones, and a sector of competent cells is generally available for transformation (Binns, 1990).

So why does *Agrobacterium* cue on phenolic compounds, sugar and acidic pH for host recognition? This study demonstrated that the signals that *Agrobacterium* recognizes are the chemicals that are released at a plant wound site and wounding plays an integral role in Agrobacterium pathogenesis. Wounding provides 1) physical access to the living cells that are buried below the wound surfaces, 2) repair processes that produce rapidly dividing dedifferentiated cells that are competent for transformation, and 3) the cell division potential to either regenerate a transformed plant or to mediate crown gall tumor formation. The hypersensitive strain YHL324 was able to overcome the barrier of vir induction at the host-pathogen interface, but unable to find competent cells for transformation at the wound sites in monocots plants.

5.4 Methods

Agrobacterium strains and plasmids

Strain construction *E. coli* strain XL1-Blue was used for plasmid construction. pYL355, which contains *virA (Y293F)* and genes flanking both sides of *virA* for specific complementation to allow double crossover, was generated by ligating the *Kpn*I fragment from pQF431 into the *Kpn*I-digested pAW162. The *BamH*I/*EcoR*I fragment was released from pYL355 and ligated with the *BamH*I/*EcoR*I digested pAW190, a derivative of pK18mobsacB, to generate pYL356. *A. tumefaciens* strains with *virA(Y293F)* substitution on the Ti plasmid was developed by pK18mobsacB-mediated homologous recombination (Schäfer et al., 1994). This strategy, which requires a two-step selection, facilitates gene deletion or gene substitution without adding additional antibiotic resistance. To generate YHL300, pYL356 was transformed into MDG165, an engineered A348 strain containing *virE::gfp* as the virulence reporter (Goulian et al., 2006). The first crossover for *virA(Y293F)* incorporation was selected by kanamycin resistance, and a single colony was chosen and cultivated overnight in LB at 28˚C. The saturated culture was 1:200, 1:1000, and 1:5000 diluted with fresh LB, and 100 μL of each dilution was spread onto LB plates containing 10% sucrose. pK18mobsacB encodes a levansucrease, which is lethal to cells grown in the presence of sucrose. Therefore, the colonies grown on sucrose plates would have successfully achieved the second crossover, which would exclude a copy of pK18mobsacB and *virA* or *virA(Y293F)*. Successful incorporation of *virA(Y293F)* was verified by sequencing (Beckman Coulter Genomics), and resulted in YHL300. YHL301 was generated by a single transformation of YHL300 with pMP7605, a plasmid

containing *tac*-driven *m-cherry*. YHL320 was generated similarly with the method described above, but a transformation of pYL356 into AB520, an A348 derivative with a deletion of the sugar transporter GguB (Zhao et al., 2011). After the selection on sucrose plates, successful creation of YHL320 was verified by sequencing, and a dual transformation of pMP7605 and pRG182, a plasmid containing *virB::gfp* as the virulence reporter, gave rise to YHL324.

Culturing, inoculation and co-cultivation

All *A. tumefaciens* inoculums were prepared by grown overnight in LB liquid medium with 100 μg/ml Gentamicin for AB650/YHL301, or 15 μg/ml kanamycin for YHL324. The overnight bacterial culture was spun down and resuspended to OD0.2 in half strength Murashige and Skoog medium (½ MS) buffered to pH5.5 with 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) and mixed with 0.005% triton, and then incubated with plant material for 10 mins (mature Nt or Zm), 5 mins (young Nt seedlings) or 1 min (Sa seedlings) to inoculate them. Then the bacterial resuspension was washed off and the plant material was placed on three types of co-cultivation medium in petri dishes: glucose plates, AS plates and glycerol plates, which contain ½ MS medium (pH5.5) supplemented with 1% glucose, 100 μM AS or 1% glycerol, respectively. Glucose plates are for measuring AS equivalents released from plant material, AS plates are for measuring glucose equivalents and glycerol plates are for measuring a combination of the signals released from the plant material (induction potential). The AS dose responses and glucose dose responses were done in ½ MS (pH5.5), liquid medium supplemented with either 1% glucose or 1% glycerol for measuring AS dose response, or supplemented with

100 μM AS or no AS for measuring glucose dose response. The range of concentration titrated was 0, 0.1, 1, 10, 100, 300 μM AS or 0, 0.01, 0.1, 1, 10, 100 mM glucose. In all treatment, 15 μg/ml or 50 μg/ml of kanamycin was added to liquid culture or agar plates of YHL324 incubation to maintain the gfp containing plasmid pRG222.

Preparation of plant material

Seeds of tobacco (*Nicotiana tabacum* cv. Havana 38) and Zea mays were purchased from LEHLE Seeds. Prior to germination, seeds were surface sterilized with 50% bleach and 0.1% triton for ten minutes, and then washed with plenty of sterile water for 4-6 times. Seedlings were grown on ½ MS solid medium until two true leaves emerged and expanded. Some seedlings were inoculated for examination and the others were transplanted to soil for obtaining mature plants. Seeds of *Striga asiatica* were received from USDA shipment. They were surface sterilized with 3% chromic acid, 50% bleach and 0.1% triton for ten minutes, 70% ethanol for two minutes, and between each treatment rinsed thoroughly with sterile water. The seeds were then incubated in sterile water at 28 °C for 7-10 days. Germination of Striga asiatica seeds was induced hydroponically in 0.1 mM KCl solution with 10 nM Strigol. Haustorium was induced hydroponically in 0.1 mM KCl solution with 10 μM DMBQ.

Wounding mature tobacco or two-month-old maize plants was done by cutting stem/vein explants with razor blades, or leaf explants with cork borer. Wounding young seedlings of tobacco or *S. asiatica* were created by pinching hypocotyl/root with pointed tweezers, or cutting the cotyledon with tiny scissors.

Flow cytometry

A population of *A. tumefaciens* was washed off the plant tissue by vortexing the co-cultivated plant tissue in ½ MS liquid medium (pH5.5). The gfp fluorescence of each bacterium was measured by flow cytometry and a population of 10,000 bacteria was counted from each sample. The average gfp fluorescence per bacterium was plotted. Gfp fluorescence was excited by a blue sapphire laser at 488 nm laser, the emission BP is 525/50 and the long pass is 505 nm; m-cherry fluorescence was excited by a green laser at 532 nm, the emission BP is 610/20 and long pass is 600 nm.

Fluorescence imaging

A Zeiss Laser Confocal Scanning Microscope LSM 510 META was used for fluorescence imaging. Argon/2 laser at 488 nm was used to excite gfp and a filter of 505-530 nm was applied for detecting gfp emission. Helium Neon of 543 nm was used to excite m-cherry and a filter of 585-615 nm was applied to detect m-cherry emission. DIC was applied to acquire bright field images. All images but Fig.1C,F are presented as merged of gfp, m-cherry and DIC channels.

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Chapter 6 - Conclusion

My dissertation research comparing a prokaryotic and a eukaryotic plant pathogen reveals the chemical and genetic strategies of plant pathogenesis as an elegant dance between wounding responses and phenylpropanoid production. Both *S. asiatica* and *A. tumefaciens* cue on host-derived phenols for host recognition and both rely on wounding responses for virulence induction. Interestingly, the sessile *S. asiatica* actively mimics a controlled wounding response to solicit virulence inducers from the host, whereas the mobile *A. tumefaciens* searches out a full-fledged wounding event in the host that exudes virulence inducers.

Agrobacterium pathogenesis requires plant cells competent for transformation. Using specifically engineered strains, I demonstrated that wounding is required for exudation of detectable virulence inducers from host plants. This study represents the first report of mapping the virulence inducing signal landscape *in vivo* and in real time. Further, we characterized the signal landscape as a function of wounding, organ/tissue type, and age, and quantified not only the concentration of wound-released phenol and sugar signals separately, but the duration of induction. Using this *in vivo* information obtained through fluorescence imaging, we were able to watch the pathogenetic behavior of Agrobacterium at each step: moving towards susceptible plant cells using chemotaxis, binding to plant cell walls, entering the apoplast through wound openings, and activating VirA/VirG detection system by wound-released phenols and sugar signals. We compared a typical

dicot host plant with a typical monocot non-host plant at each of these steps, and were able to provide direct tests of early hypotheses for the differences between hosts and non-hosts.

The hypersensitive strain of *A. tumefaciens* that overcame the suppression of virulence induction by monocots still failed to infect monocot explants, indicating that it is a downstream factor, intrinsic to monocot plants, that resists Agrobacterium infection. I propose that this is due to the inherent differences in wounding responses between dicots and monocots. Wounding in dicot plants exhibits a high capacity of cell dedifferentiation and consequently generates rapidly dividing cells that are highly competent for Agrobacterium-mediated transformation (Binns, 1990). In contrast, monocots cells lose dedifferentiation potential at a very early stage of development (Graves et al., 1988). When wounding does occur, monocots display extremely weak wounding responses, including limited cell division and rapid differentiation at the wound into a thickened cell walls that quickly seal the wound from invading Agrobacterium (Kahl, 1982; Mahalakshmi and Kurana, 1997). This difference in wounding response between dicots and monocots dictates the host range of Agrobacterium because only wounding in dicots provides dividing cells competent for transformation. My results are most consistent with Agrobacterium exploiting the up-regulation of phenylpropanoid production as a wounding response to target competent plant cells. The wide window of transformation competency in dicots is correlated with high concentration and extended period of wound-induced phenol exudation. And the low transformation competency in monocots is correlated with the low concentration and transient release of phenols in wounding

responses. The wound-induced sugar exudation, however, are very similar between dicots and monocots. This similar sugar exudation at a wound again suggests that it is the phenols that are functionally correlated with cell dedifferentiation and growth.

From an evolutionary perspective, why has it been beneficial for Agrobacterium to evolve a signal receptor (VirA) that has relatively low phenol sensitivity, but depends on a sugar signal to modulate the sensitivity to host detection? My study demonstrates that virulence induction on the pathogen's side is not the only determinant of the host range of Agrobacterium. YHL324 can be induced by monocots or young seedlings of tobacco, but these cells fail to form tumors. The hypersensitive strain is not supervirulent. Instead, the over-saturating sugar signals in the co-cultivation medium "confused" YHL320 and hampered tumor induction. Likewise, with approximately 120 kg/ha plant-derived phenols being added into grassland soil annually (Siqueira et al., 1991), a hypersensitive phenol/sugar detector could well be misled in host recognition. Therefore, Agrobacterium seems to have evolved a host sensing strategy that depends on the multiple signals specifically characteristic of wound exudates. Perhaps more importantly, the amount of phenol exudates a wound produces is quantitatively correlated with the plant cells' competence for transformation. By setting the signal detection threshold between the amount of phenol compounds in dicot wound exudates and in monocot wound exudates, Agrobacterium is able to find plant cells truly susceptible to transformation and avoid committing to virulence expression induced by plant cells that have not been primed for division.

The critical role wounding and phenylpropanoids play during plant pathogenesis is revealed again in the parasitic plant *S. asiatica*. The genetic pathways underlying haustorium development are identified via differential expression cDNA libraries, including regulation of cell division, cell elongation/expansion, vascular differentiation, wounding/defense/stress response, hormonal pathways and nutrient transport. A haustorium is a highly vascularized organ that attaches and penetrates the host root and infiltrates the host root vascular tissue. The vascular differentiation in the parasite is mediated through the phenylpropanoid pathway not only due to its biosynthetic role for lignifications, but SaCM1 is shown to play a critical role in plant growth and development. These findings, together with the correlation between phenol exudation and cellular dedifferentiation at a wound, and the earlier discovery of DCGs as growth factors (Wood et al., 1969; Wood et al., 1972; Wood et al., 1974; Binns et al., 1987b; Teutonico et al., 1991a; Black et al., 1994; Tamagnone et al., 1998b), are most consistent with phenylpropanoids playing a central role in regulating cell development.

The defense response genes in the haustorium libraries, including the ROS producing enzymes NOX and FOX for host probing, ROS detoxifying enzymes such as peroxidases, quinone detoxifying enzymes such as QR2, and other defense response genes are most consistent with *S. asiatica* mimicking a wounding response by producing a controlled mild level of ROS for host recognition. Further, quinolic haustorial inducers are shown to induce haustorial development by utilizing pre-existing auxin/cytokinin signal transduction systems. It is possible therefore that the parasitic angiosperm in the

Scrophulariaceae and *Orobanchaceae* families emerged by acquiring quinone receptors able to couple quinone perception to pre-existing auxin/cytokinin signaling pathways.

My dissertation therefore documents the discovery of the molecular mechanisms that underlie apparently vastly different pathogenesis processes of two plant pathogens in two domains of life. Through the window of these two plant pathogens and their similar host recognition mechanisms in different host ranges, we gained insight into the inherent differences in the innate immune responses that exist between dicots and monocots. The phenolic secondary metabolites are critical to the language that plants use to communicate this difference. Underlying the morphology and anatomical differences between these two branches of flowering plants, is the more fundamental difference in their cell development. The loss of cell dedifferentiation potential early in development and the absence of secondary growth define monocots as a distinct branch of flowering plants from dicots. The plasticity in dicot cell development, as seen in wound-induced immune response, may be an important factor in accounting for the greater diversity, greater number of species, and greater number of perennial species in dicots. Consistently, the susceptile monocots to Agrobacterium infection belong to *Liliales* and *Arales* orders which are composed of mostly perennial and woody monocot species, respectively. Agrobacterium capitalizes on growth-incurred wounding in dicots to provide host plant cells competent for transformation, while grasses stay undetected by Agrobacterium because wounding grass plants does not result in phenol signals. This fundamental difference in the innate plant immune responses between dicots and monocots is reflected in the host range of *S. asiatica* as well. Our prior studies indicated that a partial wounding

response is mimicked by *S. asiatica* for host recognition by producing ROS at the root tip. I propose that ROS may induce a mild wounding response in dicots to elicit a large dose of phenol exudation. Unlike grasses, wounding response in dicots involves development to generate new cells to repair the wound. The study on SaPCBER1 and SaCM1, along with the earlier discovery on DCGs, suggests that phenols may play a role in regulating plant development. The wound-induced phenol exudation from dicots may interfere with the signaling between *S. asiatica* and dicot plants after haustorial development and hamper the next step in pathogenesis, host attachment and penetration. In contrast, ROS produced at the root tip of *S. asiatica* only release a small dose of phenols from the cell walls, which is just enough to induce haustorial development but transient enough to avoid interfering with the normal downstream signaling between the parasite and the host. This understanding of plant biology, enhanced by the definition of the critical factors determining the host range of plant pathogens, may now be exploited by plant biotechnology to alter the host range of genetic engineering and to develop new strategies to control a pathogenesis that causes devastating agricultural constraints on food production.

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