TRANSCRIPTIONAL REGULATION IN COWPEA BRUCHID GUTS DURING

ADAPTATION TO A PLANT DEFENSE PROTEASE INHIBITOR AND

SCREENING OF MUTANTS THAT ARE ALTERED IN JASMONATE -

REGULATED SIGNAL TRANSDUCTION PATHWAYS USING *ARABIDOPSIS*

THALIANA

A Dissertation

by

JAEWOONG MOON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Molecular and Environmental Plant Sciences

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ABSTRACT

Transcriptional Regulation in Cowpea Bruchid Guts During Adaptation to a Plant

Defense Protease Inhibitor and Screening of Mutants That Are Altered in Jasmonate -

Regulated Signal Transduction Pathways Using *Arabidopsis thaliana*.

(December 2006)

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To study the interaction between plants and insects I performed the experiments to find out the counter-defense mechanism of insects when insects were attacked by the defense protein of plants. Jasmonate (JA) is one of the most important plant hormones that is involved in plant defense mechanism. I studied to find out the components of JA signal transduction by T-DNA insertion mutant screening.

In the first study, transcriptional regulation in cowpea bruchid guts during adaptation to a plant defense protease inhibitor, cowpea bruchid, when fed on a diet containing the soybean cysteine protease inhibitor soyacystatin N (scN), activates an array of counter-defense genes to adapt to the negative effects of the inhibitor and regain its normal rate of feeding and development. A collection of 1,920 cDNAs was obtained by differential subtraction with cDNAs prepared from guts of the 4th instar larvae of scN-adapted (reared on scN-containing diet) and scN-unadapted (reared on regular scN-free diet) cowpea bruchids. Subsequent expression profiling using DNA microarray and

northern blot analyses identified 94 transcript species from this collection that are responsive to dietary scN. The full-length cDNA of an scN-inducible cathepsin B-like cysteine protease was obtained. Its transcriptional response to scN during larval development contrasts with the pattern of the cathepsin L family, the major digestive enzymes. These results suggest cathepsin B-like cysteine proteases may play a crucial role in cowpea bruchid adaptation to dietary scN.

In the second study, screening of mutants that are altered in jasmonate-regulated signal transduction pathways using *Arabidopsis thaliana* was performed. Mutant screening strategy using T-DNA insertion mutagenesis and *AVP-LUC* as a reporter enabled to find JA-signal transduction mutants of *Arabidopsis thaliana*, 9 under-regulated mutants and 6 over-regulated mutants. 20B15 showed reduced *VSP1*, *THI2.1* expression and increased *PDF1.2* expression as compared to wild type when treated with JA. These data strongly suggested that 20B15 is a JA signaling mutant. 49R1, 49R2 and 49R3 had same T-DNA insertion site (At1g53540) and showed about 10-fold higher *AVP-LUC* expression level than wild type when JA was treated. Genetic analysis showed the mutation of these plants was recessive and tight linkage between mutant phenotype and T-DNA insertion in At1g53540.

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CHAPTER I

INTRODUCTION

Herbivorous insects are diverse group of animals that attack the plants, from leaves, stems and roots to flowers, fruits and seeds. Plants are in a war with these chewing, sucking and piercing insects. Studies on host plant resistance have established that plants possess many genes encoding defense-related proteins, including resistance genes involved in gene-for-gene interactions with insects, genes encoding signal transduction proteins, and downstream effector genes encoding proteins that protect plants against insect feeding or invasion (Farmer and Ryan, 1992; McGurl et al., 1994, Bogre et al., 1997; Koiwa et al., 1998; Rossi et al., 1998; Vos et al., 1998; Walling, 2000; de Bruxelles and Roberts, 2001; Kessler and Baldwin, 2002; Moran et al., 2002).

Insects provide many examples of what appears to be antagonistic insect-plant coevolution (Kareiva, 1999). Antagonistic coevolution is defined by the following events: The insect increases its fitness by attacking and successfully exploiting a plant. The reduced fitness of the attacked plant favors the selection of a novel defense that spreads through the plant population. This reduces the fitness of the insect and selects for a virulent insect genotype that spreads through the insect population. The rates at which defense and virulence reciprocally evolve depend on the genetics and ecology of the plant and insect, as well as the costs of defense and virulence. Plant defenses include the production of chemicals that inhibit feeding and digestion as well as the production of volatile signals that attract the natural enemies of the insect pest (De Moares, 1998).

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This dissertation follows the style and format of *Insect Molecular Biology*.

Plants produce endogenous signal molecules including stress hormones, such as jasmonic acid (JA), ethylene, abscisic acid (ABA) and salicylic acid (SA) to regulate signal transduction cascades in plant cells, leading to activation and modulation of defense-related genes. The plant's wound response plays a central role in plant defense and functions through the action of JA.

In the production of food, we might create a "smart" plant that combines the agronomic traits desired by humans, i.e., yield and quality, with enhanced defense against a variety of enemies. The development of model plant systems has started to bring together the disciplines studying plant defense. Molecular biologists, plant biochemists, and plant pathologists collaborate in studies on the defense systems of the model plant *Arabidopsis thaliana*. The result has been significant progress toward understanding many features of plant defense including the structure and function of plant disease resistance genes (Ellis, 2000) and the genetic architecture of disease resistance (Young, 2000).

CHAPTER II

TRANSCRIPTIONAL REGULATION IN COWPEA BRUCHID GUTS DURING

ADAPTATION TO A PLANT DEFENSE PROTEASE INHIBITOR*

2.1. Introduction

The cowpea bruchid *Callosobruchus maculatus* is a worldwide storage pest of cowpea *Vigna unguiculata* and other legumes (Taylor, 1981). Each female adult lays 40 to 60 eggs on the surface of cowpea seeds. Larvae feed inside the seeds, where they complete the entire four-instar larval development and pupate. Cowpea bruchids utilize cysteine proteases as their major digestive enzymes, as do many coleopteran insects (Kitch & Murdock, 1986; Murdock et al., 1987). Sequence alignment indicated that these digestive cysteine proteases are cathepsin L-like (Zhu-Salzman & Salzman, 2001). When the soybean cysteine protease inhibitor soyacystatin N (scN) was incorporated into the diet, developmental delay and increased mortality of cowpea bruchids and other coleopterans was observed in a dose-dependent manner (Koiwa et al., 1998). Supplementing free amino acids to scN-containing diet reversed the negative impact of scN on development and mortality, which establishes that the effect of protease inhibitor is via depletion of the supply of free amino acids needed for normal growth and development (Amirhusin, personal communication). The anti-insect activity of scN

suggests a potential for use in biotechnology-based insect pest control. Insect adaptation to plant defense is a major obstacle in pest management. Insects often possess multiple protease genes encoding various protease isoforms with different sensitivities to a particular inhibitor. These genes are differentially regulated in response to stresses or developmentally (Orr et al., 1994; Zhu-Salzman et al., 2003). Available molecular and biochemical studies have revealed several diverse strategies used by insect herbivores. They may (i) overproduce the existing digestive enzymes to "titer out" the inhibitors (De Leo et al., 1998); (ii) express different enzymes, which are insensitive to the protease inhibitors (Bolter & Jongsma, 1995; Jongsma et al., 1995; Bown et al., 1997; Cloutier et al., 2000; Mazumdar-Leighton & Broadway, 2001); or (iii) activate expression of hydrolyzing enzymes that fragment the inhibitors (Michaud et al., 1995; Giri et al., 1998). Cowpea bruchids apparently are able to employ all three of these strategies when challenged by dietary scN at a dosage level equivalent to the natural plant defense response when attacked by insect herbivores (Zhu-Salzman et al., 2003). Despite a significant delay in development, monitoring of insect feeding behavior revealed that scN only impacted the earlier developmental stages (1st, 2nd and 3rd instars). The 4th instar larvae were able to regain normal feeding and development prior to pupation. The adaptive changes were induced by dietary scN, rather than by programmed developmental processes. Cloning and sequencing of major digestive enzymes from the 4th instar larvae led to identification of two subfamilies of cathepsin L-like cysteine proteases, *CmCPA*s and *CmCPB*s. While *CmCPA* transcripts were 2.5-fold higher in scN-adapted than in scN-unadapted insect gut tissue, *CmCPB*s were over 100-fold higher (Zhu-Salzman et al., 2003). Differential response to dietary scN suggests that they play

distinct roles in the adaptation process. Although significant progress has been made in understanding molecular responses of insect digestive proteases that are directly targeted by the protease inhibitors (Bolter & Jongsma, 1995; Jongsma et al., 1995; Michaud et al., 1995; Bown et al., 1997; Jongsma & Bolter, 1997; Giri et al., 1998; Cloutier et al., 2000; Mazumdar-Leighton & Broadway, 2001; Zhu-Salzman et al., 2003), little is known about broader transcriptional regulation of insect digestive systems when challenged by plant defensive protease inhibitors. Alimentary tracts of herbivorous insects are constantly exposed to plant defense compounds and pathogens in ingested foods. Thus it is reasonable to assume that insects were selected for complex counter-defense mechanisms in cells lining the digestive tract, involving a large number of genes, to evade plant defense and defeat pathogen infection. DNA microarray studies indicated that the model insect *Drosophila* involves 400 genes in defense against bacterial and fungal infections (De Gregorio et al., 2001). In this study, we aimed at understanding the transcriptional response of cowpea bruchid guts to dietary scN challenge beyond the targeted cysteine proteases that we have previously reported. We first generated a cDNA collection enriched in scN-regulated genes by subtractive hybridization. We then performed high-throughput DNA microarray analyses to identify scN-responsive genes. Among up-regulated genes, we selectively cloned a full-length cDNA encoding a cathepsin B-like cysteine protease, which may play a role in cowpea bruchids in coping with the inhibitory activity of dietary scN. Our results indicate that insects are able to modulate expression of numerous genes to overcome effects of plant protease inhibitors.

2.2. Materials and methods

Expression of recombinant scN and testing for residual bacterial cell wall fragments

Bacterially expressed scN was purified using Ni^{2+}-chelate affinity chromatography as described by Koiwa et al. (1998). To ensure the purity of the recombinant protein, we used the Pro-Q^{TM} Emerald 300 Lipopolysaccharide Gel Stain kit (Molecular Probes), a fluorescent method to determine whether residual peptidoglycans or lipopolysaccharides derived from scN-expressing host bacteria were present in the scN. This method is highly sensitive and can detect as little as 200 pg of lipopolysaccharides. The manufacturer's instructions were followed. The Pro-Q^{TM} Emerald 300 dye reacts with periodate-oxidized carbohydrate groups separated in the SDS polyacrylamide gel. The green fluorescence was visualized using a UV transilluminator.

Obtaining insect gut materials from scN-adapted and -unadapted cowpea bruchids

Insect feeding treatments were done following Shade et al. (1986). Decorticated cowpea seeds, California Blackeye No.5, were ground into fine powder, and scN was incorporated to a final concentration of 0.2% (w/w) to make 200 mg artificial seeds. Control seeds were made in a similar manner without scN. Five to eight cowpea bruchid eggs were used to infest each artificial seed. The infested seeds were placed in a growth chamber at $26^{o}C$ and 60% R.H. When translucent, black windows appeared on the

artificial seeds, the seeds were cracked open, and the 4th instar larvae removed. Insect guts were collected according to Kitch & Murdock (1986), frozen in liquid nitrogen and stored in -80^oC till use. For study of gene expression during development, guts were dissected from the 2nd, 3rd and 4th instar larvae respectively, based on the biomonitor results (Zhu-Salzman et al., 2003).

Construction of the subtractive cDNA library

Cowpea bruchid guts, scN-adapted and -unadapted respectively, were homogenized in mRNA extraction buffer. mRNA was extracted using a QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech). The PCR-Select cDNA Subtraction kit (Clontech) was used to obtain cDNAs corresponding to genes differentially regulated by scN. Both forward and reverse subtractions were performed; the forward subtraction used tester cDNA obtained from mRNA of scN-adapted bruchid guts and driver cDNA from control guts, and vice versa in the reverse subtraction. Subtracted cDNAs were subjected to two rounds of PCR amplification to normalize cDNA populations. The PCR products were ligated into pCRII-TOPO[®] vector (Invitrogen), and transformed into *E. coli*, DH10B[TM] cells (Invitrogen). A total of 1,920 bacterial clones harboring cDNA inserts were inoculated, amplified and maintained in freezer medium (50mM potassium phosphate, 2.0 mM sodium citrate, 0.8 mM $MgSO_4$, 7.0 mM $(NH_4)_2SO_4$, pH 7.5) with carbenicillin (50 μg/mL) in 96-well microplates.

DNA microarray and probe preparation

cDNA inserts of the subtracted collections were PCR-amplified in 150 mL

reactions in microplate format, using M13 forward and M13 reverse universal primers.

PCR products confirmed by gel electrophoresis were precipitated by addition of 15 µL of

3 M NaOAc, pH 5.2, and 150 µL isopropanol per reaction at -20°C overnight.

Microplates were centrifuged at 3,200 rpm for 2 hr at 4°C. Pellets were washed with

70% EtOH, vacuum dried, and resuspended in 35 µL spotting solution (2x SSC, 0.1%

sarkosyl) each. DNA clones were printed onto polyL lysine-coated glass slides (CEL

Associates, Houston TX) using an Affymetrix 417 arrayer. Following printing, DNA

was UV crosslinked at 550 mJ, and slides were further processed by blocking in 0.2%

SDS for 10 min at 25°C, followed by DNA denaturation in boiling H_2O for 2 min, and

treatment with -20°C ethanol (95%) for 2 min.

Microarray probes were synthesized from equal amounts of total RNA extracted

from scN-adapted and -unadapted cowpea bruchid guts, respectively, using the 3DNA

expression array system (Genisphere, Hatfield, PA). Briefly, primers containing oligo dT

and a proprietary "capture sequence" for Cy3 or Cy5, respectively, were used with

reverse transcriptase to synthesize cDNA probes. These probes (derived from 5 µg input

RNA per channel for each microarray) were mixed with an equal volume of hybridization

buffer (Express-hyb, Clontech), added to the microarray and covered with a coverslip,

and the slides were sealed in aluminum hybridization chambers (Monterrey Industries,

Monterrey, CA) for hybridization at 65°C overnight. After washing (2x SSC/0.1% SDS

for 10min at 65°C, followed by 10 min in 2x SSC and then 10 min in 0.2x SSC, both at

25°C with shaking), a second hybridization was conducted to incorporate Cy3 or Cy5 fluor, respectively, which were coupled to an oligonucleotide complementary to the "capture sequence". Thus, cDNA probes bound to the microarray were fluor-labeled by hybridization.

Data acquisition and analysis

Slides were scanned with a Packard Scanarray 5000 4-laser confocal scanner (Packard BioChip Technologies) using the Scanarray program. Scanning parameters were adjusted to obtain balanced signals on the two channels using the line-scan function. Image analysis was done with the Quantarray Program (Packard BioScience). Data from both channels were background subtracted and normalized. The normalization factor was generated using the Quantarray Data Reduction Macro in Microsoft Excel. Means and standard deviations of normalized and background-subtracted fold-change values from replicate experiments were derived in Microsoft Excel, and are presented in Table 1. For each given cDNA spot, gene expression was considered to be changed by treatments if the spot had an average fold change ratio of >1.50 or <0.67 over the two treatments, and gave a signal intensity of >5000 in at least one of the two channels (control or treatment) in both replicates. Fold-cutoffs for valid induction and suppression ratios were established by a series of self vs self microarray experiments in which Cy3- and Cy5- labeled probes made from the same RNA were co-hybridized to the DNA arrays. When using 1.5-fold induction and suppression ratio cutoffs and a signal intensity cutoff of 5000, 1.6% of the whole array are expected to be miscategorized, based on the self vs self

error model for the array. This characteristic behavior was also observed in self vs self

tests using RNAs from diverse treatments (data not shown). Further, a series of

preliminary dye-swap experiments demonstrated that the labeling system used resulted in

negligible dye bias, as has been reported elsewhere (Yu et al., 2002). We therefore

adopted the convention of using Cy5 fluor to label the control probe in all experiments.

DNA sequencing and data analyses

Differentially expressed cDNAs were subjected to dideoxy terminator cycle

sequencing using the ABI BigDye sequencing kit (PE Biosystems), and analysed on an

ABI Prism 3100 DNA sequencer. Sequencer[TM] software (Gene Codes Corporation) was

used to trim the vector sequence from raw sequence data, and to assemble contigs.

cDNA identities were determined by sequence comparison to the GenBank database

using BLASTX.

Cloning of full-length cathepsin B-like cysteine proteases

A 187 bp cDNA fragment that was strongly induced by scN was detected by

DNA microarray. Subsequent sequence analysis indicated it shared significant homology

with cathepsin B-like cysteine proteases. The Marathon cDNA Amplification kit

(Clontech) was used to clone the full-length cDNA. The gene specific primer 5'-

CGCCGCCAGATCCAGTTGGAAATTATC-3' and adaptor primer AP1 were used for

3'RACE, and another gene specific primer 5'-

CCCAACCAATTATCCTTACTGCATGGCC-3' and adaptor primer (AP1) for 5'RACE.

The full-length cDNA was PCR amplified, cloned into pCR 2.1-TOPO vector

(Invitrogen), and the sequence of both strands of cDNA inserts was determined.

Northern blot analyses and signal quantitation

Seven μg of total RNA from each selected sample (except the 2nd instar control

and scN-treated gut tissue where 3.5 μg RNA was used) were separated on 1.2% agarose

formaldehyde gels, transferred to Hybond-N nylon membrane and hybridized with ^{32}P-

labeled cDNA probes. Blots were washed (2x SSC/0.1% SDS, 1x SSC/0.1% SDS, 0.1%

SSC/0.1% SDS at 65°C), and exposed to X-ray film. The rRNAs were used to visually

ensure even sample loading.

2.3. Results and discussion

Isolation of scN-responsive genes from cowpea bruchid alimentary tract

In order to obtain a molecular profile of the response of the cowpea bruchid

digestive system to dietary plant protease inhibitors, we used a combination of

subtractive hybridization and cDNA microarray techniques to detect scN-responsive

genes in cowpea bruchid guts. Following forward and reverse subtractions of cDNAs

from the 4th instar larval guts of scN-adapted (reared on scN diet) and -unadapted (reared

on scN-free diet) insects, a total of 1,920 cDNA fragments enriched in scN-induced or -

suppressed genes were collected, and their amplified products arrayed onto glass slides. The cDNA microarrays were hybridized with probe pairs prepared from total RNAs of scN-adapted and -unadapted insect guts (Fig. 2-1A). A signal intensity of 5,000 or higher in at least one channel and a fold-change ratio cutoff of 1.5 were applied to determine the cDNA populations that were differentially regulated by dietary scN. When this threshold was used in control vs control validation experiments (i.e. both Cy3- and Cy5-labeled probes were made from the same input RNA and co-hybridized to arrays), only 1.6% of the array spots showed 1.5-fold or greater signal differences (Fig. 2-1B), indicating that about 30 clones could be potentially miscategorized in the control vs scN treatment arrays. The two cysteine protease genes that were previously known to be induced by scN (*CmCPA1* and *CmCPB1*) were included on the array as controls, and were found to be up-regulated in the microarray, as expected. To ensure reproducibility, replicate microarrays for each treatment were conducted with separate RNA preparations. Further, selected clones showing altered expression on microarrays were subjected to northern blot analysis (Fig.2-1C).
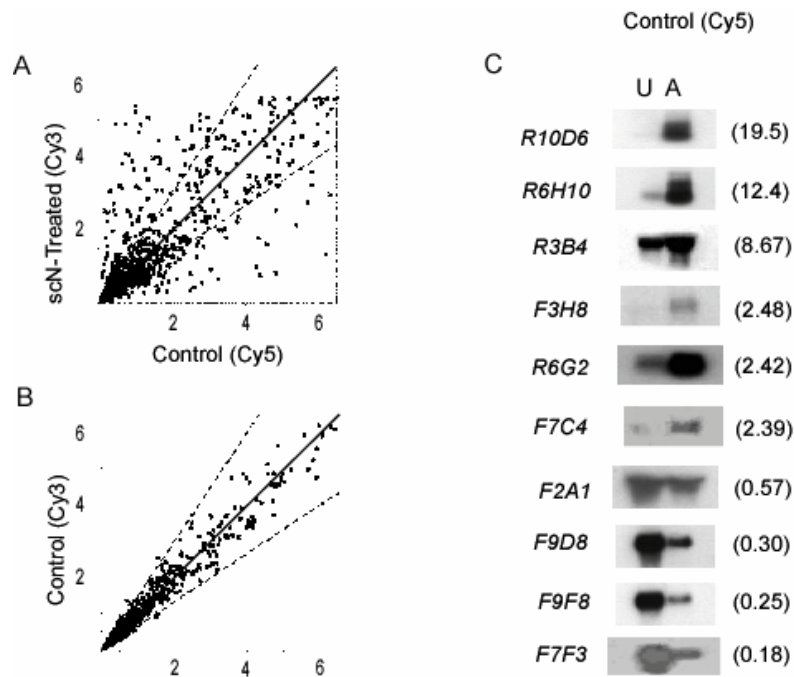
Figure 2-1. cDNA microarray and northern blot analyses of cowpea bruchid gut gene expression in response to dietary scN. Scatter plots of signal intensity distribution patterns of 1,920 ESTs after microarray hybridization. A. cDNA probes were prepared from mRNA of scN-adapted and -unadapted cowpea bruchid guts, respectively. B. The self vs self array was carried out by cohybridizing Cy3- and Cy5-labeled probes made from the same RNA (unadapted cowpea bruchid guts). Values on X and Y axes are signal intensity $\times 10^{-4}$. Solid lines correspond to a 1:1 ratio of signal intensity. Upper and lower dashed lines represent a 1.5-fold induction or suppression of gene expression, respectively. C. Northern blot validation of selected cDNA clones identified by microarrays. *R10D6*: cathepsin B-like cysteine protease; *R6H10*: cathepsin L-like cysteine protease; *R3B4*: endopolygalacturonase; *F3H8*: cytochrome P450; *R6G2*: antimicrobial peptide; *F7C4*: lysosomal thiol reductase; *F2A1*: cytochrome oxidase subunit I; *F9D8*: unknown; *F9F8*: unknown; *F7F3*: unknown. Numbers in parentheses indicate fold induction or suppression in microarrays. Total RNA was extracted from bruchid guts dissected from 4th instar larvae of scN-unadapted (U) and -adapted (A) cowpea bruchid guts, respectively. Respective cDNA fragments were labeled as probes.

A total of 151 cDNA clones exhibiting 1.5-fold or greater induction or suppression by scN in both replicates were analyzed by DNA sequencing, and found to form 94 contigs, representing 94 different genes (Table 2-1). Of the 63 scN up-regulated genes, we found genes encoding protein and carbohydrate digestive enzymes, detoxification proteins and antimicrobial peptides. Among 31 down-regulated genes were cytochrome C oxidase subunit, heat shock protein, mucin, serine protease inhibitor, thymidylate synthase antisense RNA alpha and COP9 signalosome complex subunit genes (Table 2-1).

scN activates protein and carbohydrate digestive enzymes

Numerous cathepsin L-like cysteine protease genes were among the genes induced most strongly by dietary scN (Table 2-1). Total gut proteolytic activity was indeed higher in the insect group that was reared on scN-containing diet (scN-adapted) than in the insect group reared on scN-free control diet (scN-unadapted) (Zhu-Salzman et al., 2003). These observations all support our previous conclusion that one of the adaptive strategies is to overproduce existing major digestive enzymes to out-number the scN inhibitor molecules. Up-regulation of multiple members of the cathepsin L family also serves as validation of the reproducibility of the microarray results.

Table 2-1. scN-responsive genes identified by microarray from cowpea bruchid alimentary tract

Putative function/homology[a]	No. of Clones	No. of Contigs	Ratios of signal intensity[b]
1. Proteases			
Cathepsin L-like cysteine protease	23	8	12.40 ± 5.44
Cathepsin B-like cysteine protease	6	4	19.50 ± 6.36
Carboxypeptidase	1	1	2.39 ± 0.13
2. Hydrolase			
α-Amylase	3	1	1.96 ± 0.51
α-Glucosidase	3	2	2.07 ± 0.49
β-Galactosidase	2	2	2.86 ± 0.16
Endopolygalacturonase	1	1	8.67 ± 2.57
3. Defense-related			
Peptidoglycan recognition protein	1	1	1.58 ± 0.06
Antimicrobial peptide	10	5	2.42 ± 0.11
Ferritin	1	1	1.83 ± 0.42
Cytochrome P450	2	2	2.48 ± 0.83
90-kDa heat shock protein	1	1	0.56 ± 0.12
Mucin	1	1	0.55 ± 0.05
4. Others			
Lysosomal thiol reductase	3	2	2.39 ± 0.47
Coated vesicle membrane protein	1	1	2.10 ± 0.21
Dihydropyrimidine dehydrogenase	1	1	2.02 ± 0.09
Formate dehydrogenase	1	1	1.93 ± 0.66
Reverse transcriptase	1	1	1.77 ± 0.45
P8 apoptotic factor	2	1	1.58 ± 0.11
Cytochrome oxidase subunit I	2	1	0.57 ± 0.05
Cytochrome oxidase subunit II	1	1	0.57 ± 0.04
Thymidylate synthase antisense RNA	2	2	0.57 ± 0.04
Serine protease inhibitor	2	1	0.40 ± 0.02
COP9 signalosome complex subunit I	1	1	0.49 ± 0.02
5. Unknown function			
Induced	38	28	1.58 to 5.60
Suppressed	41	23	0.18 to 0.61

a: BLASTX searches were conducted to determine homologous genes and the putative functions of the cDNA fragments. The cutoff E-value used was 0.01. For those sequences that did not result in any hits using BLASTX, BLASTN searches were employed.
b: Ratios of signal intensity were determined by cDNA microarray hybridization as described in experimental procedures. Shown is the mean ± standard deviation of fluorescence ratios of two replicate microarray slides. scN-responsive genes are defined as larger than 1.5-fold change in gene expression by dietary scN. Values for up-regulated genes are >1.5, and for down-regulated are expressed as <0.67.

It is intriguing that six cathepsin B-like cysteine protease (*CmCatB*) clones, forming four contigs, were induced highly in scN-adapted cowpea bruchids. In mammals, both cathepsins B and L are lysosomal cysteine proteases that degrade unneeded proteins from intra- and extracellular sources (Uchiyama et al., 1994; Turk et al., 1997). They belong to the papain superfamily (Turk et al., 1997). Cathepsin-like cysteine proteases are common in midguts of Hemiptera, Diptera and Coleoptera (Terra & Ferreira, 1994; Yan et al., 2002; Hernandez et al., 2003). Cathepsin B is unique in this family because it has both endo- and exo-peptidase activities. X-ray crystallographic data revealed that human cathepsin B shares an overall folding pattern and an arrangement of catalytic residues similar to papain (Musil et al., 1991). The relatively larger cathepsin B protein size compared to papain leads to the formation of several insertion loops on the surface of the molecule that are absent in papain. The "occluding loop", Ser^{104} to Pro^{126} in human cathepsin B, is responsible for its peptidyldipeptidase activity, i.e. the exo-peptidase activity. Meanwhile, it also partially blocks the active site cleft, thus interfering with its interaction with substrate or inhibitor molecules (Musil et al., 1991). Removal of the loop structure by deletion mutation increased substrate and inhibitor accessibility (Illy et al., 1997). The identification of cDNAs encoding cathepsin B-like cysteine proteases from scN-adapted cowpea bruchid guts suggests that this group of cysteine proteases may have lower affinity to cysteine protease inhibitors and thus would be less sensitive to inhibition by dietary scN. On the other hand, lower affinity to substrates might mean that cathepsin B-like proteases are less efficient enzymes in an scN-free environment. However, under inhibitor challenge where the scN-sensitive cathepsin L-like major digestive enzymes are sequestered, food degradation by cathepsin

B-like enzymes, although inefficient, could temporarily alleviate a critical shortage of essential amino acids crucial for cowpea bruchid survival while the overall complement of digestive enzymes is reconfigured to regain normal feeding and development.

As an initial step toward exploring whether the cowpea bruchid cathepsin B-like cysteine proteases contain the occluding loop and whether the loop structure could play a role in insect adaptation to plant defense, we extended a cathepsin B-like cysteine protease cDNA fragment at both 5' and 3' ends and obtained a full-length cDNA. This 1,042 bp cDNA, namely *CmCatB1*, encodes a protein of 338 amino acid residues. Sequence alignment indicated that it shares 43.2%, 42.3% and 44.7% identity with human cathepsin B, *Drosophila*, and brown planthopper *Nilaparvata lugens* cathepsin B-like cysteine proteases, respectively. CmCatB1 appears to have a shorter "occluding loop" structure, i.e. 16 amino acid residues compared to 23 from its homologues (Fig. 2-2). Under dietary scN challenge, this loop may very likely render CmCatB1 less susceptible to inhibition by this cysteine protease inhibitor.

```
C.maculatus   MKLAFIALAAVVSCTFAQPELD----FLSDEYIEQLNSK-NLPWKAGRNFERDTSLYNIQ
N.lugens      MRFSICLLFAVVSAISALPDQENTVREIANKWIDAINNNPKSTWKAGHNFHPDTPMSYLQ
Drosophila    MNLLLLVATAASVAALTSGEPS--L--LSDEFIEVVRSK-AKTWTVGRNFDASVTEGHIR
human         MWQLWASLCCLLVLANARSRPS--FHPLSDELVNYVNKR-NTTWQAGHNFYN-VDMSYLK

C.maculatus   RLLSVGTINP----PSEFETIFHEDDGK--DLPEEFDARKQWSKCESIKEIRDQSGCGSC
N.lugens      GLLGVSELESNLADLDKYEEMEENEENKKIKVPKYFDARKKWKKCKSLREIRDQGNCGSC
Drosophila    RLMGVHPDAHKFALPDKREVLGDLYVNSVDELPEEFDSRKQWPNCPTIGEIRDQGSCGSC
human         RLCGTFLGGPK---PPQR-----VMFTEDLKLPASFDAREQWPQCPTIKEIRDQGSCGSC

C.maculatus   WAVSSASVMSDRICIQSDQKNQLRISAADMIECC-ESCTFSVDGCHGGIPSFTFTEWKDS
N.lugens      WAVSVAAAFADRLCIASNAKWNGHISSRELMSCC-SYCGF---GCEGGFPDAAWVFIKRH
Drosophila    WAFGAVEAMSDRVCIHSGGKVNFHFSADDLVSCC-HTCGF---GCNGGFPGAAWSYWTRK
human         WAFGAVEAISDRICIHTNAHVSVEVSAEDLLTCCGSMCGD---GCNGGYPAEAWNFWTRK

C.maculatus   GFVSGGEYNSTNGCMSYPLPRCN-------PSCKTLYDAPT--CKKECDKGSPLKYEEDK
N.lugens      GLVTGGDYHSHDGCQPYPIAPCEHHMEGSKPNCSASPTEPTPACETTCTHGSSLAYQKDR
Drosophila    GIVSGGPYGSNQGCRPYEISPCEHHVNGTRPPCAHGGRTPK--CSHVCQSGYTVDYAKDK
human         GLVSGGLYESHVGCRPYSIPPCEHHVNGSRPPCTGEGDTPK--CSKICEPGYSPTYKQDK
CmCP                           YKAK-------------------RSIC

C.maculatus   HYAKQAYRIMSKVERQIQLEIIKNGPVVASFTVYADFIHYLSGVYKFDGESKLLGGHAVR
N.lugens      QKGKSAY-LVPVGEKQTQLEIFKNGPIVAAFKVYEDFFMYKSGVYKRHPESPFRGRHAVK
Drosophila    HFGSKSY-SVRRNVREIQEEIMTNGPVEGAFTVYEDLILYKDGVYQHEHG-KELGGHAIR
human         HYGYNSY-SVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSGVYQHVTG-EMMGGHAIR

C.maculatus   IIGWGIENG-TYPYWLVSNSWNERWGDQGLFKIWRGKNECGIEEEITAGLPR--------
N.lugens      VIGWGEQNG--LPYWLVQNSWDYDWGDKGLFKIARG-NECDFEKSMTAGLPKYKK-----
Drosophila    ILGWGVWGEEKIPYWLIGNSWNTDWGDHGFFRILRGQDHCGIESSISAGLPKL-------
human         ILGWGVENG--TPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVAGIPRTDQYWEKI
```

Figure. 2-2. CmCatB1 from cowpea bruchid (*Callosobruchus maculatus*) shares significant sequence similarity with cathepsin B and cathepsin B-like cysteine proteases. Shown are human cathepsin B (NP680093) and cathepsin B-like cysteine proteases from *Nilaparvata lugens* (CAC87118) and *Drosophila melanogaster* (NP572920). Identical residues are shaded gray, and consensus residues shaded dark gray. A potential occluding loop region is boxed. Partial sequence of the CmCP is shown to indicate that cowpea bruchid cathepsin L-like cysteine proteases do not have this loop structure. The missing amino acid residues are shown with dashes. The star marks the beginning of the mature proteins. The catalytic triad is indicated by triangles. GenBank accession number for *CmCatB1* is AY429465.

To characterize developmental regulation of *CmCatB1* under scN challenge and to contrast its expression pattern with that of *CmCP*s that encode cathepsin L-like cysteine proteases, we performed northern blotting analyses using RNAs prepared from

dissected gut material from 2nd, 3rd and 4th instars (Fig. 2-3). Cathepsin L-like *CmCP*s

were readily induced as early as the 2nd instar by dietary scN, and were detectable in

bruchid guts of both scN-challenged and -unchallenged insects at every larval stage

examined. In contrast, *CmCatB1* transcript was undetectable throughout normal larval

development, despite identical washing stringency. Expression of *CmCatB1* became

strongest during the 3rd and 4th instars in insects feeding on scN-containing diet,

concordant with the occurrence of adaptation (Zhu-Salzman et al., 2003). This

expression pattern demonstrates that the cathepsin B-like protein was synthesized *de

novo* specifically in response to scN challenge. Differential expression patterns observed

between cathepsin L-like and cathepsin B-like cysteine proteases are interpreted to

indicate they have different functions in the scN-adaptation process. Over-expression of

scN-susceptible cathepsin L-like cysteine proteases may allow insects to "out number"

the inhibitor molecules, and activation of cathepsin B-like enzymes may render cowpea

bruchids less susceptible to scN inhibitory activity.
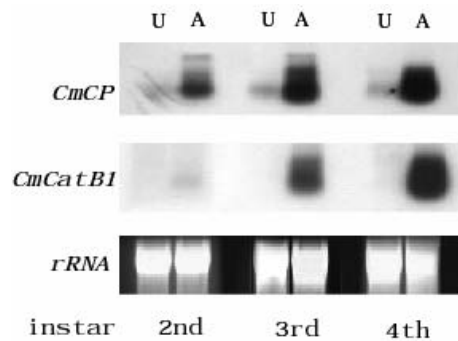
Figure 2-3. Cowpea bruchid expresses *CmCatB1* only under scN challenge. Total RNA was extracted from dissected guts of 2nd, 3rd and 4th instar larvae of unadapted (U, reared on regular scN-free diet) and adapted (A, reared on scN-containing diet) cowpea bruchids. cDNA fragments encoding CmCP, a cathepsin L-like cysteine protease, and CmCatB1 were labeled as probes. Ethidium bromide-stained rRNA bands are shown to demonstrate relative RNA loading.

We observed in our previous study that scN was truncated *in vivo* at its C-terminus when fed to cowpea bruchids (Zhu-Salzman et al., 2003). Although human cathepsin B possesses peptidyldipeptidase activity, CmCatB1 lacks the two key His residues in the occluding loop region (His[110] and His[111] in human cathepsin B; Fig. 2-2) as acceptors of the C-terminal carboxylate group (Illy et al., 1997). Thus scN truncation was not likely executed by this cathepsin B-like protease. Induction of a carboxypeptidase (Table 2-1), however, may have caused this effect. This initial truncation may facilitate further degradation and disarming of scN molecules (Zhu-Salzman et al., 2003).

In addition to proteases and peptidases, scN-adapted insects also induced amylases, glucosidases, galactosidases and a polygalacturonase, possibly to help meet its carbon and nitrogen requirements in the presence of a protease inhibitor. Up-regulation of the mRNA of a lysosomal thiol reductase-like protein may play a role in protein turnover in the cowpea bruchid digestive tract. This protein was postulated to facilitate protein denaturation/unfolding by reducing disulfide bonds (Arunachalam et al., 2000).

scN activates an antimicrobial defense pathway

Dietary scN up-regulated numerous antimicrobial peptide genes in the cowpea bruchid alimentary tract (Table 2-1). It also triggered induction of a peptidoglycan recognition protein that recognizes a bacterial cell wall component and leads to activation of the Imd pathway in *Drosophila* larvae (Takehana et al., 2002). Bacterial and fungal infection induces expression of antimicrobial peptides and proteins in plants, mammals and insects (Lehrer & Ganz, 1999). In *Drosophila*, at least seven types of antimicrobial peptides have been characterized (Tzou et al., 2002), and they are regulated through the Toll or Imd signaling pathways (Hoffmann, 1995; Lemaitre et al., 1995; Khush et al., 2001). The mechanism of activation of the insect innate immunity response is similar to the control of immune genes in mammals (Lemaitre et al., 1995; Silverman & Maniatis, 2001; Tzou et al, 2002). Although the fat body is known to be the main site of antimicrobial peptide synthesis and secretion, production of these defense peptides in the digestive tract and other tissues has also been reported (Ferrandon et al., 1998; Lehane et al., 2003; Lopez et al., 2003). Two defensins were isolated from the midgut of a blood-

feeding insect *Stomoxys calcitrans*, and their expression was increased after blood meals (Lehane et al., 1997). Gambicin, a constitutively expressed antimicrobial peptide from mosquito (*Anopheles gambiae*), was expressed predominantly in the anterior part of the midgut (Vizioli et al., 2001). In *Drosophila*, antifungal drosomycin expressed in respiratory, digestive and reproductive organs caused a local immune response, and was regulated differently from the fat body-produced drosomycin that confers a systemic response (Ferrandon et al., 1998). Signal transduction pathways that regulate scN-induced gut antimicrobial peptides from cowpea bruchid are yet to be elucidated.

We also considered the possibility that these proteins were induced in response to lipopolysaccharides or peptidoglycans present in the recombinant scN which we purified by Ni^{2+}-chelate affinity chromatography. This method produces extremely pure protein. However, to test for the presence of bacterial cell debris in the recombinant scN preparation, we applied a highly sensitive, fluorescent method that detects the presence of lipopolysaccharides or peptidoglycans (Fig. 2-4). No detectable fluorescent signal was observed even in 20 μg scN, an amount equivalent to total scN consumption from the diet through the entire life cycle of a cowpea bruchid at our experimental dosage of 0.2% (w/w). Further, since the control and scN-containing diets were processed in the same manner and were made from the same batch of cowpea flour (except for addition of scN to the later), chance microbial contamination of the original seeds (flour source) or through handling, causing higher gene expression with scN diet than with control diet is unlikely. Thus induction of antimicrobial peptide genes appears to be a direct response to dietary scN. The advantage of activating an antimicrobial pathway in response to dietary protease inhibitors is not immediately obvious. Perhaps scN indirectly activates a

defense program in response to disruption of digestion, which might be sensed by the

insect as a microbial challenge. It is also possible that cowpea bruchid digestive enzymes

play a role in degrading potential peptidoglycan elicitors derived from cell walls of

ingested bacteria. Inhibition of such digestive proteases by dietary scN may have caused

elevated peptidoglycan levels, which then could act as an elicitor to trigger the

antimicrobial defense response (Takehana et al., 2002). In any case, treating insects with

protease inhibitors, besides the obvious suppression of insect digestion, appears to

strengthen insect defense against microbes. This unintended "side effect" may represent

a potential defect in biotechnology-based plant protection strategies exploiting protease

inhibitor genes.
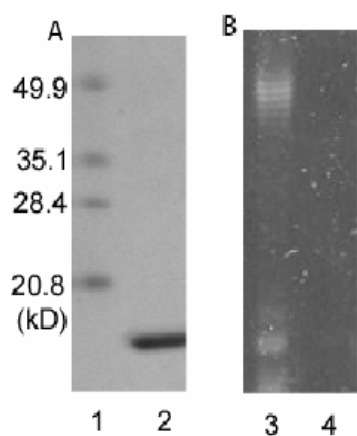
Figure 2-4. Bacterially expressed recombinant scN is free of detectable lipopolysaccharide or peptidoglycan contaminants. SDS polyacrylamide gels were stained with Coomassie brilliant blue R (A) or with fluorescent Pro-Q^{TM} Emerald 300 dye (B), respectively. Lane 1, protein molecular weight standards. Lane 2, recombinant scN. Lane 3, *E. coli* lipopolysaccharides (0.5 μg) are visible as multiple bands at different molecular masses. Lane 4, recombinant scN. Manufacturer's instructions were followed (Molecular Probes). The fluorescence was visualized using a UV transilluminator.

scN activates proteins functioning in detoxification

Strong activation of detoxifying cytochrome P450 and ferritin genes by scN (Table 2-1) may reflect yet another general defense mechanism. Insect P450s are important in detoxification of chemical insecticides (Berge et al., 1998; Scott, 1999) and plant toxins (Schuler, 1996; Danielson et al., 1997). Specific P450s are activated not only in response to plant secondary allelochemicals, but also to the plant defense signaling molecules jasmonic acid and salicylic acid that lead to biosynthesis of allelochemicals (Li et al., 2002). However, no studies have shown that plant protease inhibitors could induce expression of P450s. A ferritin gene encoding an iron-sequestering protein was induced by scN. Increase in ferritin mRNA was observed in *Drosophila* when its diet was rich in iron (Georgieva et al., 1999). Ferritin induction also occurs in mammals and plants in response to pathogen infection (Mata et al., 2001). Assuming induction of the polygalaturonase in cowpea bruchid (Table 2-1) could lead to production of reactive oxygen species that, in plants, further contribute to activation of antimicrobial defense (Alvarez et al., 1998), the reactive oxygen species could also react with iron to form hydroxyl radicals (Halliwell & Gutteridge, 1984; Harrison & Arosio, 1996). In this scenario, it would be beneficial to insects to increase expression of ferritins to prevent lipid peroxidation, protein denaturation and degradation and DNA mutation.

scN Down-regulated genes

Sequencing analysis of scN down-regulated genes appears to reflect gene regulation that facilitates a counter-defense response (Table 2-1). Serine proteases

activate the innate immune response in mosquitoes and induce production of antimicrobial peptides (Gorman & Paskewitz, 2001). Thus, suppression of serine protease inhibitors in cowpea bruchid may contribute to the increased production of antimicrobial peptides. Decreased cytochrome C oxidase expression could be the result of the insect's metabolic adjustment to the inhibition of its digestion. It is highly possible that mounting effective counter-defense would compromise some metabolic pathways.

Genes of unknown function

A significant number of genes, 28 up-regulated and 23 down-regulated, either failed to match any sequences in Genbank, or matched genes encoding proteins with unknown function (Table 2-1). Studies in humans and yeast indicated that gene clusters sharing similar expression patterns also tend to share similar functions (Eisen et al., 1998). DNA microarray has the potential to establish correlation between unknown or poorly characterized genes and genes with known functions, based on gene expression patterns. Thus, clustering analysis can provide clues to functionality of the unknown genes.

CHAPTER III

**SCREENING AND CHARACTERIZATION OF MUTANTS THAT ARE
ALTERED IN JASMONATE-REGULATED SIGNAL TRANSDUCTION
PATHWAYS USING *ARABIDOPSIS THALIANA***

3.1. Introduction

Jasmonates (abbreviated, JA) are fatty acid-derived signaling molecules involved in the regulation of many physiological and developmental processes in plants, including root growth, tuberization, fruit ripening, senescence, tendril coiling, and pollen development. They are also the key plant hormones for defense responses such as ozone exposure, wounding, water deficit, and increased susceptibility to pathogens (McConn and Browse, 1996; Berger, 2002; Turner et al., 2002; Farmer et al., 2003). JA is a terminal product of the octadecanoid pathway. JA synthesis is induced by a number of biotic and abiotic stresses, such as wounding, drought, and pathogen infection (Creelman and Mullet, 1995). JA induces the expression of wound responsive genes including vegetative storage proteins (Benedetti et al. 1995) and thionins (Epple et al. 1995).

In spite of the importance of JA as plant growth and stress regulator, current knowledge about the JA signaling pathway is limited. Understanding of jasmonate signaling is complicated by the presence of multiple oxylipins (oxidation products derived from the catabolism of fatty acids). Also, a precursor of JA (OPDA) itself is a potent regulator of gene expression in the wound response (Stintzi et al., 2001).

Mutant screening based on insensitivity to coronatine (a JA analog) identified the *COI1* gene (Xie et al. 1998). *COI1* encodes a protein with leucine rich repeats and an F-box motif. F-box proteins are components of SCF (SKP1, CDC53p/CUL1 F-box protein) complexes (Bai et al., 1996) where they function as specific receptors targeting proteins to ubiquitin-mediated proteolysis (Glickman and Ciechanover, 2002). This indicates that COI1 is part of the SCF-ubiquitin ligase machinery involved in targeting proteins for destruction by the proteosome. JA signaling may involve degradation of transcriptional repressors as well as post-translational modification of transcriptional activators. The *coi1* suppressor, *cos1*, restores the expression of JA-inducible genes (*VSP* and *LOX2*) and JA-dependent senescence-associated genes (*SEN4* and *SAG12*) to the *coi1* mutant (Xiao et al., 2004). *COS1* encodes a lumazine synthase, the enzyme involved in the penultimate step of vitamin B2 biosynthesis, suggesting a role for the riboflavin pathway in JA signaling. In addition to *coi1*, other JA-insensitive mutants identified to date include *jar1* (Staswick et al., 1992), *jin1* and *jin4* (Berger et al., 1996), and *jue1*, *jue2*, and *jue3* (Jenson et al., 2002). Among these JA-insensitive mutants, *jar1*, which is allelic to *jin4*, and *jin1* have been identified at the molecular level. *JAR1* encodes an adenylate-forming enzyme specific for JA involved in formation of JA-amino acid conjugates,, indicating that this modification modulates JA signal transduction (Staswick et al., 2002). *JIN1* encodes MYC2, a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor (Lorenzo et al., 2004).

Mutants with constitutive or enhanced responses to JA include *cev1* (Ellis and Turner, 2001), *cex1* (Hilpert et al., 2001), *cet1* to *9* (Xu et al., 2001), and *joe1/2* (Jensen et al., 2002). Among them, only *cev1* has been molecularly characterized and the

mutation lies on the cellulose synthase gene. Evidence has been shown that the inhibition of cellulose synthesis activates JA- and ethylene-dependent stress responses. The author proposed that the cell wall could mediate JA- and ethylene-dependent stress and defense responses. JA is one of the key hormones for defense responses in plants. Understanding the function and regulation of genes in JA signal transduction no doubt will ultimately lead to crop improvement. However, we know very little about JA signaling. Many components in JA signaling pathways are unknown, including the JA receptor(s). Identifying these components is important for better understanding of JA signaling and plant defense regulation.
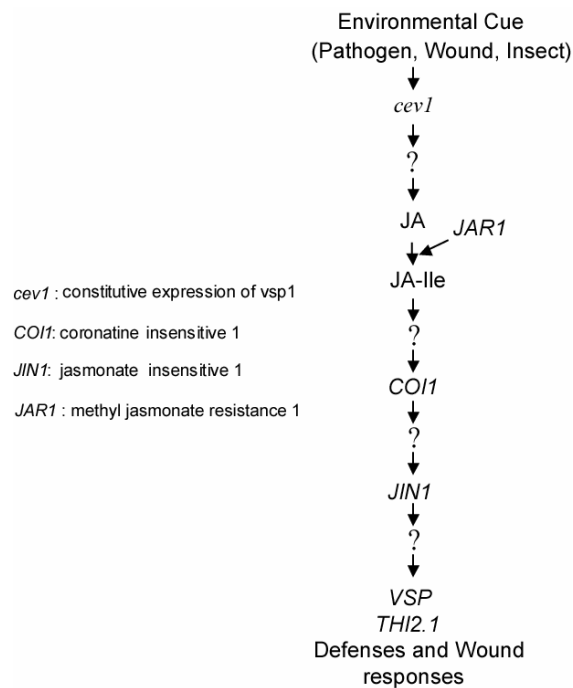
Figure 3-1. JA-signaling model. Many components of JA-signaling are unknown.

One of the JA-responsive genes is the vegetative storage protein gene (*VSP*) (Fig.3-1). VSPs were first identified in soybean (Wittenbach, 1983). They were so named originally due to the fact that they are localized in vacuoles of paraveinal mesophyll cells and accumulate in leaves upon depodding (Wittenbach, 1983; Mason and Mullet, 1990). They are presumed to act as temporary storage of amino acids when growth is inhibited by lack of water (Mason and Mullet, 1990). Recently, it was shown that Arabidopsis VSP2 (AtVSP2) is an anti-insect acid phosphatase (Liu et al., 2005). AtVSP2 significantly delayed development of several insects and increased their mortality. *VSP* gene expression was induced by wounding, water deficit and JA (Mason and Mullet, 1990), and synergistically co-regulated by JA and soluble sugars (Mason et al., 1992). The soybean *VSP* promoter is well characterized: JA-, sugar- and phosphate-response domains are defined (Tang et al., 2001). The *AtVSP* was shown to be regulated similarly to soybean VSP (Berger, et al., 1995). VSP expression is well characterized and suitable for dissection of signal transduction pathways as VSP can be activated by mechanical wounding, insect feeding and JA treatment (Berger et al., 1995: Stotz et al., 2000). Plants with reduced expression of *AtLOX2*,that is involved in the biosynthesis of JA, showed lower accumulation of endogenous JA and lower *AtVSP* expression after wounding (Bell et al., 1995). The *VSP1* promoter was activated constitutively in *cev1*, a constitutive JA-signaling mutant plant (Ellis and Turner, 2001). The induction of *VSP* by JA was at least 4 fold less in *jar1* compared to the induction of *VSP1* in wild type (Staswick et al., 1992). *AtVSP* expression was regulated by *COI1* (Benedetti et al., 1995; Xiao et al., 2004). The knockout mutations in the *AtJIN1* (same as *AtMYC2* gene) have much reduced levels of

expression of *VSP* in response to JA treatment (Boter et al., 2004). These results show *VSP* is the target gene of JA. Though some components of JA signaling were known, still there are missing components in JA signaling (Fig.3-1). Exploiting *VSP* as a target gene of JA is a good way to find JA signaling components.

In this study, *Arabidopsis* transformed with an AtVSP promoter-reporter fusion was used for subsequent mutagenesis and isolation of JA signaling mutants. The reporter gene encodes firefly luciferase (LUC). LUC gene has an advantage as its expression in plants can be measured non-invasively by using a low light imaging system, which can be detected by the thermoelectrically cooled Charge-Coupled Device (CCD) camera (Ishitani et al., 1997). T-DNA insertion was the means for gene knockout or activation mutation, as it provides a straightforward way to clone the identified mutant genes (Jenks and Feldmann, 1996).

The isolation and characterization of mutants that alter the JA signaling pathway would allow a more detailed analysis of signaling events and interaction of the JA pathway with other defense reactions such as those mediated by ethylene and SA. In this study, nine JA under-regulated mutants and six over-regulated mutants are described. Figure 3-2 shows the flow chart of JA-signaling mutant screening.

T0 Plant (Homozygous, single copy *AVP* -*LUC* transformed plant

↓

T1 Plant, selected based on glufosinate (pSupertag transformed on T0)

↓

T2 seeds (next generation of T1)

↓

Screening for jasmonate signal transduction mutants with T2 seedlings

↓

Transfer the seedlings (putative mutants) to soil

↓

PCR detection of the presence of *LUC* and *BAR* in T2 plants

↓

Harvest T3 seeds

↓

Second screening for jasmonate signal transduction mutantwith T3 seedlings

↓

TAIL-PCR with T3 plant genomic DNA to know T-DNA insertion site

↓

Diagnostic PCR with T3 plant gnomic DNA to know
homozygous or heterozygous T-DNA insertion

↓

Genetic analysis:
Backcross of mutant plant (T4 plant) with wild type
F1 generation analysis to know the mutation is dominant or recessive
F2 generation anaysis (co-segregation analysis)

Figure 3-2. Flow chart of JA-signaling mutant screening

3.2. Materials and methods

Seed sterilization and plant growth condition

Seeds were sterilized by soaking in 70% ethanol for 30 sec in 1.5ml microcentifuge tube, treating with 10% bleach (0.6%HOCl) in 0.05% Triton X-100 for 7 min, and then washing six times with sterile distilled water. After 3-day cold treatment, seedlings were germinated in sterile conditions on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 0.8% agar which was supplemented with sucrose. All plants were grown at 25ºC under 16h light/8h dark cycle. The light intensity was 150 μE $s^{-1}m^{-2}$ for Petri dish grown plants and 50 μE $s^{-1}m^{-2}$ for soil grown plants.

T-DNA tagging

To incorporate a reporter gene in the *Arabidopsis* line, the AtVSP promoter-Luciferase (*AVP-LUC*) cassette was ligated into a plant transformation vector (Fig. 3-3). The construct was transformed into *Arabidopsis* ecotype WS4 using *Agrobacterium* (Clough and Bent, 1998). A homozygous plant with single copy of the transgene was used to generate T-DNA insertion mutant lines. The modified activation T-DNA (Supertag (Koiwa et al., 2006)) vector of pSKI015 (Weigel et al., 2000) containing the bar gene as a selection marker was used to generate an insertion mutant population (T1) in the genetic background of *Arabidopsis thaliana* WS4 transformed with *AVP-LUC*.

These T-DNA insertion lines were selected based on glufosinate (BASTA™) herbicide selection. Seeds (T2) were used for mutant screening.

Screening for jasmonate signal transduction mutants

Seeds were sown on agar plates (300 seeds/plate). Seven-day old seedlings were treated with 0.2mM JA for 24hrs, followed by a spraying of 1mM luciferin in 0.01% Triton X-100 water solution. Immediately after the spray, the seedlings were placed in the camera chamber for a fluorescence image, followed by 5 min incubation in the dark before imaging for luciferase expression (luminescence image) with a CCD camera. Seedlings with lower or higher luminescence levels relative to wild type indicate disruption of positive and negative regulators, respectively. Constitutive mutants were excluded from this study as they have already been screened (Ellis and Turner, 2001). All putative mutants were transferred from plates to soil. Fifteen to twenty T3 seeds from each mutant line were re-screened as per above to eliminate false positives.

Genomic DNA preparation

Genomic DNA for *Arabidopsis* was prepared as described (Edwards et al., 1998). One fresh leaf or one seedling was harvested and ground in a 1.5ml microcentrifuge tube, followed by addition of 200µl extraction buffer (200mM Tris-HCl, 250mM NaCl, 25mM EDTA, 0.5% SDS) and further grinding. The pestle was rinsed with 600µl extraction

buffer and the ground tissue was mixed well by inverting the tube several times. The

tissue debris was removed by centrifugation for 10min at 15,000rpm at room temperature

and the supernatant was transferred to a new tube with a equal volume of isopropanol.

After an overnight precipitation at -20°C, the genomic DNA was obtained by

centrifugation for 10 min at 15,000 rpm at room temperature. The DNA pellet was rinsed

with 70% ethanol, dried in the air and dissolved with 100μl distilled water.

PCR detection of presence of LUC and BAR

Insertions of *AVP-LUC* and supertag T-DNA into the *Arabidopsis* genome were

confirmed by PCR (Fig.3-3). Following *LUC* PCR, *BAR* PCR was subsequently

performed in LUC-positive lines. The false positive contaminants (i.e. non-*AVP-LUC*

transgenic lines that were confirmed by *LUC* PCR using AtvP1B and LUCA1 primers

(Table 3-1)) were eliminated. *BAR* PCR using BAR1 and BAR2 primers (Table 3-1) was

carried out to confirm Supertag T-DNA insertion in the plant genome.
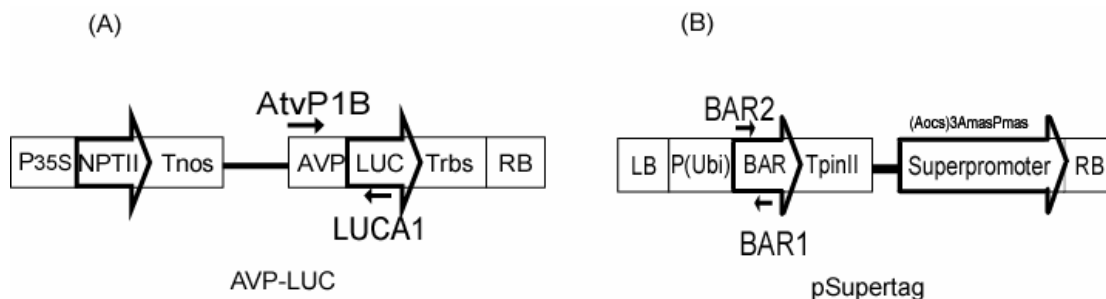
Figure 3-3. Maps of T-DNAs for making mutant library. (A) *AVP-LUC* (*AtVSP* promoter-luciferase) reporter cassette construct for Arabidopsis transformation. LB and RB: left and right border of T-DNA. Ori: Origin of replication. *AVP*: *AtVSP* promoter. *LUC*: firefly luciferase coding region. P35S: CaMV 35S promoter. NPTII: kanamycin resistance marker gene. (B) Supertag T-DNA for insertional mutagenesis: Modified activation T-DNA vector pSKI015 (Weigel et al., 2000) containing bar gene as a selection marker. Super promoter (Aocs)₃AmasPmas: Trimer of the octopine synthase (ocs) activator affixed to the mannopine synthase (mas) activator plus promoter (Ni et al., 1995). Trbs: ribosome terminator. BAR: Basta^R selection marker. P(ubi): Ubiquitin promoter. TpinII: potato protease inhibitor II terminator.

Table 3-1. LUC PCR and BAR PCR primer sequences

PCR	Primer	Sequence	PCR product (bp)
LUC	AtvP1B	5'-GGA TCC CCT AAA ATC AAC ATA AGA ACT CCA AAT TTT AAG C-3'	807
	LUCA1	5'-TTC CAT CCT CTA GAG GAT AGA ATG-3'	
BAR	BAR1	5'-AAA CCC ACG TCA TGC CAG TTC-3'	421
	BAR2	5'-CCA TCG TCA ACC ACT ACA TCG AGA-3'	

TAIL-PCR

To obtain the T-DNA flanking region of genomic DNA, thermal asymmetric

interlaced-PCR (TAIL PCR) was performed with T3 plant genomic DNA. Prior to this,

LUC and *BAR* PCR were performed with the T3 plant to confirm T-DNA was present in

the genomic DNA. Genomic DNA templates obtained as described above were further

extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and washed with 70%

ethanol, air dried and resupended in 100 µl. A nested set of three primers (LB1, LB2,

LB3) (Fig.3- 4) corresponding to sequences flanking the left border of Supertag T-DNA

were used for TAIL PCR. The arbitrary degenerate primers (AD1, AD2) were used for

TAIL-PCR reaction. The primer sequences of TAIL PCR are shown in Table 3-2.
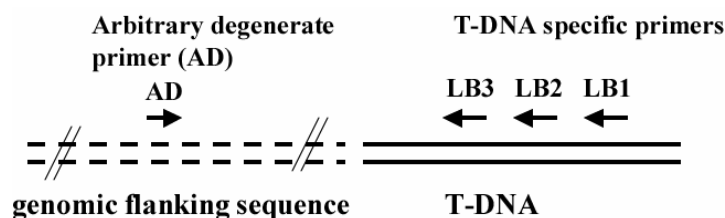
Figure 3-4. Recovery of genomic sequences flanking T-DNA by TAIL-PCR using
three left border primers (LB1, LB2, LB3) and arbitrary degenerate primer (AD).

Table 3-2. Primer sequences for TAIL-PCR

Primer	Sequence
LB1	A$_{(4790*)}$TA CGA CGG ATC GTA ATT TGT C$_{(4769)}$
LB2	T$_{(4741)}$AA TAA CGC TGC GGA CAT CTA C$_{(4720)}$
LB3	T$_{(4670)}$TG ACC ATC ATA CTC ATT GCT G$_{(4649)}$
AD1	WGC NAG TNA GWA NAA G (W=A/T, N=A/T/G/C)
AD2	AWG CAN GNC WGA NAT A (W=A/T, N=A/T/G/C)

*: Base number in pSupertag

Three PCR reactions were carried out sequentially to amplify target sequences using nested T-DNA-specific primers (LB1, LB2, LB3) on one side and an AD primer on the other (Fig.3-4). Genomic DNA (1µl aliquot out of 100µl, approximately 1ng) was used in the primary TAIL-PCR (20µl) using LB1-AD1 or LB1-AD2 primer set, 1µl from 5-fold dilutions of the primary PCR products was applied directly to secondary TAIL-PCR reactions (20µl) using LB2-AD1 or LB2-AD2 primer set. The secondary TAIL-PCR products (1µl) were amplified in 50µl tertiary TAIL-PCR reactions using LB3-AD1 or LB3-AD2 primer set. Table 3-3 shows the cycle setting of TAIL-PCR.

Table 3-3. Cycle setting used for TAIL-PCR

Reaction	Cycle no.	Thermal settings
Primary	1	93°C 1 min; 94°C 1 min 30 sec.
	5	94°C 30 sec; 58°C 1 min; 72°C 2min
	1	94°C 30 sec; 25°C 3 min; ramp to 72°C over 3 min
		Stay at 72°C after ramping for 2 min 30 sec
	15	94°C 10 sec; 64°C 1 min; 72°C 2 min
		94°C 10 sec; 64°C 1 min; 72°C 2 min
		94°C 10 sec; 44°C 1 min; 72°C 2 min
	1	72°C 5 min
Secondary	1	94°C 1 min 30 sec
	15	94°C 10 sec; 64°C 1 min; 72°C 1 min 30 sec
		94°C 10 sec; 64°C 1 min; 72°C 1 min 30 sec
		94°C 10 sec; 44°C 1 min; 72°C 1 min 30 sec
	1	72°C 5 min
Tertiary	1	94°C 1 min 30 sec
	25	94°C 15 sec; 44°C 1 min; 72°C 1 min 30 sec
	1	72°C 5 min

Cloning and sequencing of TAIL-PCR products

The TAIL-PCR products were cloned using a "T-vector" (pCR2.1, invitrogen or home-made T-vector of pBluescript excised at EcoRV site). The nucleotide sequences of the TAIL-PCR product were determined by dideoxy terminator cycle sequencing using the ABI BigDye sequencing kit (PE Biosystems), and analyzed on an ABI Prism 3100 DNA sequencer. Flanking sequences were compared with the Arabidopsis database sequence information (http://signal.salk.edu/cgi-bin/tdnaexpress).

Diagnostic PCR

The visible phenotypes of recessive mutants should be linked to homozygous T-DNA insertions, while the phenotypes of dominant mutants can be linked to homozygous or heterozygous T-DNA insertions. In the cases of recessive mutation and dominant homozygous T-DNA insertion, all of the plants should show mutant phenotype and all of the plants should have homozygous T-DNA insertion genotype. In the cases of dominant heterozygous mutation, T3 generation is expected to show 75% mutant and 25% wild type phenotypes. The genotype of T-DNA should be 1:2:1 ratio (homozygous T-DNA insertion: heterozygous T-DNA insertion: wild type). To verify that the T-DNA insertion was linked to mutant phenotype, diagnostic PCR (Fig. 3-5) was performed with T3 plant seedlings using two-genomic-primer set (LP/ RP) and one genomic primer (LP or RP)-left border primer (LB) set. The primers were designed in such a way that wild type (no insertion) should result in a single product from the LP/RP primer combination, while T-DNA insertion lines should give a band from LB/LP or LB/RP primer sets depending on the T-DNA insertion orientation. The size of PCR-product by LP and RP primers was designed to be 800bp and the PCR product size by T-DNA primer (LB) and genomic primer was about 500bp. PCR reactions using LP and RP on T-DNA insertion lines were not expected to result in any bands due to the large size (9.4 kb) of the T-DNA, and the mere 1 min extension time.
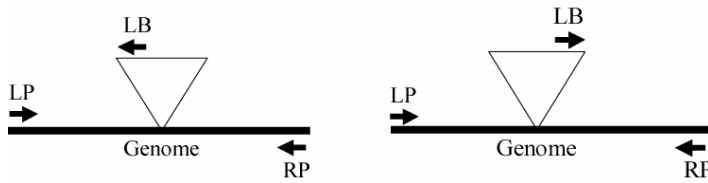
Figure 3-5. T-DNA primer design for diagnostic PCR.
LB: left border primer of T-DNA. LP: left genomic primer. RP: right genomic primer.
For the PCR product size by using the three primers, wild type will get a product of
about 800 bps (from LP to RP), homozygote will get a band of about 500 bps (from
LB to LP or LB to RP depending on T-DNA insertion direction), and heterozygote
will get both bands.

Plants without T-DNA should give only one PCR product from the primer set

LP+RP. Homozygous T-DNA insertion lines will give only one PCR product from the

primer set LB+LP or LB+RP (depending on the insertion direction). Accordingly,

heterozygous lines will produce both bands. The LB3 primer was used as a T-DNA

primer in the diagnostic PCR. Table 3-4 shows the genomic primer sequences for

diagnostic PCR of each putative mutant line.

Table 3-4. Sequence of diagnostic PCR primers

Primer*	Sequence
2R1-LP	CTT CAG ATA TCA GAG GAA GAA CTC AGG
2R1-RP	TTT ATT GAG CAA GCA GAG TTT TTT AAA
4B4-LP	CAG ACT GGT AGA AGA GGA GAG
4B4-RP	TCT ATT AAA CAA CTA CCA TTG AGG
5B29A-LP	TCC CAA TGG AGG AAG AGA AG
5B29A-RP	ATT ACG TGT GGA GTT TTG CTC
5B29-B-LP	AGC ATA TAC AAG TAT GTC GGA AGC
5B29-B-RP	CAA GTA ATA TAG TCC ATG AGG TAG
10B6A-LP	TTT CTT GAT CAA TAA TTC ATA AAG ATA
10B6A-RP	CAT GTG CCT TGT ATT CGA ATA AC
10B6B-LP	ATA TAA ATT GTT CTT CTA ACA AAT CAA TCT
10B6B-RP	TGA AGT TAG GCT TAT TAT CTC TG
20B15-LP	TCA CAC GAC CTA TAT ATT CTA TGC
20B15-RP	ATG ATC CGT ACG ACC GAT ATG
23B13-LP	CAT TAG TGC ATG GTA ATA AGA CAA G
23B13-RP	CCC ATA AAG AAC TTT GAT TTT CTT TGC
45R1-LP	GTG ACG GTT TTG TGT TGA AGT TGA AAA
45R1-RP	TGT TTA CTT CTT GGA ATG GAT GAC AC
49RH-LP	CGA TCC GTT CGA AGG TTT CTT GAC
49RH-RP	AAC CTA ATT TTC AAC CTA TCT TTA TAT TGC GT
49RF-LP	CCA TTT AAA CAA CTT GGG CCA CAA CAA
49RF-RP	TGT TAT CAA CAA GTC TTC TGT CCC CA
82B4A-LP	ACT TAA TTT GGT CTG AAG GTG GAT G
82B4A-RP	AAA GGT ACA TTG AAT CAC GTG GCT

*Primer name (A, B, H, F: See Table 3-8)

Genetic analysis

Backcrossing of homozygous mutants with wild type reveals whether the mutation is dominant or recessive. In the case of recessive gene, backcrossing F1 heterozygous plant should have a wild type phenotype. The backcrossed plant is analyzed by diagnostic PCR to confirm heterozygous T-DNA insertion. Selfing of the backcrossed plant confirms co-segregation. F2 progenies should give rise to 25% mutant phenotype and 75% wild type phenotype. Two thirds of the wild type progenies should be T-DNA heterozygous and one third should contain no T-DNA. Mutant phenotype plant should be T-DNA homogeneous.

In dominant mutations, if a homozygous T-DNA insertion plant is backcrossed with a wild type plant, F1 plants should have the mutant phenotype and be heterozygous for T-DNA insertion. F2 progenies should show 75 % mutant phenotype and 25 % wild type phenotype segregation. One third of mutant progenies should be T-DNA homozygous and two thirds should be T-DNA heterozygous. Wild type phenotype plants should have no T-DNA insertion. If a dominant-heterozygous T-DNA insertion plant is backcrossed with a wild type plant, F1 plants should show segregation: 50% mutant phenotype and 50% wild type phenotype. The mutant phenotype plants should be heterozygous for T-DNA insertion and wild type phenotype plants should have no T-DNA. F2 progenies derived by selfing of heterozygous T-DNA plant (mutant phenotype plant) should show 75% mutant phenotype and 25 % wild type phenotype. Out of the mutant phenotype, the genotype of T-DNA should show a 1:2 ratio (homozygous T-DNA insertion : heterozygous T-DNA insertion).

Mutant characterization

1) Phosphate starvation

To evaluate effects of phosphate starvation on *AtVSP* expression, wild type and mutant seeds were sterilized and plated on a layer of nylon mesh on solid MS media (0.8% agar, 3% sucrose, 1x MS micronutrients, 0.2x macronutrients, 2.5mM MES, pH 5.7). Plating density was 75 seeds per 100x25 mm plate. Five days after plating, the seedlings on the nylon mesh were transferred to the growth medium with 1.25mM KH_2PO_4 or no phosphate (respectively). Samples were collected after 48hr. The response of *AVP-LUC* to phosphate starvation in the putative mutants was evaluated by the measuring the *AVP-LUC* expression level using the CCD camera.

2) Sucrose treatment

To screen for sucrose effect on the *AtVSP* expression, wild type and mutant seeds were sterilized and plated on a layer of nylon mesh on solid MS media (0.8% agar, 1% sucrose, 1x MS media, pH5.7 with KOH). Plating density was 50-100 seeds per 100x25 mm plate. Seven days after plating, the seedlings on the nylon mesh were transferred to the growth medium containing 0, 1, and 4% sucrose respectively. The response of *AVP-LUC* of the putative mutants to various sucrose concentration was tested by the measuring of *AVP-LUC* expression level.

3) Effects of jasmonate and phosphate starvation on root growth

JA inhibited root elongation to some extent (Berger et al., 1996; Staswick et al., 1992). Phosphate starvation also causes primary root growth inhibition (Williamson et al., 2001; Sanchez-Calderon et al., 2005). Seedlings were grown on MS agar plates supplemented with MeJA (3, 6, 9μM respectively) and incubated vertically. Root lengths were determined after an 8-day incubation. The JA-insensitive mutant *jar1*-1 (kindly provided by Dr. Paul E. Staswick) was used as a positive control.

For phosphate starvation treatment, 3-day-old seedlings were transferred to basal medium containing 1x MS micronutrients, 0.2x MS macronutrients without KH_2PO_4, 3% sucrose, 2.5 mM MES, and 1.5% agar. A one-way analysis of variance test was used to analyze the data, with seven to eleven replicates. Scheffe multiple means comparison test (p=0.05) was used for mean separation.

4) Reverse transcriptase (RT)-PCR and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) from control and treated plants, and cDNAs were prepared from 1 μg of total RNA per sample using Superscript II reverse transcriptase (Invitrogen). PCR was carried out by using sense- and antisense-primers of JA-responsive genes (Table 3-5). Gene-specific primers (Table 3-5) were synthesized using the ABI Prism Primer Express software (Applied Biosystems). *Actin1* gene-specific primers (5'-ATGCTGGTATCCATGAAACCACCT-

3', 5'-CCTGTGAACAATCGATGGACCTGA-3') were used to amplify a positive control PCR product.

Table 3-5. RT-PCR primer sequences of JA-responsive genes

Gene	Primer	Sequence	RT-PCR product size (bp)
VSP	Sense	5' GTC GAT GGA TCC ATG AAA ATC CTC TCA CTT TCA C 3'	836
	Anti-sense	5' CGT GCG CTC GAG TTA AGA AGG TAC GTA GTA GAG T 3'	
LOX2	Sense	5' AAG ACG ATG TAA GGT CAC CGC TTC 3'	431
	Anti-sense	5' CAA CGG ACT TGG GGG CCA CCC 3'	
MYC2	Sense	5' GAG CGT GTG ATA CAC GTG CGA 3'	817
	Anti-sense	5' CTT GCT CTG AGC TGT TGT TGC 3'	
bCHI	Sense	5' GAT GGG CTA CAG CAC CAG AC 3'	500
	Anti-sense	5' GTA ACA ATC AAG ATT ACC ACC AGG 3'	
PDF1.2	Sense	5' TCA TGG CTA AGT TTG CTT CC 3'	282
	Anti-sense	5' AAT ACA CAC GAT TTA GCA CC 3'	
THI2.1	Sense	5' GGT CAT GGC ACA AGT TCA AGT A 3'	413
	Anti-sense	5' GGT GGG ACT ACA TAG CTC TTG G 3'	
ERF1	Sense	5' CGG CGG AGA GAG TTC AAG AGT C 3'	234
	Anti-sense	5' TCC CAC TAT TTT CAG AAG AAC CC 3'	
LUC	Sense	5' CTG CCT GCG TCA GAT TCT CG 3'	802
	Anti-sense	5' GAA GTT CAC CGG CGT CAT 3'	

The mRNA levels were compared between treated and control samples by quantitative real-time PCR using the ABI prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reactions (95ºC for 15 sec, 60ºC for 1 min, 47 cycles), following an initial incubation of 50ºC for 2 min and 95ºC for 10 min, were performed with 2x SYBR Green Master Mix (Applied Biosystems). Real time PCR was

performed using *VSP1* and *VSP2* gene-specific primers (Table 3-6). PCR amplification of

18S rRNA was used for normalization of input cDNA between samples. Each PCR

reaction was run in duplicate. Mean induction folds were calculated as $2^{\wedge}(\Delta\ Ct)$.

Standard deviation range of replicate reactions were calculated by the following equation.

Upper error bar = $2^{\wedge}(\ \Delta\ \Delta Ct + s)$

Lower error bar = $2^{\wedge}(\ \Delta\ \Delta Ct - s)$

$\Delta\ \Delta Ct$ = (ΔCt control cDNA) – (ΔCt treatment cDNA)

ΔCt = (mean Ct cDNA test primers) – (mean Ct cDNA ribosomal primers)

$s = \sqrt{((\text{Std dev of Ct test primers}^2) + (\text{Std dev of Ct ribosomal primers})^2)}$

ΔCt control = mean Ct untreated sample – mean Ct untreated ribosomal

ΔCt treatment = mean Ct treated sample – mean Ct treated ribosomal

Table 3-6. Real time PCR primer sequences of *VSP1* and *VSP2* gene-specific primers

Gene	Primer*	Sequence	PCR product size (bp)
VSP1	sense primer	5' TAG CCT TGT GAA GAA AGG GTA CAA C 3'	111
	anti-sense primer	5' AAG TAG AGT GGA TTT GGG AGC TTA AA 3'	
VSP2	sense primer	5' GTT AGG GAC CGG AGC ATC AA 3'	74
	anti-sense primer	5' TCA ATC CCG AGC TCT ATG ATG TT 3'	

* These four primers were also used for Reverse Transcriptase PCR (RT-PCR)

SALK T-DNA insertion lines

SALK line seeds (Alonso et. al., 2003) were obtained from the Arabidopsis

Biological Resource Center (ABRC) at Ohio State University

(http://www.arabidopsis.org/servlets/Order). Using SALK T-DNA insertion lines

enabledus to determine whether the TAIL-PCR identified genes are involved in the

change of *AVP-LUC* expression. The same growth phenotype and VSP expression level

should be observed in the SALK line and the putative T-DNA tagged mutant, as the same

gene (or the region near to it) was disrupted in the T-DNA insertion and the SALK lines.

T-DNA insertion was confirmed by diagnostic PCR. Further, JA signaling mutant

phenotypes should not be recovered after the cross between two mutants (putative mutant

and SALK line). Table 3-7 shows the primer sequences of SALK line diagnostic PCR.

SALK lines which have T-DNA insertion in the genes (At5g15400 and At5g04500

encoding U-box domain-containing protein and glycosyltransferase family protein,

respectively) were ordered in the Arabidopsis Information Resource Center (TAIR) web

site (http://www.arabidopsis.org/servlets/Order). SALK T-DNA verification primer was

designed in the SIGnAL web site (http://signal.salk.edu/tdnaprimers.html).

Table 3-7. Primer sequences of SALK line diagnostic PCR

T-DNA insertion gene	SALK line	Primer	Sequence
23B13 (At5g15400)	SALK_036847	LP	TTC AAA GTC ATG GCA AGC TCA
		RP	GCG AGC TCT GTC TTT CTA TCG CC
	SALK_059513	LP	TGA CGT TGA ATG ATG GGA CGA
		RP	CGA CAT GAA GCT GGC AAA TGA
		RP	CTT CTC TTC GCT TGC TGA CGG
	SALK_054084	LP	CTT TCA TCA ACG GAA CGT CGC
		RP	GGT TGC CAG GAC AAC GCT AAG
20B15 (At5g04500)	SALK_077298	LP	TTG AAG ACT TTT ATT CTG TAA ACG C
		RP	TTT TTC TTC CTC CTC TCG GCG
	SALK_002825	LP	GGG ACA TGG AAG CCA CAC AAG
		RP	GCG GAG TCT AGC TCG GAC AGG

LBa1*: TGG TTC ACG TAG TGG GCC ATC G (Tm= 66°C)
LBb1*: GCG TGG ACC GCT TGC TGC AAC T (Tm= 68°C)
  * left border primers in the T-DNA insertion of SALK

Genetic complementation

Two methods were used to clone the genomic fragment containing the small heat shock protein (At1g53540): (i) ligation of EcoRI-restricted fragment into pENTR2B (Invitrogen), and (ii) recombination of *att*B-flanked PCR product with pDONR221 (Invitrogen).

(i) For the cloning into pENTR2B vector, a 4.2 kb genomic fragment containing the At1g53540 (small heat shock protein) gene in BAC DNA (T3F20, from Arabidopsis Biological Resource Center (www.arabidopsis.org) was amplified using primers

at1g53540-FE (forward primer containing EcoRI restriction site: 5'-GCGCGC<u>GAATTC</u>TAAGCGGTGTATGATTGACGAGTTGGGATG-3') and at1g53540-R (reverse primer: 5'-CTTGCTGCTTACGTTTTCTCATGATGAATTAAA-3') by the PCR enzyme BD Advantage2 (BD Biosciences). PCR conditions were as follows, 95°C for 0.5 min, 68°C for 4 min, for 35 cycles in 50μl volume. The PCR product was cleaned with PCR purification kit (Qiagen) and digested with EcoRI for 4hrs in 30μl volume. The EcoRI digested fragment (3.9-kb) of PCR product was purified from the agarose gel with Qiaquick^{TM} gel extraction kit (Qiagen) and transferred into alkaline phosphatase-treated pENTR2B (Invitrogen). The ligation reaction was carried out using 50ng of pENTR2B and EcoRI fragment for overnight at room temperature. The DNA amount was estimated by comparing band intensity with known amount of size marker (1kb ladder) after electrophoresis through agarose gel. DH10B *E.coli* host cells were used used for transformation of pENTR-2B-small heat shock protein gene construct. Electroporation was used for transformation. Cells were then incubated in 37C for 1hr, and plated on LB plates containing 50μg/ml kanamycin.

(ii) For cloning into the pDONR221 vector, a genomic fragment containing the *sHSP* gene (At1g53540) in BAC DNA (T3F20) was also amplified using primers 49R-attB1(forward primer containing attB1 sequence: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGCGGTGTATGATTGACGAGT TGGGATG-3') and 49R-attB2 (reverse primer containing attB2 sequence: 5'-GGGACCACTTTGTACAAGAAAGCTGGGTCTTGCTGCTTACGTTTTCTCATGAT GAATTA-3') by the PCR enzyme BD Advantage2 (BD Biosciences). The PCR conditions were as described in (i). This 4.2-kb PCR product was transferred into

pDONR221 (Invitrogen) by using BP ClonaseII ^{TM} (Invitrogen). The PCR product was cleaned with PCR purification kit (Qiagen) and ethanol precipitated. The residual ethanol was removed completely by vacuum drying (ethanol inhibits recombination reaction). The DNA pellet was resuspended in TE buffer (pH 8.0). 70ng (25fmole) of purified sHSP fragment and 75ng of pDONR211 vector, estimated by agarose gel, were used for the BP recombination. The BP recombination reaction mixture (5 μl volume) was incubated overnight at room temperature. One μl (out of 5 μl reaction mixture) was transferred to DH10B *E.coli* via electroporation. Cells were then incubated in 37C for 1hr, and plated on LB plates containing 50 μg/ml kanamycin. *ccd*B gene acted as a negative selection marker to ensure recombination.

DNA flanked by recombination sites (att) can be transferred into vectors that contain compatible recombination sites (attB × attP or attL × attR) in a reaction mediated by the GATEWAY™ BP Clonase™ II or LR Clonase™ II Enzyme Mix (Invitrogen) (Fig. 3-6) (Karimi et al., 2002). The BP reaction is a recombination reaction between an attB-flanked PCR product and Donor (pDONR) vector (which has attP sequence). The LR reaction is a recombination reaction between entry vector (which has attL sequence) and a destination (pBSVirHygGW) vector (which has attR sequence).
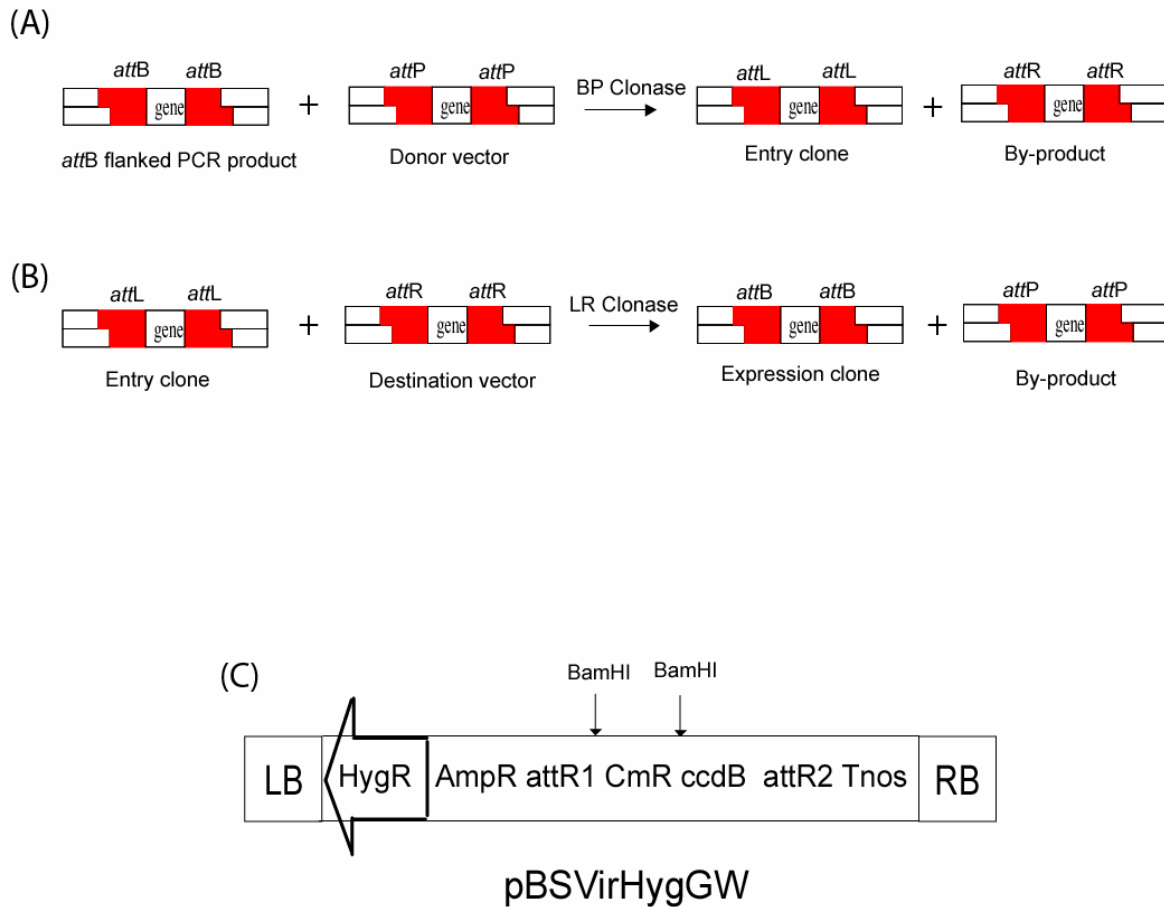
Figure 3-6 Two recombination reactions in gateway technology. (A) BP reaction facilitates recombination of *att*B substrate (*att*B flanked PCR product) with an *att*P substrate (donor vector) to create *att*L containing entry clone. This reaction is catalyzed by BP clonase II. (B) LR reaction facilitates recombination of *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create *att*B containing expression clone. This reaction is catalyzed by LR clonase II. Red region represents *att* sequence. (C) Map of pBSVirHygGW for complementation. HygR: hygromycin resistance gene. Cm R: Chloramphenicol resistance gene. Amp R: Ampicillin resistance gene. attR1, attR2: site for gateway transfer of DNA from entry vector (pENTR2B^{TM}, Invitrogen) or donor vector (pDONR221, Invitrogen). ccdB; gene of bacterial toxin that acts on DNA gyrase. Tnos: Nos terminator. Linearization site with BamHI is showed. Linearization does not disrupt *att*R site or *ccd*B gene.

pBSVirHygGW vector contains the hygromycin resistance gene for the selection on the antibiotic hygromycin and ampicillin marker for transformed *E.coli* selection. The 3.9-kb and 4.2-kb fragments in entry vector were then transferred into pBSVirHygGW vector (Fig. 3-6C) by using LR Clonase^{TM} (Invitrogen) for *Arabidopsis* transformation for complementation analysis of At1g53540. To facilitate exposure of the att sites for recombination, destination vector (pBSVirHygGW) was linearized via BamHI digestion. The restriction reaction was ethanol precipitated, and the DNA pellet was vacuum dried and resuspended in TE buffer (pH 8.0). Seventy-five nanograms of supercoiled Entry clone (which has complementation fragment) and linearized pBSVirHygGW were used for the LR reaction. The LR recombination reaction mixture was incubated overnight at room temperature. The clonase enzymes were then degraded, thus the reaction terminated, by incubation with 0.5μl of Proteinase K for 10 min at 37°C. One μl of LR reaction mixture was used for transformation. Electrocompetent DH10B *E.coli* cells were used as the transformation host and the ampicillin marker and *ccd*B gene were used forselection of transformed cells. Transformants were selected on the ampicillin plate (100 μg/ml ampicillin in LB media).

The binary plasmids were introduced into *Agrobacterium tumefaciens* (GV3101 (pMP90RK) (Koncz and Schell, 1986)). Arabidopsis plants were transformed using a floral dip procedure (Clough and Bent, 1998). *Agrobacterium* was cultured in LB media containing rifampicin (10 mg/l, selects for host chromosome), gentamicin (30 mg/l, selects for helper plasmid), kanamycin (30mg/l, selects for helper plasmid) and carbenicillin (80 mg/l, selects for T-DNA plasmid). GV3101(pMP90RK) was prepared for transformation as follows: Cells from -70°C frozen stocks were streaked on an LB

plate containing appropriate antibiotics (10 µg/ml Rifampicin) and incubated 2 days at 28°C. 5 ml liquid culture was cultured 24 hr. at 28-30°C. 500ml of fresh liquid media were inoculated with 5 ml culture broth and cultured until ABS_{600}=0.5 to 1.0. The cell cultures were cooled on ice for 15-30 min. The cells were precipitated by centrifugation at 5,000 rpm at 4°C for 5 min and resuspended in 100 ml ice-cold 10% glycerol, without vortexing. The cell pellet was obtained by centrifugation at 4°C, and resuspended in 5 ml ice-cold 10% glycerol, without vortexing. The cells were rinsed with 5 ml of 10% glycerol once or twice in 450 ml tubes, and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was removed and equal (pellet) volume of ice-cold 10% glycerol was added. The cell suspensions were dispensed (20 µl per transformation) to chilled 1.5 ml tubes on ice. The cell-containing tubes were frozen at -20°C for 1 hour then transferred to -80°C for storage. Transformation of GV3101 cells protocol is as follows: The competent cells were thawed in hand and mixed gently. Two µl miniprep DNA was mixed with cells, and put in the electroporation cuvettes on ice. . BRL cell porator was used for Agrobacterium transformation. 380kV with 2mm cuvette was used. The setting of the Genepulser was as follows: High Ω, Capacitance: 330, Charge rate: fast, Voltage Booster: 4 kΩ. After pulsing, 1ml LB was gently added. The cells were transferred gently to a glass culture tube, and incubated at 28°C for 2 hours. An aliquot of 50 and 500 µl per plate on selective media (YEP media containing rifampicin (10 mg/l), gentamicin (30 mg/l) and carbenicillin (80 mg/l), and incubated until colonies appeared (3 days).

1ml LB was gently added. The cells were transferred gently to a glass culture tube, and incubated at 28°C with gentle shaking (200 rpm) for 2 hours. An aliquot of 200-350 µl cells were plated per small plate, 300-500 µl per large plate on selective

media (YEP media containing rifampicin (10 mg/l), gentamicin (30 mg/l), and carbenicillin (80 mg/l), and incubated until colonies appeared (3 days). The Plant transformation protocol was as follows: The plants were raised in long day conditions (16hr day time, 8hr dark). The first bolt was cut off. Plants were ready to transform in 4 – 6 days. Optimal plants have many immature flower clusters, but few fertilized siliques. The fresh 10ml liquid culture in YEP (containing gentamicin, rifampicin, and carbenicillin) from an Agrobacterium plate is started early in the day and cultured all day long and overnight at 28°C, 225 rpm. 150ml of fresh liquid media were inoculated with 10 ml culture broth and cultured at 28°C until ABS_{600}=0.6 to 1.6. The cells were harvested by centrifugation at 5,000rpm for 6 min. and resuspended in 5% sucrose solution to a final ABS_{600}=0.8. Silwet L-77 was added to cells immediately before dipping, (final concentration was 0.03%). The flowering plant should be well watered prior to dipping. The above ground parts of the plant were dipped for 3 to 5 seconds with gentle agitation. Under a humidome, the plants were laid on the paper towels to drain excess sucrose solution. The plants are placed in humidome for 16 to 20 hrs. The humidone was cracked for 4 hrs, and then removed. The plants were watered and raised normally.

Bioassay of mutant plants

Mutant plants were subjected to bioassay to measure the resistance toWestern flower thrips, *Frankliniella occidentalis*. Wild type, 49R2, 49R3 and 20B15 plants (3-week-old, 12 plants respectively) were put into the transparent cage (45×35×34 cm) and

were infestated by adult female thrips. The number of thrips was 3 insects per plant at the

first day of infestation, 1 insects per plant at the third day of infestation, 1 insects per

plant at the fifth day of infestation and 3 insects per plant at the 25th day of infestation.

Plants without thrips were used as control to make sure the plant damage in thrips

treatment experiment was from thrips. Damage of plants was observed to measure

susceptibility to thrips. In each day, the damage level (damage level 1 to 5) of plant was

observed. Damage level 1, 2, 3, 4, 5 indicated no damage, less than 50% damage, more

than 50% damage, just before dead and dead (100% damage) respectively. The damage

index $[(\sum \text{damage level} \times \text{number of plants})/6]$ was calculated and compared between

wild type and mutant plant to represent susceptibility to thrips. The damage index were

compared and analyzed by T-test or one-way ANOVA.

3.3. Results

Isolation of mutants

A screen of 224,000 T2 seedlings from 11,200 T-DNA insertion lines, yielded 15

putative mutants, confirmed in the T3 screening. Out of the 15, 9 mutants were under-

regulation mutants and 6 were over-regulation mutants (Fig.3-7; B and R represent

under-regulation and over-regulation of JA-signaling mutant respectively based on *AVP-*

LUC expression level. Fig.3-7 shows the luminescence intensities of T3 seedlings of

putative mutants. All over regulation mutants had no constitutive expression of *AVP-*

LUC (Fig. 3-7).
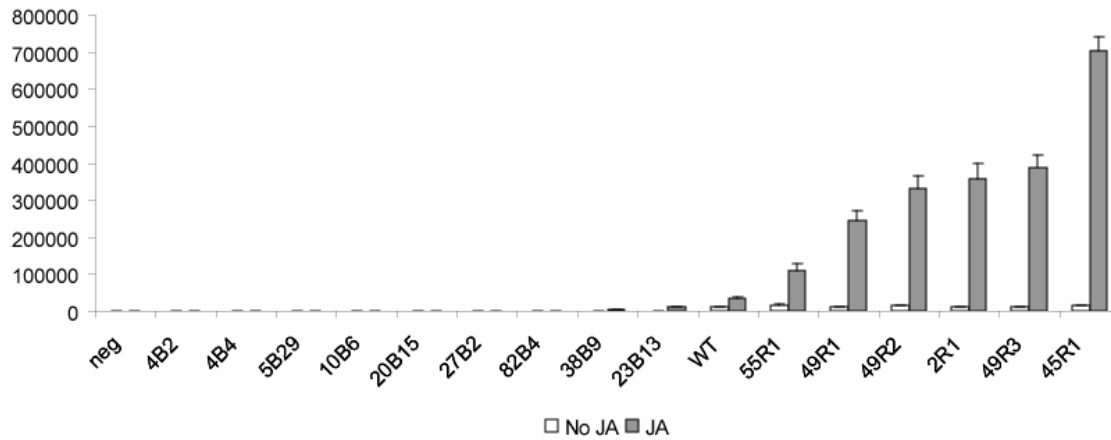
Figure 3-7. Putative JA signaling mutants. Mean value of luminescence intensities of 10 to 15 T3 seedlings after JA treatment were determined by quantitative analysis of the image recorded with CCD camera (JA). Mean value of luminescence intensities of over regulation mutants without JA treatment (No JA). Error bar represents standard error of the mean. Values of y-axis are luminescence intensities.
neg: negative control (no *AVM-LUC*), WT: *AVP-LUC* transformed plant
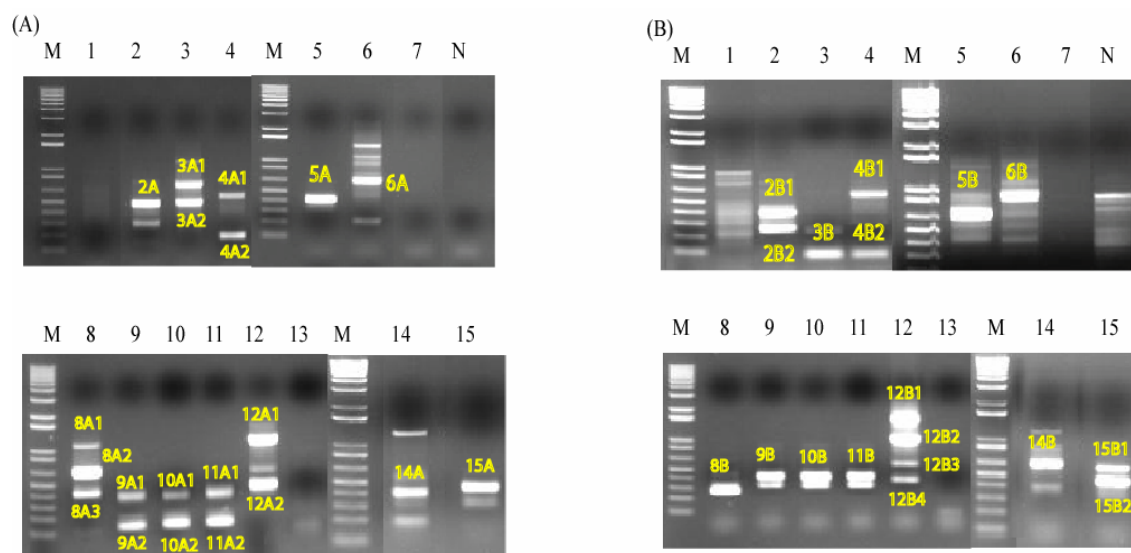
Figure 3-8. TAIL-PCR to obtain flanking region of T-DNA.
(A) Tertiary TAIL-PCR products using LB3 and AD1 primer.
(B) Tertiary TAIL-PCR products using LB3 and AD2 primer.
M: 1kb ladder size marker 1: 4B2, 2: 4B4, 3: 5B29, 4:10B6, 5: 20B15, 6: 23B13, 7: 27B2, 8: 45R1, 9: 49R1, 10:49R2, 11: 49R3, 12: 55R1, 13: 38B9, 14: 2R1, 15: 82B4, N: wild type.

T-DNA insertion (detailed information of T-DNA insertion is in Table 2-9)
4B4: At3g28200 downstream (band 2A);
5B29A: region between At2g45190 and At2g45200 (band 3A1);
5B29B: At4g08420 (band 3A2);
10B6A: At5g05590 (band 4A1);
10B6B: At1g51250 (band 4A2);
20B15: At1g53540 (band 5A, 5B);
23B13: At5g15400 (band 6B);
45R1: At5g41820 (band 8B);
49RH: At1g53540 (band 9A2, 10A2, 11A2);
49RF: At3g49520 (band 9A1, 9B, 10A1, 11A1, 11B);
55R1: T-DNA (band 12A1, 12A2, 12B1, 12B2, 12B3, 12B4);
2R1: At1g77950 (band 14A, 14B);
82B4A:At3g49520 (band 15B1, 15B2).

Identification of flanking sequences of T-DNA Tag

TAIL-PCR was carried out to find the site of T-DNA insertion using LB1, LB2, LB3 primers as T-DNA primers and two random primers (AD1 and AD2). Fig. 3-8 shows the tertiary TAIL-PCR results of putative mutants.

Table 3-8 shows the insertion site of the T-DNA in each putative mutant. All mutant plants were *LUC* positive. 4B2 was *BAR* negative,did not survive in BASTA^{TM} containing media, and had no TAIL-PCR product. 27B2 and 38B9 were *BAR* positive but there was no TAIL-PCR product. Among them, 49R1, 49R2, and 49R3 have the same T-DNA insertion site. 55R1 has two T-DNAs inserted in a row (RB-T-DNA-LBRB-T-DNA-LB) and the TAIL-PCR product was the T-DNA sequence. Thus, the insertion site of T-DNA in 55R1 was not determined. Inverse PCR, a method for the rapid in vitro amplification of DNA sequences that flank a region of known sequence (Ochman et al., 1988) was carried out to determine T-DNA flanking genomic sequence, but was unsuccessful.

Table 3-8. Insertion sites and T-DNA diagnostic PCR results of each putative mutant

Name	*BAR* PCR	T-DNA insertion site (TAIL-PCR)	Diagnostic PCR	Back cross
2R1*	yes	At1g77950 (MADS box family protein) exon	H*	yes
4B2	no	No TAIL-PCR product.		
4B4	yes	No gene involved. At3g28200 (putative peroxidase) downstream	N*	
5B29	yes	5B29A) In between At2g45190 and At2g45200 5B29B) AT4g08420 (mutator-like transposase) exon	N*	
10B6	yes	10B6A) At5g05590 (phosphoribosylanthranilate isomerase) promoter 10B6B) At1g51250 (unknown protein) promoter	N*	
20B15*	yes	At5g04500 (glycosyltransferase) promoter	H*	yes
23B13*	yes	At5g15400 (U-box domain-containing protein) exon	H*	yes
27B2	yes	No TAIL-PCR product.		
38B9	yes	No TAIL-PCR product		
45R1	yes	At5g41820 (geranylgeranyl transferase alpha subunit-related) promoter	N*	
49R1*	yes	49RH) At1g53540 (17.6 kDa class I small heat shock protein) 3'UTR 49RF) At3g49520 (F-box family protein)	H* N*	yes
49R2*	yes	49RH) At1g53540 (17.6 kDa class I small heat shock protein) 3'UTR 49RF) At3g49520 (F-box family protein)	H* N*	yes
49R3*	yes	49RH) At1g53540 (17.6 kDa class I small heat shock protein) 3'UTR 49RF) At3g49520 (F-box family protein)	H* N*	yes
55R1	yes	Two T-DNA insertion	Not determined	
82B4	yes	82B4A) At3g49520 (F-box family protein) exon 82B4B) In between At3g62090 and At3g62100	N*	

* Homozygous insertion lines were subjected to backcross for further analysis
H*: Homozygous T-DNA insertion
N*: Non-homozygous T-DNA insertion

Once positions of the insertions in the genomic DNA had been found, diagnostic

PCR primers were designed to verify T-DNA insertions using genomic sequence

information (http://signal.salk.edu/isect.html#Region). Figure 3-9 shows representative

examples of homozygous or heterozygous T-DNA insertions. Based on diagnostic PCR,

six mutants (2R1, 20B15, 23B13, 49R1, 49R2, 49R3) had homozygous T-DNA

insertions (Table 3-8). These 6 plants were backcrossed with wild type plants and F2

generation plants were analyzed to see the relationship between T-DNA insertion and

mutant phenotype using the *AVP-LUC* reporter gene expression phenotype. All other

putative mutant plants, even though they had non-homozygous T-DNA insertions, had

lower (or higher) *AVP-LUC* expression level homogeneously which implied that they

were putative JA-signaling mutants. Therefore these heterozygous T-DNA insertion

mutants were also studied to characterize JA-related responses such as effect of

phosphate starvation or sucrose on *VSP1* expression, root growth inhibition by JA and

phosphate starvation effect on root growth. Also these phenotypes were used to

characterize the mutants by genetic analysis (backcross and F2 generation analysis).
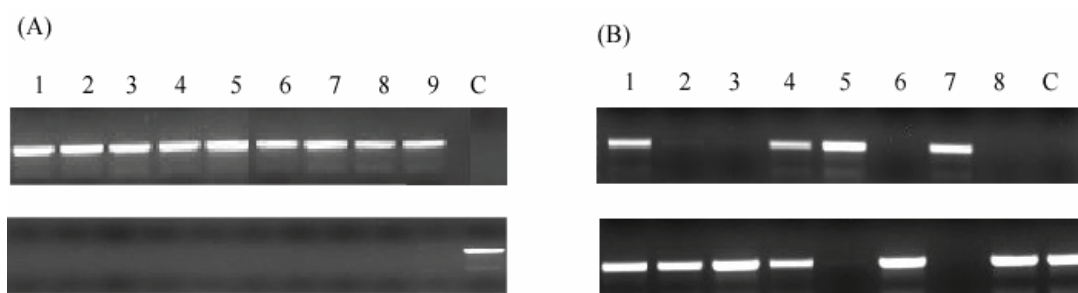
Figure 3-9. Diagnostic PCR. Left border primer and genomic primer (upper panel),
and two genomic primers (lower panel) were used. Representative example of
homozygous T-DNA insertion (A) and non-homozygous T-DNA insertion (B)
1 to 9: diagnostic PCR using genomic DNA from mutant T3 plant. C: diagnostic
PCR using genomic DNA from wild type.

Mutant characterization

1) 20B15

Stunted growth, more branching shoots and low yield was observed in the 20B15 mutant. In young plants (2-week-old seedlings), there was little growth difference compared to wild type, but in mature plants (5-week-old plants), 20B15 showed stunted growth (Fig.3-10). The length of plant, the number of shoots and seed yield of 20B15 were compared with wild type in different growth temperature (30°C, 22°C and 18°C). As expected, 18°C gave best growth in 20B15 (Fig.3-10). The length of matured 20B15 and wild type plants was measured in different growth temperatures and compared with each other. The length of 20B15 in 18°C growth conditions was longer than 22°C and 30°C, and percent length of 20B15 compared to wild type was similar in 18°C, 22°C and 30°C growth conditions (Table 3-9).

Table 3-9. The length of matured plants (6-week-old in 30°C, 7-week-old in 22°C and 8-week old in 18°C) of 20B15 and wild type

Temperature	The length of 20B15 (cm, mean ± standard deviation)	The length of wild type (cm, mean ± standard deviation)	% length of 20B15 compared to wild type
18°C	25.4 ± 2.0	45.2 ± 3.4	59.8%
22°C	19.2 ± 1.2	31.8 ± 2.7	60.3%
30°C	21.4 ± 1.9	38.0 ± 3.4	56.4%

20B15 showed more branching shoots than wild type. This phenotype was clearly observed in 18°C growth conditions (Fig.3-10). In wild type, there was one major shoot and the remaining shoots were much smaller than the major shoot, while 20B15 had a higher number of similar size shoots than wild type (Table 3-10, Fig 3-10).

Table 3-10. The number of shoots in wild type and 20B15 in 18°C growth condition

	20B15 mean ± standard deviation	Wild type mean ± standard deviation
The number of basal shoots (Shoots from base)	5.8 ± 1.1	4 ± 0.71
The number of shoots including basal shoots	7.6 ± 1.1	6 ± 0.71

The length between the nodes of 20B15 was shorter than wild type, and the silique size of 20B15 was smaller than wild type in both 30°C and 18°C growth conditions (Fig.3-11). But the number of siliques was higher in 20B15 than in wild type (Table 3-11). Both 52-day-old plant (siliques starting to mature) and 66-day-old plant (half of siliques matured) of 20B15 had more siliques than wild type. The number of retained siliques of 20B15 was reduced in 2 weeks (from day 52 to 66). The siliques of 20B15 may be weaker than wild type and more siliques of 20B15 than wild type fell down during maturation. After maturation, the seeds were harvested and the weight of seeds from 20B15 and wild type was measured. Although the number of siliques of 20B15 was higher than wild type, the weight of seeds of 20B15 was 2.5 fold lower than wild type

(Table 3-11). This can be explained by that the size of silique in 20B15 is smaller than wild type (Fig. 3-11) thus, the number of seeds per silique in 20B15 is less than wild type.
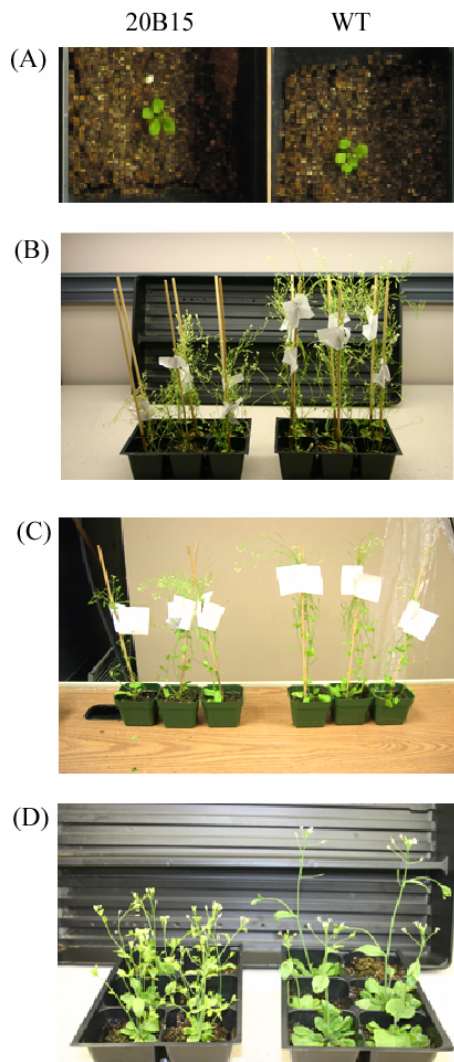
Figure 3-10. 20B15 showed stunted growth phenotype at different growth temperatures. ((B) 30°C, (C) 22°C (D) 18°C), all plants in (B), (C), (D) are 5 week old. At 18°C, the 20B15 showed better growth than at 22°C and 30°C. 2-week-old seedling shows little difference (A). 20B15 had more branching shoots than wild type.

Table 3-11. The yield of 20B15 and wild type in 18°C growth condition

	20B15 mean ± standard deviation	Wild type mean ± standard deviation
The number of siliques per plant (52-day-old)	440 ± 34.8	228.3 ± 26.8
The number of siliques per plant (66-day-old)	426.5 ± 32.5	276.8 ± 24.5
The yield of matured seeds per plant (mg)	56.1 ± 16.6	143.1 ± 47.5
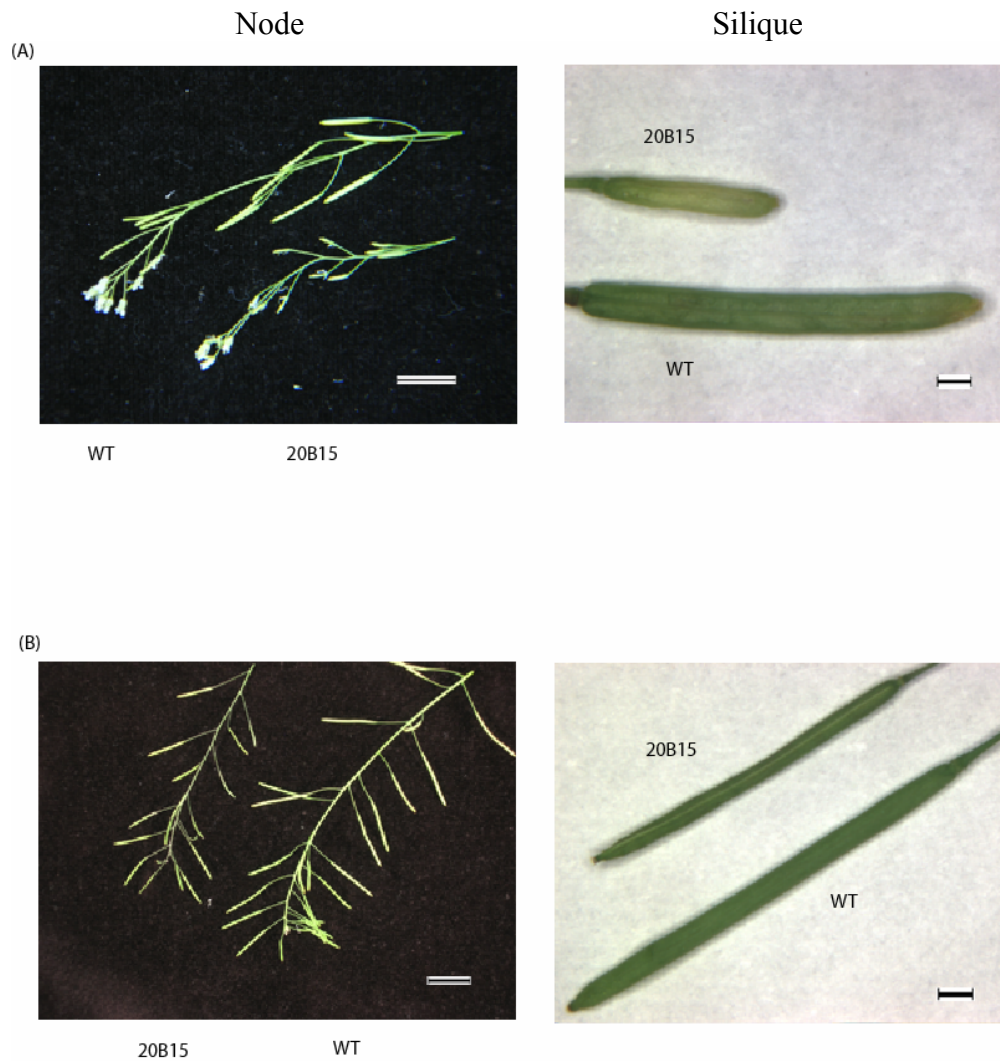
Node   Silique

Figure 3-11. 20B15 showed shorter length between the nodes and smaller silique size than wild type in both 30°C (A) and 18°C (B) growth conditions. The bar in the node picture represents 1cm and the bar in the silique picture represents 1mm.

JA-responsive gene expression in 20B15

To confirm that VSP was down-regulated in 20B15, *VSP* expression level was measured by Northern blot (Fig.3-12 A) using the entire cDNA as the probe. This primer cannot distinguish *VSP1* from *VSP2*; therefore, Northern blotting could not distinguish *VSP1* from *VSP2*. 20B15 showed lower *VSP* expression level than the wild type, also documented in the *AVP-LUC* expression using the CCD camera. VSP transcript abundance was also measured by reverse-transcriptase PCR (RT-PCR) and quantitative real-time (qRT-PCR). For reverse transcriptase analysis, 1μg of total RNA from wild type, and mutant seedlings was used as a template for reverse transcription. *AtACTIN1* gene specific primers were used to amplify a positive control PCR product. *Actin1* was expressed evenly in all cDNA samples, which means the same amount of cDNA in RT-PCR reaction (Fig.3-12 D upper panel). To show there is no contamination of genomic DNA in RNA preparation, RT-PCR using the template without reverse transcriptase was carried out. Fig.3-12 D lower panel showed there is no genomic DNA contamination in RNA preparation. To distinguish between *VSP1* and *VSP2*, gene specific primers for each gene were synthesized (Berger et al., 2002).
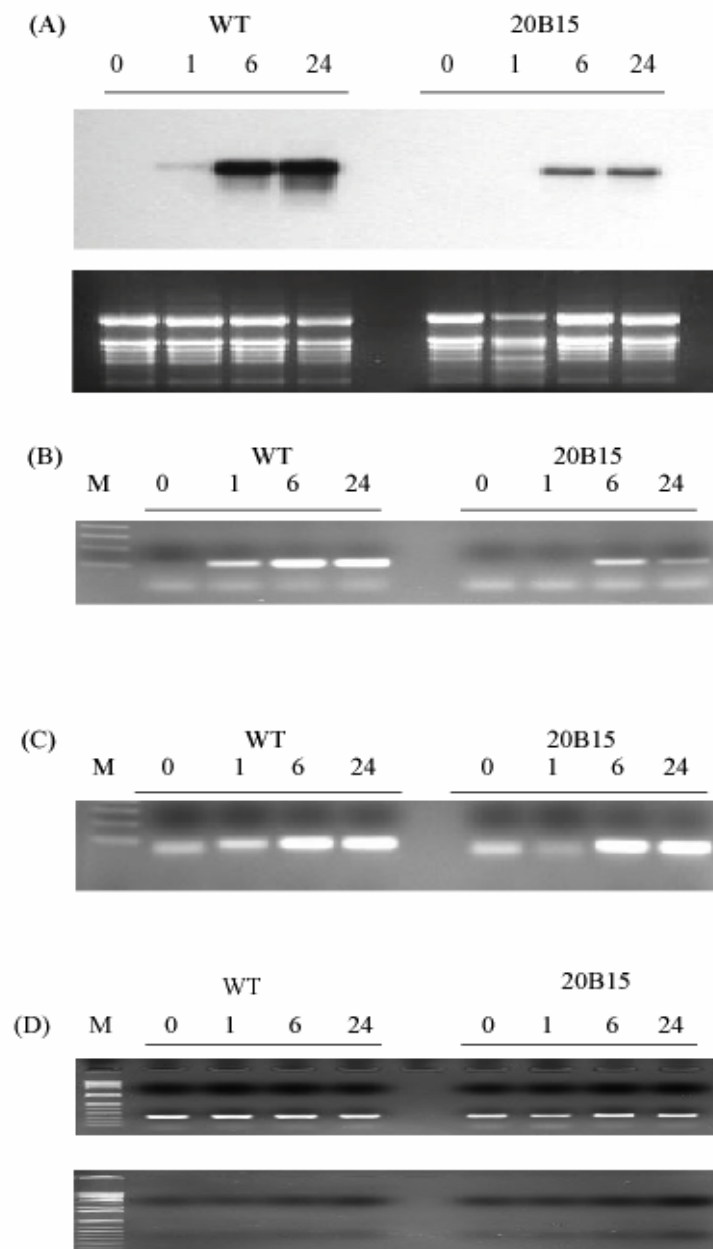
Figure 3-12. Low *VSP1* expression in 20B15. Northern Blot of *VSP* in wild type (WT) and putative JA-signaling mutant (20B15) using full length *VSP* probe(A). Lower panel of (A) shows total RNA used for Northern blot. RT-PCR analysis using *VSP1*-specific (B), and *VSP2*-specific (C) primers in wild type (WT) and 20B15. RT-PCR analysis using *Actin1*-specific primers in wild type (WT) and 20B15 using cDNA template (D upper panel), RNA template (No reverse transcriptase) (D lower panel). Numbers above lanes indicate time (hr) after JA treatment was initiated.

VSP1 and *VSP2* expression levels in 20B15 were analyzed by qRT-PCR (Fig.3-13). 20B15 showed lower VSP1 expression level compared with wild type, corresponding with *AVP-LUC* expression in the CCD camera analysis.

VSP1 expression level of 20B15 was 7.3-fold lower than wild type controls (untreated) and 100-fold lower than wild type when treated with JA when analyzed by qRT-PCR analysis (Fig.3-13A). *VSP2* expression of 20B15 was similar to that of the wild type. The SALK_002825 line, in which T-DNA insertion occurred in the exon region of At5g04500 (encoding glycosyl transferase family protein), was ordered to confirm involvement of the genes in JA-signaling and *VSP* expression. Homozygous T-DNA insertion in SALK_002825 was confirmed by diagnostic PCR. *VSP1* expression level in JA treatment of SALK_002825 was similar to the wild type, indicating that At5g04500 is not involved in *VSP1* induction. But there was a difference of *VSP1* expression in the absence of JA treatment; *VSP1* expression level in SALK_002825 was 5.8 fold higher than the wild type. Comparing *VSP1* induction by JA, SALK_002825 was similar to 20B15, in both cases VSP1 induction by JA was much lower than that in WT (Fig.3-13B). It could be thought that At5g04500 is involved in the regulation of *VSP1* induction. In the case of *VSP2* expression, wild type, 20B15 and SALK_002825 showed similar induction levels and induction fold, meaning *VSP2* was not affected in 20B15 and At5g04500 does not affect *VSP2* expression.
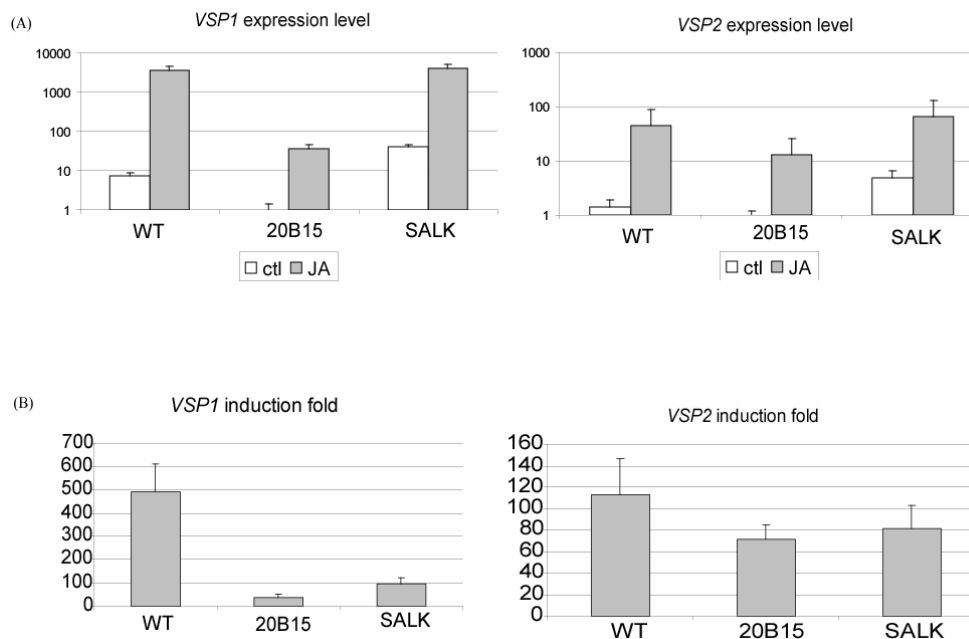
Figure 3-13. Low *VSP1* expression in 20B15 and low *VSP1* induction by JA in 20B15 and SALK (SALK_002825) by real time PCR analysis using *VSP1* and *VSP2*-specific primers. Relative transcript level (A), and induction fold (B) of wild type, SALK_002825 (T-DNA insertion line in the same gene as in 20B15) and putative JA-signaling mutant (20B15). Y-axis in (A) is logarithmic scale. 7-day-old seedlings were treated with 0.2mM JA for 6 hrs (JA) and compared with untreated seedlings (ctl). Error bar represents standard deviation.

To confirm that the mutant plant is a JA-signaling mutant, and to discover mutant phenotypes besides *VSP* expression, another JA-responsive gene characterization study was performed. In addition to *VSP*, the differential expressions of other JA-responsive genes can be the measured in JA-signaling mutants. *LOX2*, *PDF2.1*, *THI1.2* and *MYC2* genes were selected, as these are well-known JA-responsive genes (Tiryaki and Staswick, 2002; Jensen et al., 2002; Epple et al., 1995; Lorenzo et al., 2004). *LOX2* is involved in

JA biosynthesis, and *PDF2.1*, *THI1.2* are JA-inducible defense genes. *MYC2* is a helix-loop-helix-leucine zipper-type transcription factor that regulates the JA and ethylene signaling pathway (Lorenzo et al., 2004). By measuring the expression level of these JA-responsive genes, the gene(s) affected in the mutant plant may be found.

To measure the expression levels of the JA responsive genes (*LOX2*, *PDF2.1*, *THI1.2* and *MYC2*) as well as *VSP*, RT-PCR analysis of each JA responsive gene were performed (Fig.3-14). Table 3-7 shows the RT-PCR primer sequence of JA-responsive genes. Under JA treatment, *LOX2* expression was similar in 20B15 and wild type. 20B15 showed lower *VSP* expression (Fig.3-12, 3-13), but this occurred only in *VSP1*, not *VSP2*. *Thi2.1* induction level was also lower in 20B15 than wild type. *VSP* and *Thi2.1* were induced by *MYC2* (Lorenzo et al., 2004; Fig.3-15). *MYC2* expression level was same in WT and 20B15.

PDF1.2, *VSP* and *Thi2.1* are regulated by *MYC2* and *ERF1*. *PDF1.2* is induced by *ERF1* and repressed by *MYC2*. *VSP* and *Thi2.1* are induced by *MYC2* and repressed by *ERF1* (Fig.3-15). *PDF1.2* induction level was higher in 20B15 than wild type. On the contrary, *VSP* and *Thi2.1* expression were lower in 20B15 than wild type. Considering *MYC2* expression level is similar in 20B15 and wild type in 6hr and 24hr after JA treatment, *ERF1* could regulate the expression level of these three genes (Fig.3-15). Based on the results of *VSP* and since *Thi2.1* level is down-regulated and *PDF2.1* is up-regulated in 20B15, *ERF1* expression level could be higher in 20B15 than in wild type.
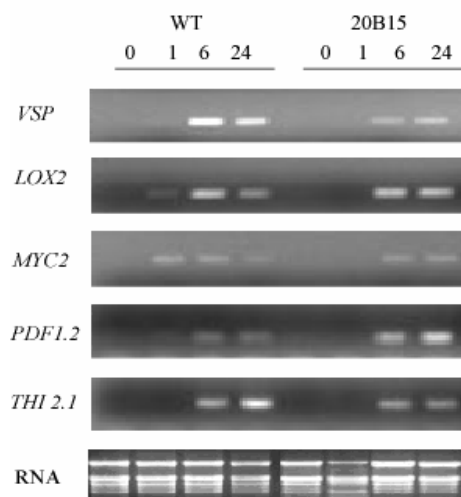
Figure 3-14. Low expression of *VSP* and *Thi2.1* and high expression of *PDF1.2* in 20B15. RT-PCR analysis using *VSP*, *LOX2*, *MYC2*, *PDF1.2*, and *Thi2.1* -specific primers in wild type (WT) and 20B15. Numbers above lanes indicate time (hr) after JA treatment was initiated.
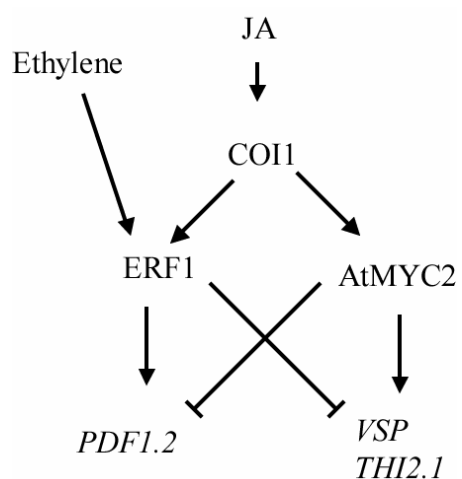
Figure 3-15. Schematic representation of ERF1- and AtMYC2-dependent activation of JA-responsive genes. *VSP* and *THI2.1* expression is induced by AtMYC2 and repressed by ERF1 (Lorenzo et al., 2004).

Genetic analysis of 20B15

When backcrossed to wild type, F1 showed a partial dominant phenotype; higher luminescence than 20B15, but 4.5-fold lower than wild type (Fig.3-16). This result shows that 20B15 has incomplete dominance. The mutant F2 generation were analyzed by diagnostic PCR. Out of 194 F2 plants, 27 plants showed wild type luminescence, 63 plants showed mutant phenotype (no luminescence) and 70 plants showed medium luminescence (less luminescence than wild type but higher luminescence than mutant). In 46 low luminescence mutant phenotype plants, 11 plants were homozygous for T-DNA insertions, 18 plants were heterozygous for T-DNA insertions, 14 plants had no T-DNA insertions and 3 plants had no PCR products. This means low luminescence mutant phenotype is not linked to T-DNA insertion. Out of 46 low luminescence mutant phenotype plants, only 20 plants showed a stunted growth phenotype. 26 plants showed wild type growth pattern. Out of 20 stunted growth plants, 5 plants showed the same growth pattern as 20B15 (dwarf level 1), 8 plants (dwarf level 2) were larger than dwarf level 1 but smaller than 7 dwarf plants (dwarf level 3). This also means the stunted growth mutant phenotype is not linked T-DNA insertion. To know the number of T-DNA insertion in one genome of 20B15, segregation analysis in the BASTA^{TM} media was carried out using F2 generation seedlings of backcross. Out of 428 seedlings, 328 seedlings were BASTA^{TM} sensitive (wild type) and 107 seedlings were resistant to BASTA^{TM} (3:1 (wild type:T-DNA transformed) ratio, χ^2 value=1.44) . This result showed that 1 copy of T-DNA was inserted in the 20B15 plant.
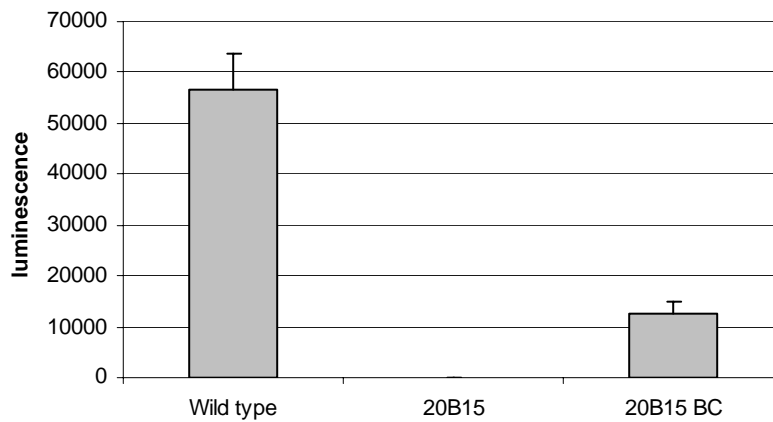
Figure 3-16. 20B15 shows intermediate phenotype in backcrossed plants. The luminescence intensities of 15 backcross lines showed the luminescence was lower than wild type and higher than 20B15 mutant.

Effect of phosphate starvation or sucrose on VSP1 expression in 20B15

Phosphate and sugar are involved in VSP expression. In research on soybean, VSP expression is shown to be regulated by phosphate and sugar (Sadka et al., 1994). Soybean has two VSP genes: VSPA and VSPB. VSP mRNA levels are induced by soluble sugars, which act in a synergistic manner with JA (Mason et al., 1992). The sugar-mediated activation of VSPB expression was inhibited by phosphate (Sadka et al., 1994). Promoter studies demonstrated that VSPB promoter sequences between −536 and −484 were identified as important for phosphate responses, whereas the region between −486 and −427 mediated sugar responses (Tang et al., 2001). In Arabidopsis, AtVSP was also regulated similarly by sugars and phosphate (Berger et al., 1995). Therefore, it is

worthwhile to study the effect of phosphate and sugar on VSP expression of putative JA-

signaling mutants.
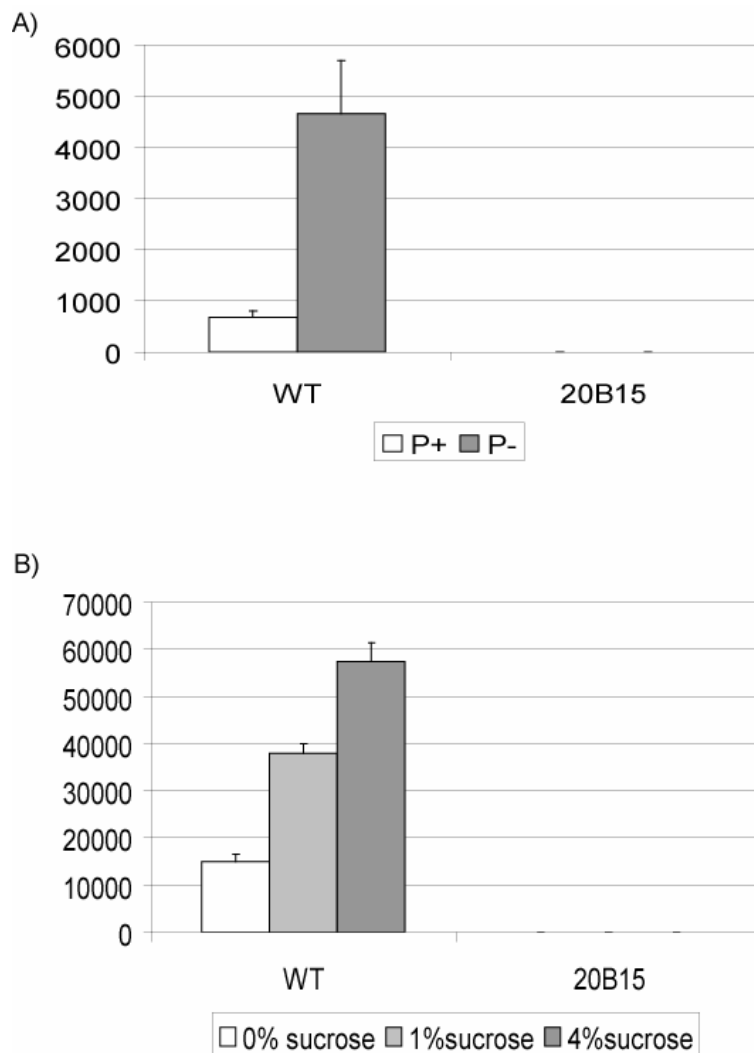
Figure 3-17. Phosphate starvation and sucrose concentration effect on *AVP-LUC* expression in 20B15. *AVP-LUC* was induced by phosphate starvation in wild type but not expressed in 20B15 (A). *AVP-LUC* was induced by increasing sucrose concentration in wild type but not expressed in 20B15 (B). Values of y-axis are luminescence intensities. Error bar represents standard error.

In this experiment, *AVP-LUC* expression was induced by phosphate starvation in wild type as shown in soybean VSP. In 20B15 mutant, the *AVP-LUC* expression level was not expressed by the phosphate starvation and in all sugar concentrations (0 to 4% sucrose).

Root growth inhibition by jasmonate in 20B15

JA induces inhibition of root growth (Staswick et al., 1992, Berger et al., 1996). We investigated whether root growth inhibition by JA was affected in 20B15 plants. Seeds of 20B15 mutant and wild type plants were germinated on JA plates, and root length was measured after 8 day growth. *jar1* which is known as JA-insensitive was used as a positive control. Similar root growth was observed for wild type and the mutants. Therefore, it is thought that root growth inhibition by JA in 20B15 was not affected.

20B15 showed less sensitivity to phosphate starvation in root growth

Phosphate starvation causes primary root growth inhibition. Plants have evolved developmental and biochemical adaptations to low and unevenly distributed phosphate supply (Raghothama, 1999; Abel et al., 2002; Rausch and Bucher, 2002; Vance et al., 2003). Developmental responses mostly involve changes in root architecture that enhance the root surface/soil volume ratio and, consequently, the ability of the plant to access soil phosphate. These include increases in the root-to-shoot growth ratio, in the number of lateral roots and in the number and length of root hairs (Bates and Lynch, 1996; López-Bucio et al., 2002; Ma et al., 2001; Williamson et al., 2001). In phosphate starvation

conditions, normal primary root growth is inhibited (Lenin et al., 2005; Williamson et al., 2001). Considering the presence of a phosphate responsive element in VSP promoter in soybean (Tang et al., 2001) and phosphate starvation response of VSP in Arabidopsis thaliana (Berger et al., 1995), the phosphate starvation effect on root growth in putative mutants was tested. 20B15 showed less sensitivity to phosphate starvation in root growth. (Fig.3-18).
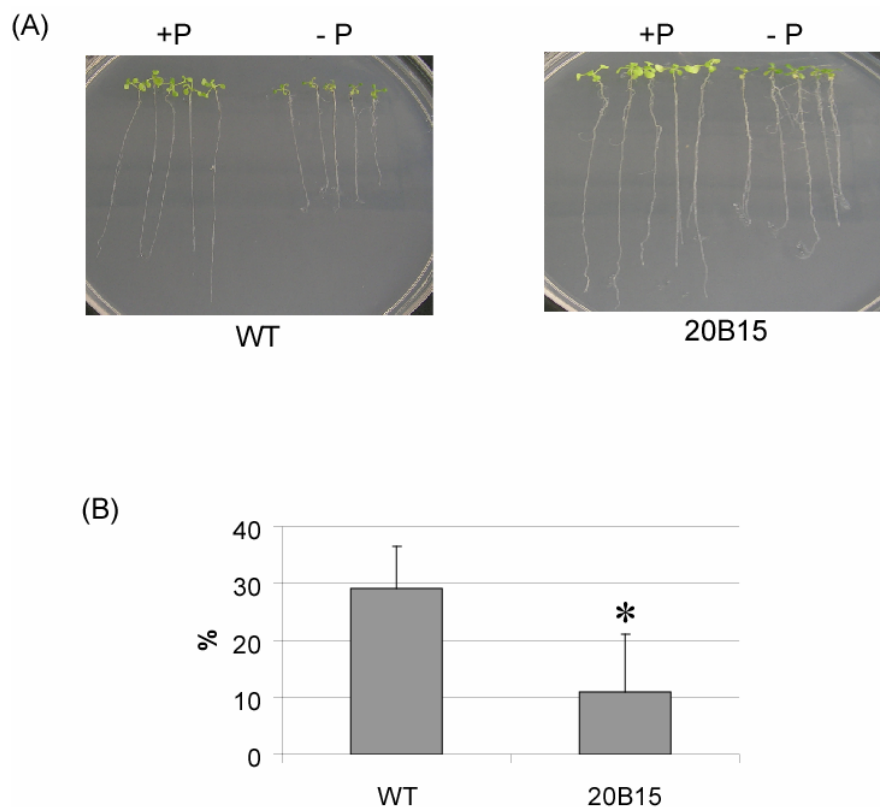
Figure 3-18. Root length growth inhibition by phosphate starvation. (A) Picture of wild type (WT) and 20B15 grown on MS media free of phosphate (-P) comparing the root length of seedlings grown on MS media containing phosphate (1.25mM, +P) are shown. (B) Percent reduction of root length of indicated seedlings by phosphate starvation. The effect of phosphate starvation on root growth was analyzed by one-way ANOVA and Scheffe multiple means comparisons tests. Error bars indicated standard deviation. Asterisk shows the significantly difference from WT at p=0.05. 20B15 showed more sensitive than wild type to phosphate starvation in root growth.

20B15 is more susceptible to thrips feeding than wild type

Since 20B15 is an under-regulation JA signaling mutant, I suspected 20B15 would be more susceptible to thrips. To determine whether 20B15 has more susceptibility when infested by thrips, thrips were treated to the 20B15 and wild type. Thrips feed inside developing flower buds and in newly expanding leaves of plants and is one of the most common greenhouse problems (Bush et al, 2006). Assessments of damage index of damaged plants suggest that 20B15 is more susceptible to thrips than wild type (Fig. 3-19). There was a significant different susceptibility from day 13 to day 20 and day 28. LSD analysis indicated there were significant differences between wild type and 20B15 (Fig. 3-19B). On day 13, the damage index of 20B15 was 4.08 (damage index 4 is just before dead) and the damage index of wild type was 3.25 (damage index 3 is more than 50% damage). On day 20, the damage index of wild type was 4.08, therefore 20B15 was dead about 7 day earlier than wild type. More damaging and earlier dead in 20B15 indicated the mutation in 20B15 makes plant more susceptible to insects and dead early. These effects are likely due to the under-regulation of JA signaling, which leads to lower production of defense protein such as PDF1.2 and THI 2.1 as well as VSP1.

(A)

(B)

day	Probability
4	0.698
6	0.152
8	0.328
11	0.075
13	0.023*
14	0.041*
15	0.025*
16	0.029*
17	0.019*
18	0.006*
19	0.006*
20	0.037*
22	0.054
24	0.054
26	0.054
28	0.008*
30	0.187
32	0.151

(C)
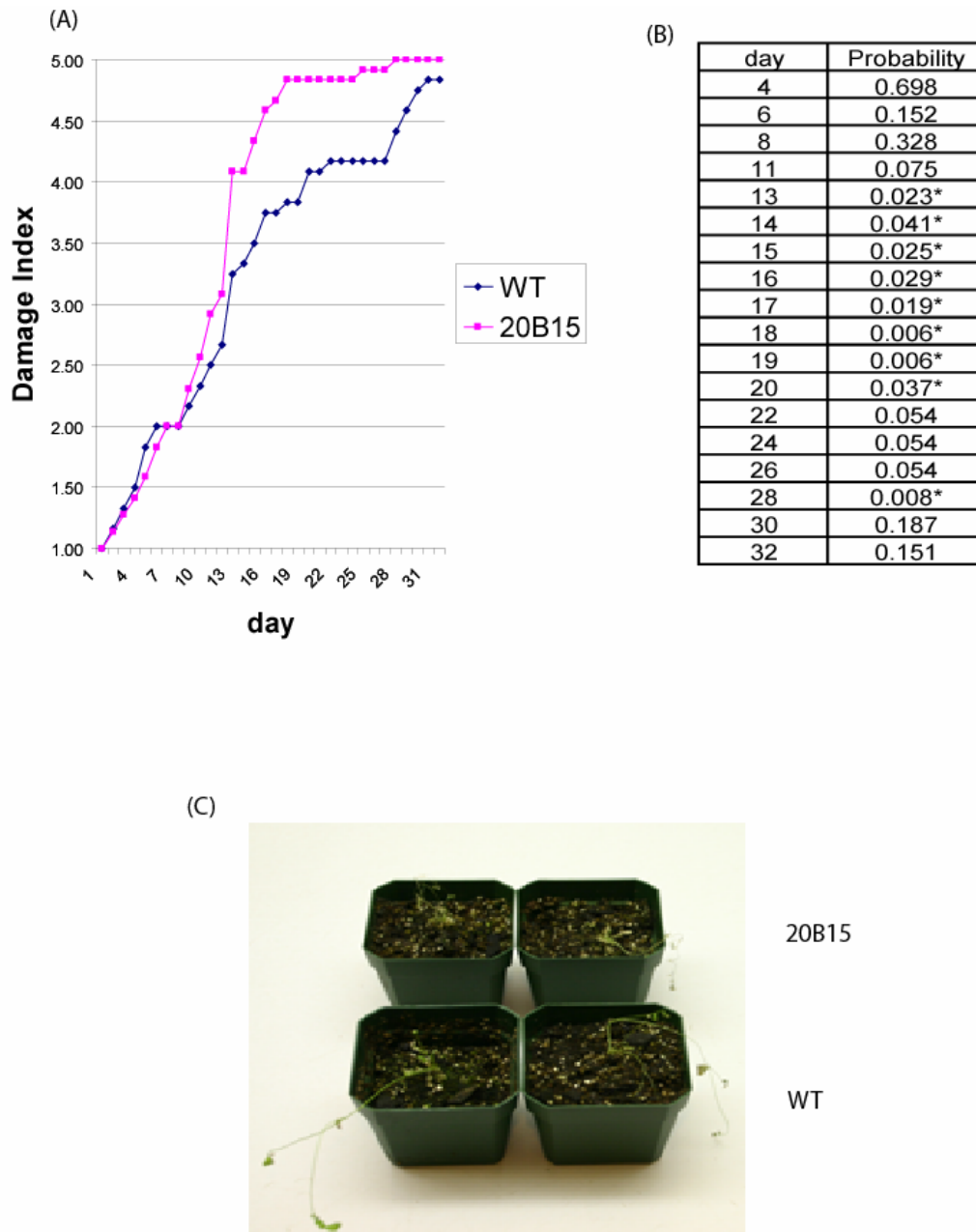
Figure 3-19. 20B15 shows more susceptibility to thrips than wild type (WT). (A) Damage index versus time (day) plot. (B) A probability of 0.05 was used to determine statistical significance. The effect of thrips on plant damage was analyzed by T-test. Asterisk (*) shows the significant difference from WT at p=0.05. (C) Picture of damaged plants (wild type and 20B15) by thrips for15 days. 20B15 has more damage than wild type.

1) 49R mutant (49R1, 49R2 and 49R3)

49R1, 49R2 and 49R3 have same T-DNA insertions in At1g53540 and the mutant

phenotypes were similar. Among these, 49R2 and 49R3 were backcrossed with wild type

and further studied.

Phenotype of mutant: higher expression of AVP-LUC

49R2 and 49R3 showed about 10-fold higher *AVP-LUC* expression level than

wild type when JA treated (Fig.3-20), and were homozygous for T-DNA insertion. TAIL-

PCR revealed the same T-DNA insertion site (exon region of At1g53540, annotated as

class I small heat shock protein).
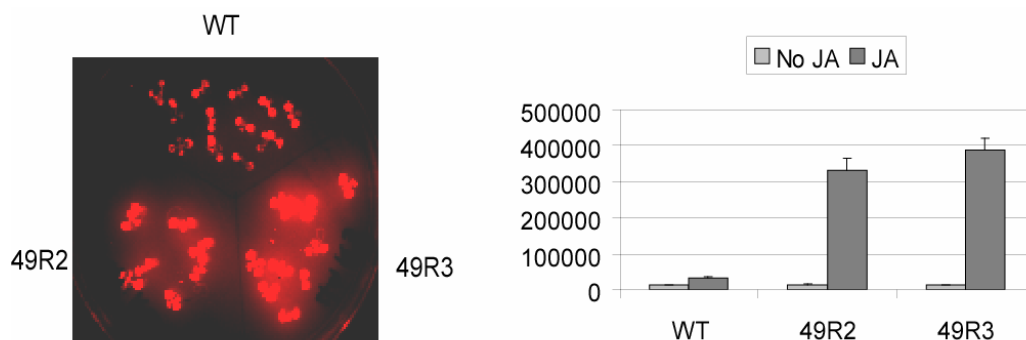
Figure 3-20. 49R2 and 49R3 shows10 fold higher luminescence than wild type.
Mean value of luminescence intensities of 10 to 15 T3 seedlings after JA
treatment were determined by quantitative analysis of the image recorded with
CCD camera with JA and without JA (No JA) treatment. Error bar represents
standard error of the mean. Values of y-axis are luminescence intensities.

Genetic analysis

In 49R2, out of 144 F2 plants, luminescence level of 26 plants was similar to wild type, 118 plants showed mutant phenotype (3:1 (wild type:T-DNA transformed) ratio, χ^2 value=3.7). In 49R3, out of 143 F2 plants, luminescence level of 28 plants was similar to wild type, 115 plants showed mutant phenotype (3:1 (wild type:T-DNA transformed) ratio, χ^2 value=2.24). Among the mutant plants, all plants were homozygous for T-DNA insertion except one of F2 plants of 49R2 backcross line. This result suggests that the mutant phenotype is linked to T-DNA insertion.

Diagnostic PCR of T3 genomic DNA showed homozygous T-DNA insertion in At1g53540. Both LB+LP and LB+RP produced homozygous T-DNA bands. This implies two or more T-DNAs are inserted in tandem repeat manner. The sequences of LB+LP and LB+RP diagnostic PCR products were determined using the ABI BigDye sequencing kit (PE Biosystems), and analyzed on an ABI Prism 3100 DNA sequencer. The diagnostic PCR products were parts of At1g53540. This result confirms T-DNA insertion was occurred in 3'UTR region of At1g53540 and also gives another supporting data about tandem T-DNA insertion (Fig.3-21). Fig.3-22 shows the detailed sequence information of T-DNA insertion of 49R2 and 49R3 mutant. The 49R mutant contains a 3-bp insertion of unknown origin between the T-DNA left border (LB) and genomic sequence out of T-DNA 5' end, and a 32-bp insertion of unknown origin between the T-DNA LB and genomic sequence out of T-DNA 3' end. This phenomena was also observed in other's work (Koiwa et al., 2002).
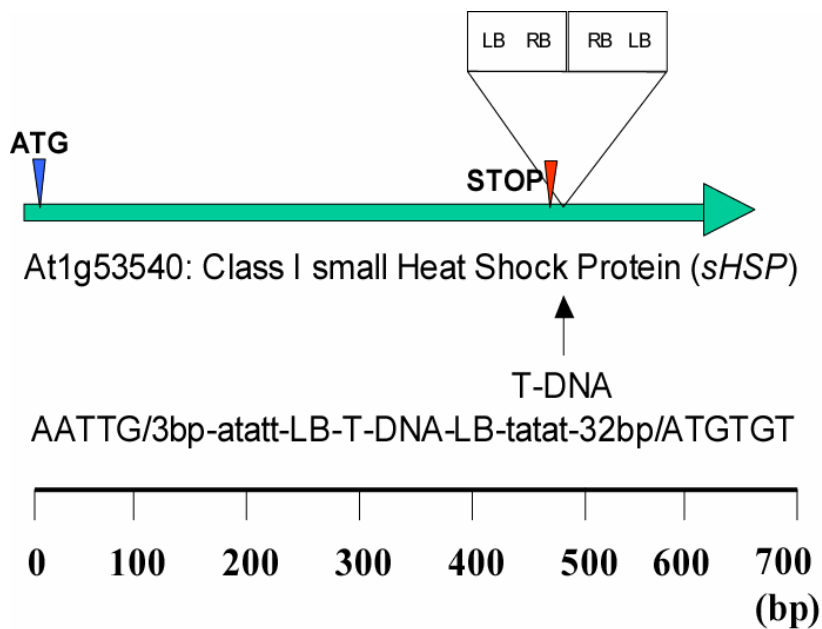
Figure 3-21. Scheme illustrates the location of T-DNA insertion in 49R1,R2, and R3. T-DNA insertion is indicated by open triangle. Sequences at the T-DNA left border (lowercase)-genome (uppercase) junctions were determined by TAIL-PCR analysis. The mutant contains a 3-bp insertion of unknown origin between the T-DNA left border (LB) and genomic sequence out of T-DNA 5' end, and a 32-bp insertion of unknown origin between the T-DNA LB and genomic sequence out of T-DNA 3' end. At1g53540 has one exon (green arrow). The length of exon is 646bp (48bp of 5'UTR, 474bp of ORF and 124bp of 3'UTR).

AAAACAGAGCAAACGCAAAGAGTAGCAATCACAAGAGGAA

GTGAAACGATGTCTCTAATTCCAAGCATCTTCGGAGGAAG

AAGAACAAACGTTTTCGATCCGTTCTCGCTGGATGTTTTC

GATCCGTTCGAAGGTTTCTTGACGCCGTCAGGATTGGCAA

ACGCACCCGCTATGGATGTGGCAGCGTTCACAAACGCTAA

AGTGGATTGGAGGGAGACACCTGAGGCGCACGTGTTCAAG

GCGGATTTACCGGGACTGAGGAAGGAAGAGGTGAAAGTGG

AGGTTGAGGATGGCAACATACTTCAGATAAGTGGAGAGAG

GAGCAATGAGAATGAAGAGAAGAATGACAAGTGGCACCGT

GTGGAGCGGTCGAGTGGGAAGTTCACGAGGAGGTTTAGGC

TGCCGGAGAATGCTAAGATGGAGGAAATCAAAGCCAGTAT

GGAAAATGGTGTGTTGTCGGTTACCGTGCCCAAGGTGCCA

GAGAAAAAGCCAGAGGTTAAGTCCATTGATATCTCTGGTT

AAAGGGAATTG-3bases unrelated sequence-LB-T-DNA –

TTTAAGACCAAAA-T-DNA –LB-ATGTGTGATGGTAGTGA

AATAATTGGTGTGTGATGTGTTTTTCTTGTAATCATGAAT

CTCGTTGTATCATTATCATAAAATCTTAATAAGAGTGTGC

TTTGT

Figure 3-22. cDNA sequence of At1g53540 and detailed sequence information of 49R1,R2, and R3 mutant. At least two T-DNAs were inserted in the 3'UTR region of At1g53540 in tandem manner. START and STOP codon are underlined. UTR sequences are red. LB: left border of T-DNA. The DNA sequence information is from TAIR web site (http://www.arabidopsis.org).

LUC transcript level was measured by RT-PCR using luciferase gene specific primers (LUC5': 5'-CTGCCTGCGTCAGATTCTCG-3', LUC3': 5'-GAAGTTCACCGGCGTCAT-3') (Fig. 3-23). *LUC* transcript level of 49R2 and 49R3 was higher than wild type. This result shows the consistency between luminescence intensity in CCD camera analysis and luciferase gene expression level in the plants.

VSP1 transcript level was also measured by RT-PCR using *VSP1* gene specific primers (Fig.3-23). However, no difference was detected between wild type and 49R mutants. The discrepancy (*VSP1* vs *AVP-LUC*) can be explained by mRNA stability. If the stabilities of the endogenous *VSP1* in 49R2 and 49R3 are lower than wild type, *VSP1* expression level can be lowered in this plant. The stability of translation products also can affect this difference. Western blot analysis using antibodies of VSP1 after JA treatment can show the stabilities of these two proteins. Another possible reason for the difference is the positional effect of the reporter gene. The environment of a reporter gene can affect its expression level, but it is difficult to describe the exact mechanism of the regulation of this environmental effect without knowing the exact environment of the insertion site. Open/active or rigid/inactive nucleosome structures can affect gene expression level. In addition, some other modulation system, such as variation in the characteristics of nuclear matrix attachment, would be involved in the gene expression (Kato et al., 2000).
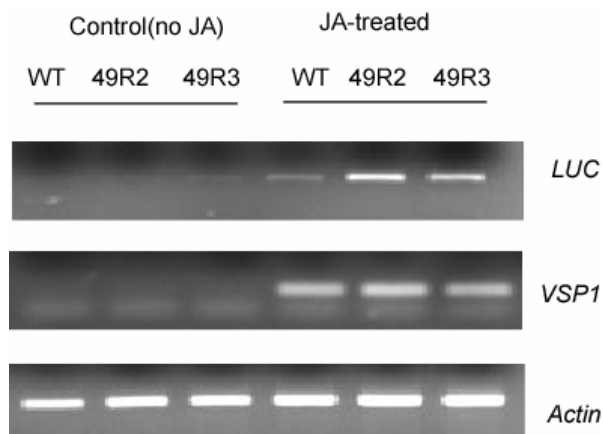
Figure 3-23. Higher expressions of *luciferase* in 49R2 and 49R3 than in wild type. Seedlings were treated with 0.2 mM JA by spaying for 6 hr before harvest. cDNA from total RNA (1µg) was used as template for RT-PCR analysis. Luciferase gene specific primers were used. *Actin* was used for control for equal amount of cDNA.

Sequence alignment of these sHSPs (Fig.3-24A) showed significant similarity among them though they have different functions. Since the overall sequence of At1g53540 is very similar to At3g46230 (89.2% amino acid identity), their functions may be similar to each other. Sequence alignment of all of the *Arabidopsis* sHSPs (Fig.3-24B) showed class I sHSPs (including At1g53540) have sequence similarity but class I sHSPs have lower similarities when compared to other sHSPs of other classes (class II, III and IV) (Fig. 3-24B).
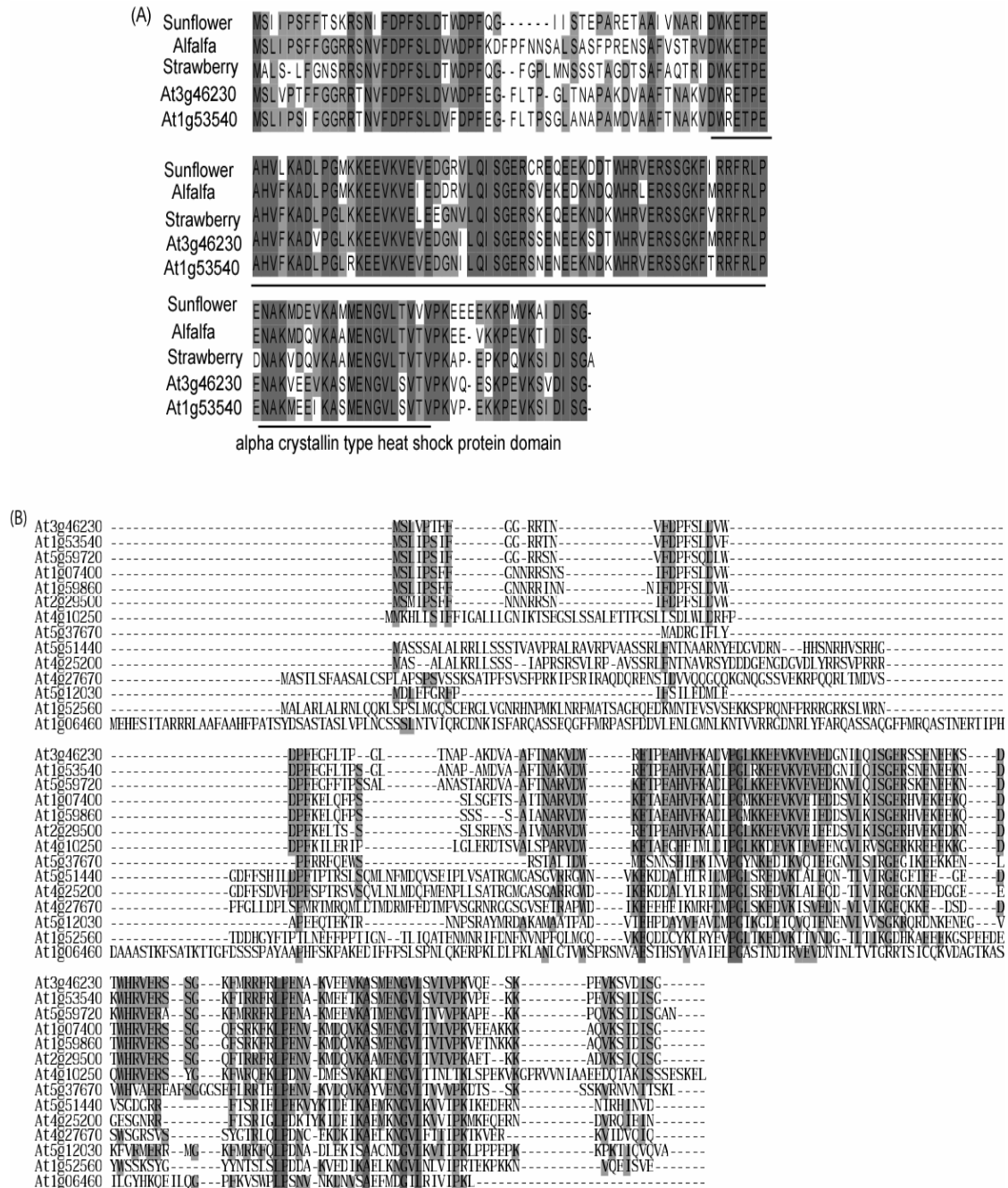
Figure 3-24. Sequence alignment of small heat shock proteins. (A) At1g53540 shows significant similarity with other small heat shock protein. Sunflower (*Hahsp17.6G1*, CAB08441), Alfalfa (CAA41547), Strawberry (AAC39360) and Arabidopsis (At3g46230, At1g53540) class I small heat shock protein. (B) Sequence alignment of Arabidopsis sHSPs. class I sHSP (At1g46230, At1g53540, At5g59720, At1g07400, At1g59860, At2g29500), class II sHSP (At5g12030), class III sHSP (At4g10250, At5g51440), classIV (At1g06460, At4g10250, At5g51440) and class I-like sHSP (At1g52560, At5g37670) . Light grey: completely conserved residues. Dark grey: identical residues.

Plants sHSPs consist of five families (class I to V) based on the sequence similarity, immunological cross reactivity and intracellular localization (Waters et al., 1996). Plant sHSPs are targeted to not only the cytoplasm but also to the chloroplast, mitochondria and endoplasmic reticulum (Waters et al., 1996). sHSP is a member of class I small heat shock protein. Six class I small heat shock proteins were listed in The Arabidopsis Information Resource (TAIR, www.arabidopsis.org), and all show similar DNA sequence (Fig.3-25). To find sHSP gene specific sequences, all the class I small heat shock protein genes and a homologue of class I small heat shock protein were aligned, and distinguished sequences were found (underlined in Fig.3-25). These gene specific sequences were used to design At1g53540 gene-specific primers. The At1g53540 sHSP promoter region was analyzed using TFSEARCH: Searching Transcription Factor Binding Sites (http://www.cbrc.jp/research/db/TFSEARCH.html). In addition to a heat shock transcription binding site, many putative transcription factor binding sites were found (Fig.3-26).

```
at1g59860  --------------------------------------------------------------------------------------------------------------
at1g07400  ------------------------------------------------AATCTTTTCATCGTTTTCGTGTGAAGTTTAAAGAAGTTTGGGACCCGTAAATT
at2g29500  --------------------------------------------------------------------------------------------------------------
at3g46230  --------------------------------------------------------------------------------------------------------------
at1g53540  --------------------------------------------------------------------------------------------------------------
at5g59720  --------------------------------------------------------------------------------------------------------------
at3g46220  GCTTCCAGGGGTATATTTGTTCCTTCCAATTTGTCGGCATTGTGGGCACCATTGCAACAGTTGGTACAAGAAACGAATGGAGCCAGTGGAGTTGCTGTTGAGAATTCATTTTTCCA

at1g59860  ----------------------------------------------------ATCATAAATTCGAAATTCAATTCGTC--
at1g07400  AAGCTCTGGAGATTTCTTGTTCACCACTATAAATACATCATCCGAGTCACCAGCTTTGCTCAGAAGTAGAAATCGAACTCGCCGGAAAATCGTAATTAGCTATCTCAGCATTCC--
at2g29500  ----------------------------------------------------ATCAAAAATCGAAAAATCAAAGTTCTCA
at3g46230  ----------------------------CTCTACCATCATTTCTCCCTATAAATACGCCAACGCACATAAGTGTTTGCATTCGAAGAGAGTTCTAGCAAA
at1g53540  ---------------------------------------------AAAACAG----AGCAAA
at5g59720  ------------------------------------------AGATCAAATCAGC---AGGAAA
at3g46220  ATATTTAACCGACTGTTGAAGGAAGAGGAGATGCTTGGATCACTACGTGCTGGAACCCATTGGACTCCTAGTGCTTTTGCAACTGCTCAAAAGGAATGCGTAGACTCTTCTTTTTC

at1g59860  AAATTGTTCAGATTTCGAAATCAACCGA-GAAAT----GTCGCTGATTCCAAGC-TCTTCGGCAACAACAGGCGAATCAACAACAACATATTCGATCCATTC-TCACTCGACGT
at1g07400  CATCTCTTTCGCTTTCGAAATCAAATC--GAGAAT----GTCGCTTATTCCAAGCT-TCTTCGGCAACAACAGGCGAA--GCAACAGCATCTTCGACCCGTTC-TCACTCGACGT
at2g29500  GATATCAACAAAAAAAAAGTAAATTCTTTAAAT----GTCGATGATTCCAAGTT-TCTTCAACAACAACAGACGAA------GCAACATCTTTGATCCATTC-TCTCTTGACGT
at3g46230  AACAAAACAGAGCAAACAGAGTAAGCGA-AACGAT----GTCTCTAGTTCCGAGCT-TTTTGGAGGCCGAAGAACAA--------ACGTGTTCGACCCATTT-TCACTAGACGT
at1g53540  AAGAGTAGCAATCACA-AGAGGAAGTGA-AACGAT----GTCTCTAATTCCAAGCA-TCTTCGGAGGAAGAAGAACAA--------ACGTTTTCGATCCGTTC-TCGCTGGATGT
at5g59720  AGAACCAAAAGTCTCCCGAAAAGCAACG-AACAAT----GTCTCTCATTCCAAGCA-TTTTTGGAGGACGCAGATCAA--------ACGTCTTTGATCCGTTC-TCGCAAGACTT
at3g46220  AATTCCTACATCTCCTATGGAGCATGCAGAAGCTTGGGATTTCTCAAGCGTGTGCAGTTCTTACAGATACCCAGATGGCACACCTTTAGCTGCTGTATTTATTCATTCCTCAATGA

at1g59860  GATCC--ATTCAAGGAACTTCAATTCCCGTC----------------ATCGT-----CTTC---------GTCGGCGATAGCAAACGCACGTGTGGATTGGAAAGAGACGGCGGAGG
at1g07400  GATCC--ATTCAAGGAACTGCAATTCCCGTC----------GTCGC-----TCTCCGGAGAAACATCGGCGATAACGAACGCACGTGTGGACTGGAAAGAGACGGCGGAGG
at2g29500  GATCC--ATTCAAGGAACTAACAT--CATC----------ATCAC-----TTTCTCGTGAGAACTCAGCGATCGTGAACGCACGTGTGGACTGGAGAGAGACGCCGTGAGG
at3g46230  GATCC--GTTCGAAGGATTCTTGACGCCC------GGGTTGAC--AAACGC----ACCTGCCAAGGACGTGGCAGCCGTTCACAAACGCTAAAGTGGACTGGAGGGGAGACACCTGAAC
at1g53540  GATCC--GTTCGAAGGTTTCTTGACGCCGTCA---GGATTGGC--AAACGC---ACCCGCTATGGATGTGGCAGCCGTTCACAAACGCTAAAGTGGATTGGAGGGGAGACACCTGAGG
at5g59720  GATCC--ATTCGAAGGATTCTTCACGCCATCTTCTGCGTTGGC--AAACGCTTCAACCGCTCGTGATGTGGCAGCCGTTTACAAACGCAAGAGTGGATTGGAAGGAAACGCCGGAGC
at3g46220  GATGCTGGATTCTGCAACTGAGGATGCTATTGAACAAACAGTTGGATCGATTCCCTCTCTGTATTACCTTCATCGTTCACGT-CACAGGATGCAAACAAGATGTTGCTTCTTTGT

at1g59860  CGTGTTCAAGGCGGATTTACCGGGGATGAAGAAAGAAGAGGTTAAAGTAGAGATTGAGGAT--GATAGTGTGCTTAAGATCAGCGGAGAGAGACACGTTGAGAAAGAGG-AGAAGC
at1g07400  CGTGTTCAAGGCCGATCTGCCCGGGGATGAAGAAAGAGGAGGTTAAAGTGGAGATAGAGGAC--GACAGTGTGCTCAAGATCAGTGGAGAGAGACACGTGGAGAAAGAGG-AGAAGC
at2g29500  CCGTGTTTAAAGCTGACTTGCCTGGATTGAAGAAGGAAGGAAGTTAAAGTTGAGATTGAGGAG--GATATGTGTTTGAAGATCAGTGGAGAAGAAGAAG-ATAAGA
at3g46230  TGTGTTCAAGGCGGACGTGCCTGGGCTTAAGAAGGAAGAGGTGAAGTGGAGGTTGAAGAT--GGTAACATACTTCAGATAAGCGGTGAGAGGAGCAGTGAGAATGAAG-AGAAGA
at1g53540  CGTGTTCAAGGCGGATTTACCGGGACTGAGGAAGGAAGAGGTGAAAGTGGAGGTTGAGGAT--GGCAACATACTTCAGATAAGTGGAGAGGAGGAGCAATGAGAATGAAG-AGAAGA
at5g59720  TGTGTTCAAAGCGGACTTGCCCAGGCCTGAAGAAGGAAGAAGTCAAGGTTGAGGTTGAAGAC--AAGAACGTTCTGCAGATTAGCGGAGAGAGGAGCAAGGAGAACGAAG-AGAAGA
at3g46220  C-TGTTCAA----TCAGCTCTCAAGGCTGAGAAAGCACTTTATCTTGGGCGAATCCTATGTCCTAAGCAGTGGCTTTATCAAGGGAATATATGATCAAATTGAGAAAGAGGCAGATGC

at1g59860  CACGTGGCATCGTGTAGAGAGA-TCGAGTGGGGGATTCTCG-AGGAAGTTTAGGTTACCGGAGAATGTGAAGATGGATCAAGTTAAAGCTTCAATGGAGAATGGTGTTTGACTGT
at1g07400  CACGTGGCATCGTGTGGAGAGG-TCGAGCGGGCAATTCTCG-AGGAAGTTTAAGCTGCCGGAGAATGTGAAGATGGATCAAGTCAAGGCTTCCATGGAGAACGGAGTTCTGACTGT
at2g29500  CACGTGGCACCGTGTGGGAGAAA-TCGAGTGGGCAGTTTACG-AGGAAGTTTAGGTTGCCGGAGAATGTGAAGATGGATCAGGTTAAGGCTGCGGAGAATGGTGTTGACTGT
at3g46230  CACATGGCACCGTGTGGAGCGG-TCAAGTGGGAAGTTCATG-AGGAGGTTTGCCAGAGAATGCAAAGGTGGAGGAAGTAAAGCGGAGTATGGAGAATGGGGTGTTGTCGGT
at1g53540  CAAGTGGCACCGTGTGGAGCGG-TCGAGTGGGAAGTTCACG-AGGAGGTTTAGGCTGCCGGAGAATGCTAAGATGGAGGAAATCAAAGCCAGTATGGAAAATGGTGTTGTTGTCGGT
at5g59720  TAAGTGGCACCGTGTGGAGAGA-GCTAGTGGGAAGTTTATG-AGAAGGTTTAGGTTACCGGAGAATGCAAAGATGGAAGAAGTGAAGGCAACAATGGAGAATTGGTGTGCTTACGGT
at3g46220  AGCATCCAAGCTTCTACTGCTACTTTGATACATCCTTCAAGCAAGTCGTCAGAGTCGACAGAAAGCATACCTGCCAATACAGATAAAGGGTCAAAGAAGAAAAAAAGGAAAGTCTGC

at1g59860  --GACTGTGCCTAAAGTGGAGACTAATAAGAAGAAGGCTCAAGTTAAGTCTATTGATATCTCTGGCTAAGCCAATTTAGCTTGATTTGAGTTCTCTCTGGGTCTTGACTTTGTGTG
at1g07400  --GACGGTGCCTAAAGTGGAAGAGGCTCAAGAAGAAGGCTCAAGTTAAGTCTATTGATATCTCTGGCTAAAGCCAATTAGCTTA-------------TGGGTTTCATGTTTATGCC
at2g29500  --TACGGTGCCTAAGGCTGAGACT------AAGAAGGCTGATGTTAAGTCTATTCAGATATCTCGGTTGAG--TAATGGGTTCGAGTT----------TTATCATCGGAGTTGCTTC
at3g46230  --TACGGTGCCGAAAGTTCAGGAG-----AGTAAGCCGGAGGTGAACGTCCGTTGAATCTCTGTGGTTAAG---AGGAACTCTGAAGTTGGAAA--GCTTAAGAC-AGTAATATGTG
at1g53540  --TACCGTGCCCAAGGTGCCCAGAG-----AAAAAGCCAGAGGTTAAGTCCATTGATATCTCTGGTTAAA---GGGAATT---------GTTTAAGACCAAAAATGTGTG
at5g59720  --TGTGGTTCCGAAAGCGCCTGAG------AAGAAGCCTCAAGTCAAGTCCATCGATATCTCCGGGGCTA---ATTGAATATGAA----------GATGAAGATGAAATATTTCGGT
at3g46220  ACGAAGGCAGCTACCGTTGAAACTGTTC--CAGATGATGAAGAGGATGCCCGTCCAAAATCTAAGAGAAACCGAAGAAAGGAAG--GGATTCATCTTCATCACAGAAGTTGGATT

at1g59860  TGTCTCTGATGATTATGGTTCTTCGTTTGATTGTAGTTTT-AAACTAATAATCAATGGAAGC-TAGTAACTTGTGTGACAAGTAAATGTATGGTGGAGAA-ATGTGTGTATTTGGA
at1g07400  AGACTCTTCTGATTATGGCTCTTGATTTGAATATGGCTCT-GTGTT--TTGGTAATGATGTA-TTATAACTGTTAATCCTAATAATTA------AGCAAG-GCCTTTATATT----
at2g29500  TTTTTGTCATGGTTATGGTTCATGTTTTTACTTGAGTGTGT-GAGTACTCTATCTAAATTATAATAATCTCCGATTGAGCTATGAATTATGATGTATCGGATACATTTGATCCTAAT
at3g46230  T-GTGAAATAAATGGTGCCAATTTCCTTTCTTCTTTTTCTTGTTGTAATCACCAATCTCGTTGTA-TCAATAAA-GAGTATCG--TAATAAAGAGTGTTTTTGATCCATTGATAGTTAC
at1g53540  TAGTGAAATAATTGGTGTG-------TGATGTGTTTTTCTTGTAATCATGAATCTCGTTGTA-TCATTATCATAAAATCT--TAATAAGAGTTGTGCTTTG-
at5g59720  CAAATAAAAAGCTTGTGTGCTTAAGTTTGTGTTTTTTTCTTG-----GCTTGTTGTGTGTATG-AATTTGTGGCTTTTTCTAATATTAAATGAATGTAAGATCTCATTATAATGAAT
at3g46220  AGCAGGAGGCAAGAAAGAG-------TCAGTTAAGGCGCAAGAA---AGTAATAACATTATTCCGCCAGACGAATGGGTGATGAAAAAGATCGTCGACTCTGTACCTGAATTTGAA
```

Figure 3-25. cDNA sequence alignment of class I small heat shock proteins and a homologue (at3g46220) of class I small heat shock protein in *Arabidopsis*. Gene specific primer sequences of At1g53540 are underlined. Light grey: completely conserved residues. Dark grey: identical residues.
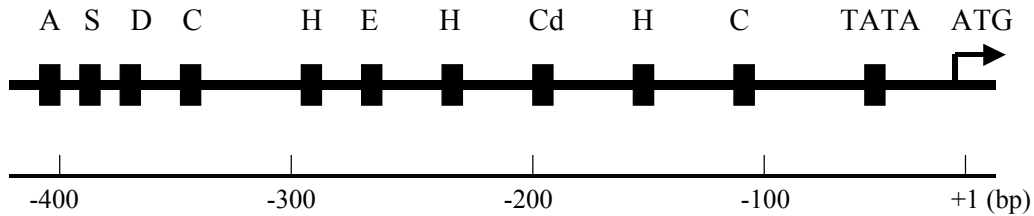
Figure 3-26. Promoter analysis of At1g53540. A: ADR1(alcohol dehydrogenase gene regulator1). S: Sox-5 (involved in development of sperm). H: HSF (Heat shock factor). C: CdxA, D: Dfd, E: deltaEF1 and Cd: CDP are homeogene responsive elements. TATA: TATA box. ATG: translation initiation (+1bp)

At1g53540 (*sHSP*) was induced by JA (Fig.3-27). Heat shock proteins were shown to be induced by JA. Class I and class II sHSP mRNAs were increased by JA in tomato (Ding et al., 2001). *sHSPs* and *HSP70* are induced in untreated leaves when distant leaves are treated with JA in tobacco (Hamilton et al., 2001). In my experiment, RT-PCR analysis showed *sHSP* was also induced by JA (Fig.3-27). In 49R2 and 49R3 mutant, *sHSP* was induced by JA but at a decreased level (Fig.3-27). To confirm the RT-PCR primer is At1g53540 gene-specific, the DNA sequences of RT-PCR products of JA-treated wild type and 49R2 mutant were subjected to dideoxy terminator cycle sequencing using the ABI BigDye sequencing kit (PE Biosystems), and analyzed on an ABI Prism 3100 DNA sequencer. Both RT-PCR products from wild type and 49R2 mutant were At1g53540.
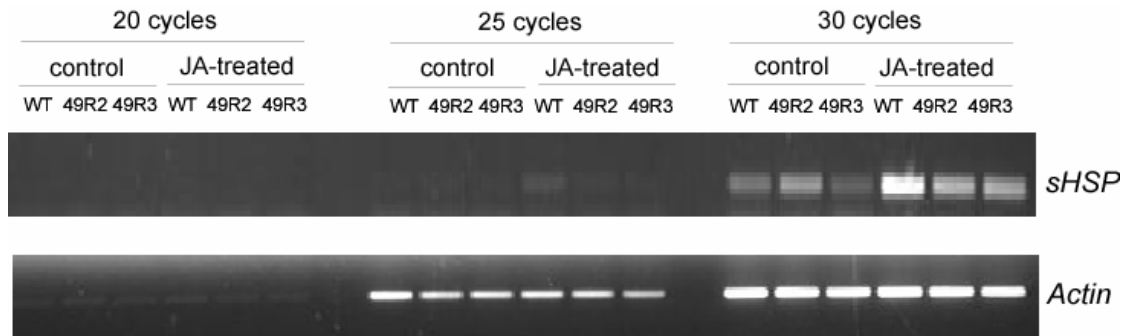
Figure 3-27. Induction of *sHSP* by JA. *sHSP* (At1g53540) was induced by JA in wild type. In 49R2 and 49R3, *sHSP* was also induced by JA but less than wild type. Seedlings were treated with JA for 6 hr before harvest. cDNA from total RNA (1µg) was used as template for 20, 25 and 30 cycles of RT-PCR analysis. *sHSP* gene specific primers were used. *Actin* was used for contol for equal amount of cDNA.

Effect of phosphate starvation or sucrose on VSP1 expression

In 49R2 and 49R3 mutants, the *AVP-LUC* expression level was increased by phosphate starvation. The fold induction of *AVP-LUC* by phosphate starvation when compared to no phosphate starvation was lower in 49R than in wild type (6.8 fold induction in wild type and 3.1 fold induction in 49R). The basal level of *AVP-LUC* expression was 6.4 fold higher in 49R mutant than wild type (Fig.3-28A).

A)

B)
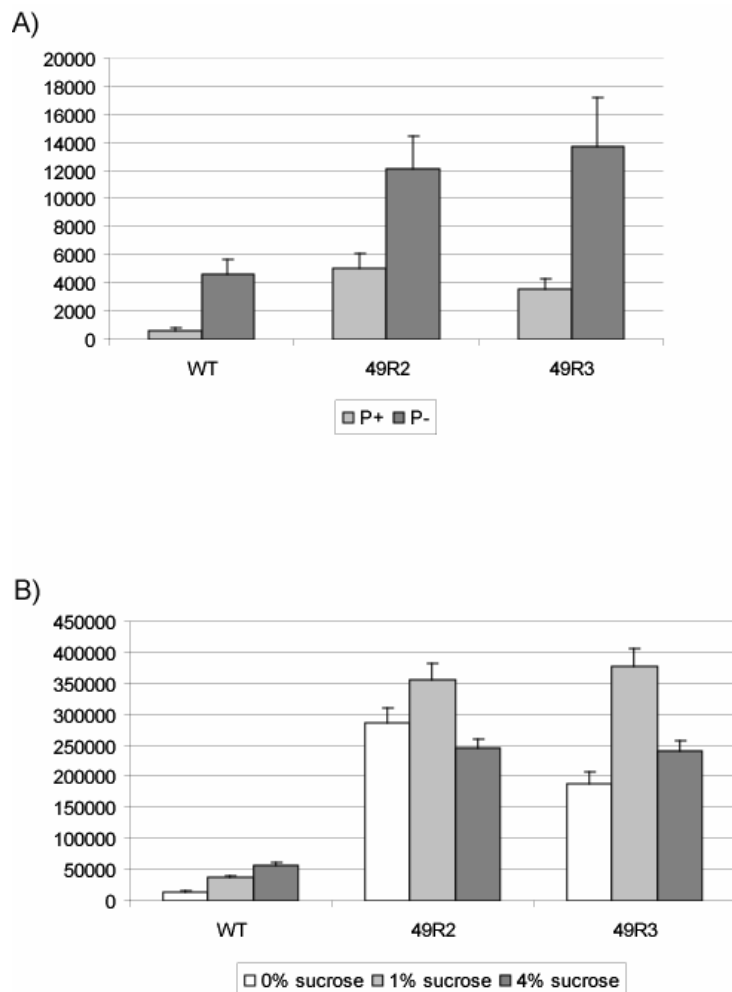
Figure 3-28. A) Phosphate starvation effect on *AVP-LUC* expression in 49R (49R2, 49R3). *AVP-LUC* was induced by phosphate starvation in wild type and 49R. B) Sucrose concentration effect on *AVP-LUC* expression in wild type (WT) and in 49R (49R2, 49R3). *AVP-LUC* was induced by increasing sucrose concentration in wild type and in49R. 49R showed that *AVP-LUC* expression was reduced by increasing sucrose concentration. Values of y-axis are luminescence intensities. Error bar represents standard error.

AVP-LUC expression in wild type plants was increased when sucrose

concentration was increased from 0 to 4%. This is the same result reported by Berger et

al. (1995). The 49R mutant showed a similar pattern as the wild type, that is the *AVP-LUC* expression level was increased by the increase of sucrose concentration. The induction fold of *AVP-LUV* of 1% sucrose when compared 0% sucrose was similar (2.5 fold induction in wild type and 1.6 fold induction in 49R). But in the 49R mutant, there was a difference in *AVP-LUC* fold induction between wild type and 49R at 4% sucrose concentration (3.8 fold induction in wild type and 1.1 fold induction in 49R). And the basal level of *AVP-LUC* expression was 15.8 fold higher in 49R mutant than wild type (Fig.3-28B).

Since *sHSP* was disrupted in the 49R mutant by T-DNA insertion, higher expression of *AVP-LUC* in the 49R mutant than wild type in these phosphate starvation and treatment of different concentrations of sucrose experiments suggest that sHSP reduces phosphate starvation and sucrose responses of *AVP-LUC*.

Root growth inhibition by jasmonate in 49R mutant

JA induces inhibition of root growth (Staswick et al., 1992, Berger et al., 1996). We investigated whether root growth inhibition by JA was affected in 49R mutant plants. Seeds of 49R2, 49R3 and wild type plants were germinated on JA plates, and root length was measured after 8 days growth. *jar1* Which is known JA-insensitive was used as a positive control. Similar root growth was observed for wild type and the mutants. Therefore, it is thought that root growth inhibition by JA in 49R mutant was not affected.

(A)

(B)
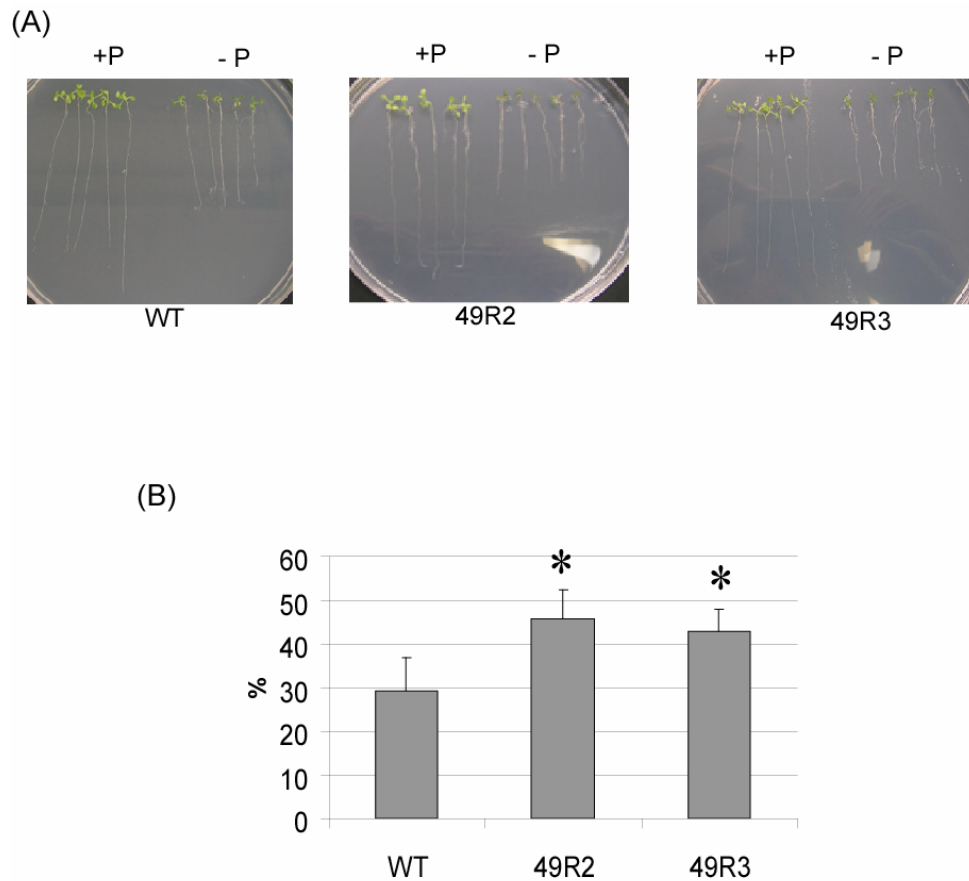
Figure 3-29. Root growth inhibition by phosphate starvation. (A) Picture of wild type (WT) and 49Rmutant grown on MS media free of phosphate (-P) compared to the root length of seedlings grown on MS media containing phosphate (1.25mM, +P) are shown. (B) Percent reduction of root length of indicated seedlings by phosphate starvation by phosphate starvation. The effect of phosphate starvation on root growth was analyzed by one-way ANOVA and Scheffe multiple means comparisons tests
 Error bars indicated standard deviation. Asterisk shows the significantly difference from WT at p=0.05. 49R2 and 49R3 showed more sensitive than wild type to phosphate starvation in root growth.

49R showed more sensitivity to phosphate starvation in root growth

The 49R2 and 49R3 mutants showed more sensitivity to phosphate starvation in root growth. (Fig.3-29). Therefore, sHSP gene could be involved in the root growth phenotype in phosphate starvation.

49R2 and 49R3 showed similar susceptibility to thrips compared to wild type

Since 49R2 and 49R3 are an over-regulation JA signaling mutant, I suspected 49R2 and 49R3 would be more resistant to pathogen. To test the hypothesis, 49R2, 49R3 and wild type were infested with thrips. Assessments of damage index of damaged plants suggest that 20B15 has similar susceptibility to thrips compared to wild type (Fig. 3-30). There was no significant different susceptibility in all of the test days. Similar susceptibility between wild type and 49R mutant indicated the *sHSP* disruption in 49R2 and 49R3 have little effect on insect susceptibility.

(A)

(B)

day	Probability	
	49R2	49R3
4	0.208	0.208
6	0.244	0.085
8	1.000	0.078
11	0.328	0.469
13	0.464	0.065
14	0.485	0.129
15	0.527	0.128
16	0.552	0.174
17	0.540	0.255
18	0.356	0.203
19	0.370	0.370
20	0.562	0.217
22	0.869	0.123
24	0.861	0.183
26	0.851	0.504
28	0.558	0.213
30	0.244	0.073
32	0.191	0.073

(C)
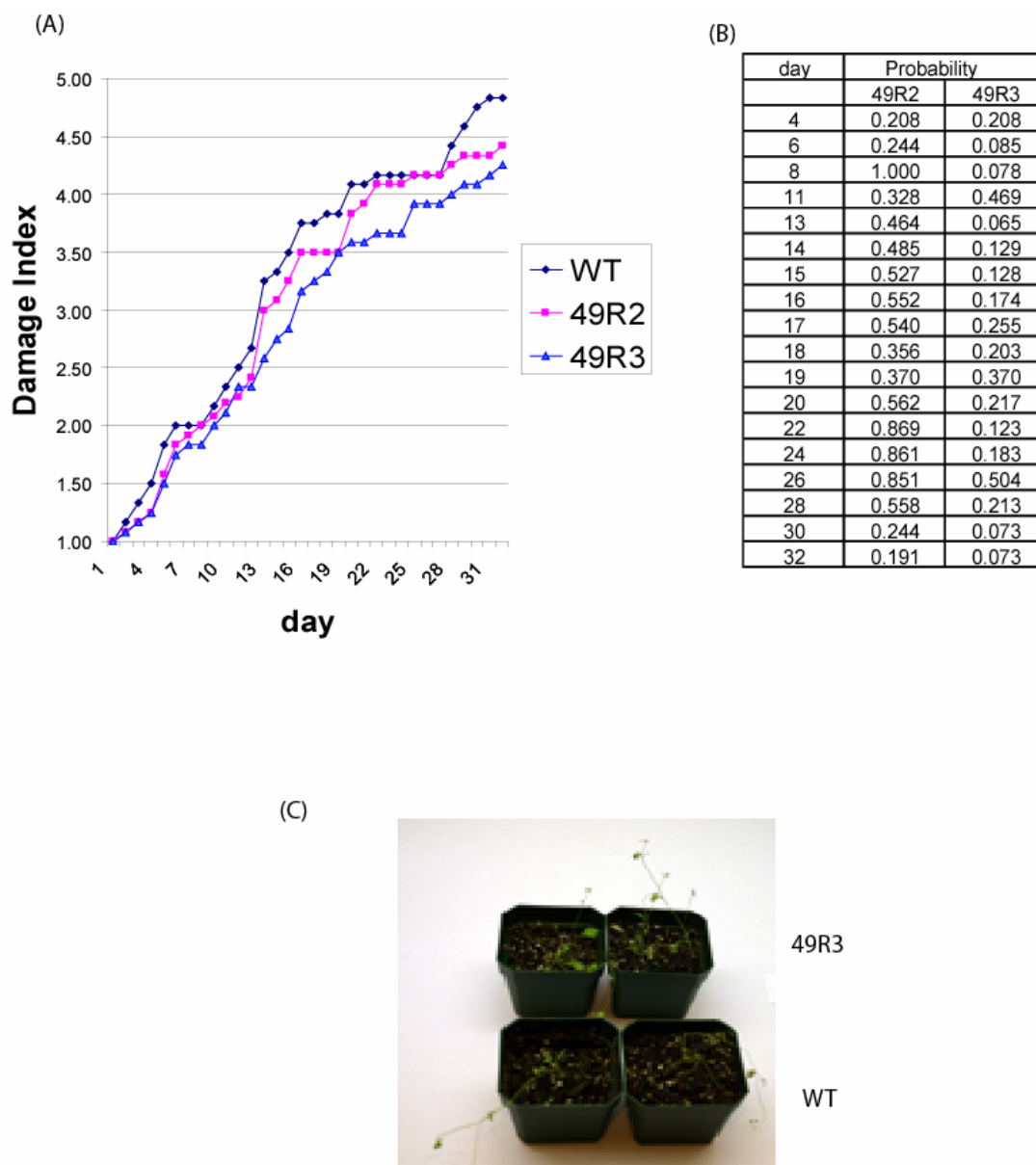
Figure 3-30. 49R2 and 49R3 shows no significant difference in susceptibility to thrips compared to wild type (WT). (A) Damage index versus time (day) plot. (B) A probability of 0.05 was used to determine statistical significance. 49R2 and 49R3 shows there is no significant difference from WT at p=0.05. (C) Picture of damaged plants (wild type and 20B15) by thrips for15 days. 49R3 have similar damage with wild type.

Complementation analysis of At1g53540

TAIL-PCR analysis showed that 49R1, 49R2 and 49R3 had the same T-DNA insertion. The T-DNA insertion site was the exon region of At1g53540. They were all *AVP-LUC* over-regulation mutant phenotypes (10 fold higher luminescence than wild type) in the T3 generation and showed homozygous T-DNA insertion by diagnostic PCR. Backcrossing with wild types showed this mutation was recessive. The F2 generation in the backcross line showed a 3:1 segregation ratio (wild type: mutant). All mutant phenotype plants of the F2 generation had homozygous T-DNA insertion in At1g53540. These results suggest the At1g53540 gene is responsible for *AVP-LUC* over-regulation mutant phenotype. To prove this, complementation analysis was carried out.

T3F20 BAC DNA contains At1g53540 genomic sequence (Fig.3-31). Parts of At1g53540-adjacent region were included in complementation fragment to make sure all components for the gene expression were included. Complementation analysis can give evidence that At1g53540 is responsible for the mutant phenotypes. Figure 3-32 shows the detailed information about At1g53540 complementation fragment. A genomic fragment

(Fig.3-31) containing the *sHSP* gene (At1g53540) in BAC DNA (T3F20) was amplified using primers at1g53540-FE and at1g53540-R by the PCR enzyme BD Advantage2 (BD Biosciences). The EcoRI fragment (3.9-kb) PCR product was cloned into pENTR2B (Invitrogen). Together with this entry vector construction, A genomic fragment (Fig.3-31) containing the *sHSP* gene (At1g53540) in BAC DNA (T3F20) was amplified using primers 49R-attB1 and 49R-attB2 by the PCR enzyme BD Advantage2 (BD Biosciences). The 4.2-kb PCR product was cloned into pDONR221 (Invitrogen) by using BP ClonaseII ^{TM} (Invitrogen).

To confirm the DNA sequences of complementation fragment were not changed, DNA sequence analysis of complementation fragment containing construct was performed. The sequences from the first base of complementation fragment to the end of complementation fragment were analyzed. The complementation fragment cloned into pENTR2B has no difference from the Arabidopsis genomic sequence in TAIR web site (www.arabidopsis.org). In case of the complementation fragment cloned into pDONR221 vector, one base (770 base after stop codon of At1g53540) was different (A to G) from the Arabidopsis genomic sequence in TAIR web site.

Figure 3-31. Scheme illustrates complementation fragment of At1g53540. Complementation fragment includes part of previous gene (At1g53530) and next gene (At1g53550) to make sure all components of At1g53540 for expression are contained.

taagcggtgtatgattgacgagttgggatggtctagaaggtaaagcattgctagttactgattcaa
ggtaaaacgatgcaatttgcaaatttctagatctagagctaggtttcaattgtgtatgattgatgattt
gggatggtctagattgtggatcattgctagttattggtttcaatgaaactcaatttcagagtaattata
aaaacttatgctttttgctatcttacatctctacactgcgtccaatctctgtttttgcagcactaaagctt
gtctaataaaaggttatgtgtttacttcttcaatagGTTCCGAAAGGTCATGTTTGG
ATTCAGGGAGATAACTTGTATGCTTCAACTGATTCACGTCATTTT
GGGCCTGTACCTTACAGTCTCATTGAAGGAAAAGCTCTTCTACG
Ggtaactctttctcaaaatttctagctgtgaagttattatgaaacaatgacaataatctcatcagta
accatctgtttgcttgtctagGTCTGGCCACCAGAATACTTTGGGTCATTGA
GATGAcgattatatacacatacacattcaggtaatcaggttgtctgcctactgtatacgtcaag
agatcactccaacgattttgttgcactgtaactcatgcgagtaatgtatcattcatgtgggataagc
attttcgtctactctcctttttggcagtaatagaagaaaagccatggtcctgatattgtttctgaaacc
cgaacttcaatatcaaatgttaagattcatattgttggtatcgaaaaagctggtgcgttatgggatg
ataattgtgtccttgatacaaagttttatagatgtttttgattcatttgggggactcaagccaattgttca
atgttcaccaaaaaaataaatttagacgaaatcattaatgggcctcgatattatagcccaagaa
aaaacaaaaaaacggcaaggcgttcactcgccgacctgatgcattgcacgtcgtctcagccc
tcaacaccagaaacttcttgaaagcacctgagaagtctggtaacgtcgttaaaagaaagaaa
cgaagatccctgatttttcaaggataccatcgatcaaaataattttcaagaaactcgcagaatgtt
ctatcttactttccatactctctccctataaatatgcgaaggcctcgtagctgtacgaaccaacaag
gagcaaagagttcatagcaaagcaaagcttcaaaacagagcaaacgcaaagagtagcaat
cacaagaggaagtgaaacgATGTCTCTAATTCCAAGCATCTTCGGAGGA
AGAAGAACAAACGTTTTCGATCCGTTCTCGCTGGATGTTTTCGAT
CCGTTCGAAGGTTTCTTGACGCCGTCAGGATTGGCAAACGCACC
TGCTATGGATGTGGCAGCGTTCACAAACGCTAAAGTGGATTGGA
GGGAGACACCTGAGGCGCACGTGTTCAAGGCGGATTTACCGGG
ACTGAGGAAGGAAGAGGTGAAAGTGGAGGTTGAGGATGGCAAC
ATACTTCAGATAAGTGGAGAGAGGAGCAATGAGAATGAAGAGAA
GAATGACAAGTGGCACCGTGTGGAGCGGTCGAGTGGGAAGTTC
ACGAGGAGGTTTAGGCTGCCGGAGAATGCTAAGATGGAGGAAA
TCAAAGCCAGTATGGAAAATGGTGTGTTGTCGGTTACCGTGCCC
AAGGTGCCAGAGAAAAAGCCAGAGGTTAAGTCCATTGATATCTC
TGGTTAAagggaattgtttaagaccaaaaatgtgtgatggtagtgaaataattggtgtgtgat
gtgtttttcttgtaatcatgaatctcgttgtatcattatcataaaatcttaataagagtgtgctttgtatac
ttctgcctctcggattgtttgaagtacgtattgcgatttacaaggattccccatttcattagctaatcaa
aagagaaccggttcggtcatgtttttaaccaatgttgtgacgacaatacaagaaaaatggggct
aatagataacttataggtacgttttggaaaacttctgaagtcaaggctcttttacgcaatataaag
ataggttgaaaattaggtttaagggtaaaatagacaattcaatactgaaggttttcttcttcctcatg
ctgcttataaataaacatcgccgcgataagggtagaatccaagtgcctttgagagatcagagca
gcgagttacaatgctgttcttgttccgttgcgggcataaatcgaaacccattgatctttctttgcttcc
tcttgcaagtaatcttctttactgcaaatcggaagaaaagatctcacatttctttgatcaggaacca
aactctcttatcgtttcttttccttcttgttgttgtttttgtgattatgtttgtgatatggccgatccaaatggg
ttcgttcatctttatattagttcccaatttcacccggccgaaatttaaaatcaatttaatggagcagtct
ctctattttatgattctctacatgtatatttttcatgtcggaaatcagagattctgccctaaaatttattc
gtcaattttgtaatttatctccgtataatagagatcttagacgtaaatatttatcttttctgttttgtttgatt
tgtgatatggccgatccaaatgggttcgttcatctttatattagttcccaatttcacccggccgaaatt

At1g53530

ATG, TGA
=Translational Start/Stop
atgc = UTR
ATGC = Exon
atgc = Intron

**Putative
heat shock factor
Binding Sites**
(underlined)

taaatat: **TATA Box**

At1g53540

TAA
=Translational Start/Stop
atgc = UTR
ATGC = Exon

Figure 3-32. Detailed information of the At1g53540 complementation fragment. Putative heat shock factor (transcription factor that responsive to heat shock) binding sites are underlined and purple colored.

taaaatcaatttaatggagcagtctctctatttttatgattctctacatgtatattttttcatgtcggaaatcag
agattctgccctaaaatttattcgtcaattttgtaatttatctccgtataatagagatcttagacgtaaatatt
tatcttttctgttttgtttgattcacttaccaaacaatgttaagtttcgatgtctgagttatttgtgtagtatttaata
acaaatggattcgactatgaaagaaaaaattgtctgtaatgaatttatatgtagcattacttcaaagact
ggtttatggtagtgaatgcgtgctttatagtggaagtatgactgtaattgattttcagttggtatggaccaa
gctccgactaagcagcattcgattcatctttcacattggttgaagaagtaatgtgattgattgctgccaaa
attggagttggtaataatgaaaacgctgaacaaacatgttatgaaatcagcataagacgattttaatta
acctaataatgtcagaaatcttgtaattcacatggataagttatggtttagcattaacttacgataatctga
atggagatgctttaaaactagatcctcattctatctcaataatttgaacggagatgccaaatgataatta
caaatgttaaagattggtctacaattcgcgagtagattttgggctctatcctatgatttagcacttgggttc
atgtaatctctcctatttaaggactcaaaagaaaagcaacacaagcggacagtttaagttttatctagg
gtttcatgacatcctttatgaatttatgataagtaaacgtaagcaacaagttatcgaggataacttaacc
gtatctagttcaataaacgatatctacaatctccaaatgagaagaaatataatgctttgttctaatccctgt
tgatctcgtcatcaatattttgtcaagattgtcattaaagttgatagtcgtttttgtatcaaagttgtggtcatct
atacttttttcatctgttactatccccaaacacaaaccaaacttaactaacttcggttgagcttggttagag
ttttacaagtcttaaataaaccgaaccgcacccaaacccgaagttaaaccgaaaagtccaccctagt
ctttggtggtaacctcctttaaaaacaagagcctccctcagttactctattgcgagaacgaatcgagattt
cattaaaattagtataagacagtagttttgaccttaaagtgaattaacttaacatatcttaccaagaaaa
actgatcaatttttaaattcatgtagatgagagttatttgcttacagtggatctttagtggtatgaagacaa
gacacattgaagacttcatgaagaaccatatactggtcaattaatattaatctggctattcaaaaagat
gaatgaattcatgtagattgtattttacatatgtgtttaaacaaatgtgatatttcaatgttcaaactttaggt
ctgaggaggggcctgcaattaatttagtgatgagtaggtatcggggcctaattaagcccaacttattga
gcttggatgaagcatcagatataagacagagtcttccaattccttgatatgtaggaattattattccatac
ctcttgtttcagactgtgaccaacatcacagagccaaaatctagggttttaattcatc**ATG**AGAAAA
CGTAAGCAGCAAG

ATG
=Translational Start
ATGC = Exon

At1g53550

Figure 3-32. Continued.

The *sHSP* entry construct was then used to transfer the complementation fragment

to destination binary vector (pBSVirHygGW) by LR recombination (Fig. 3-6) by using

LR clonase[TM] (Invitrogen). pBSVirHygGW vector containing the complementation

fragment was analyzed by restriction digestion analysis using HindIII (which can cut inside the complementation fragment but cannot cut vector) and by PCR using the combination of *sHSP* gene specific primers and the primers which anneal vector sequences. The sequences of junction region between vector and the complementation fragment were also analyzed to confirm correct connection between vector and the complementation fragment. pBSVirHygGW-sHSP constructs (3.9kb and 4.2kb complementation fragment) were used for Arabidopsis transformation for complementation analysis of *sHSP*. pBSVirHygGW-sHSP constructs were transformed to *Agrobacterium* strain GV3101(pMP90RK) and transformed *Agrobacterium* was analyzed by colony PCR using sHSP complementation fragment specific primers and by restriction digestion analysis using HindIII. Floral dip method was used for Arabidopsis transformation (Clough and Bent, 1998). pBSVirHygGW vector containing *GFP* (Green fluorescent protein) was used as negative control. Complemented 49R mutant is supposed to show wild type phenotype in *AVP-LUC* expression, luminescence and *sHSP* expression when compared to negative control (*GFP* containing binary vector transformed 49R mutant) to confirm *sHSP* is responsible gene for mutant phenotype of 49R mutant.

1) Other mutants

Besides 20B15 and 49R, other mutants were studied. In theses mutants, phenotypes (low or high luminescence) were not linked to T-DNA insertion.

Genetic analysis

In 23B13, the mutant plants of the F2 generation of backcross were analyzed by diagnostic PCR. Out of 191 F2 plants, 52 plants showed wild type luminescence, 139 plants showed mutant phenotype (3:1 (wild type:T-DNA transformed) ratio, χ^2 value=0.56). In 48 mutant phenotype plants, 22 plants were homozygous T-DNA insertions, 12 plants were heterozygous T-DNA insertions, 11 plants were no T-DNA insertions and 3 plants were no PCR products. This means the mutant phenotype was not linked to the T-DNA insertion.

In 2R1, the mutant plants of the F2 generation of backcross were analyzed by diagnostic PCR. Out of 165 F2 plants, 46 plants showed wild type luminescence, 43 plants showed mutant phenotype (no luminescence) and 76 plants showed medium luminescence (higher luminescence than wild type but lower luminescence than mutant). In 46 mutant phenotype plants, 7 plants were homozygous T-DNA insertions, 18 plants were heterozygous T-DNA insertions, 18 plants had no T-DNA insertions and 3 plants gave no PCR products. This means the mutant phenotype was not linked to the T-DNA insertion.

Phenotypes of other mutants

1) Effect of phosphate starvation or sucrose on VSP1 expression

In most mutants, the pattern of phosphate starvation-induced *AVP-LUC*
expression was similar to JA–induced *AVP-LUC* expression, that is, the mutant showed
low expression of *AVP-LUC* during phosphate starvation if the plant had low expression
of *AVP-LUC* during the JA treatment. But, in the case of 38B9, *AVP-LUC* expression
level was higher than wild type in phosphate starvation, even though 38B9 is an under-
regulation mutant (Fig.3-33). One possible reason is the negative response element or
negative regulator of phosphate starvation response could be mutated in this mutant.
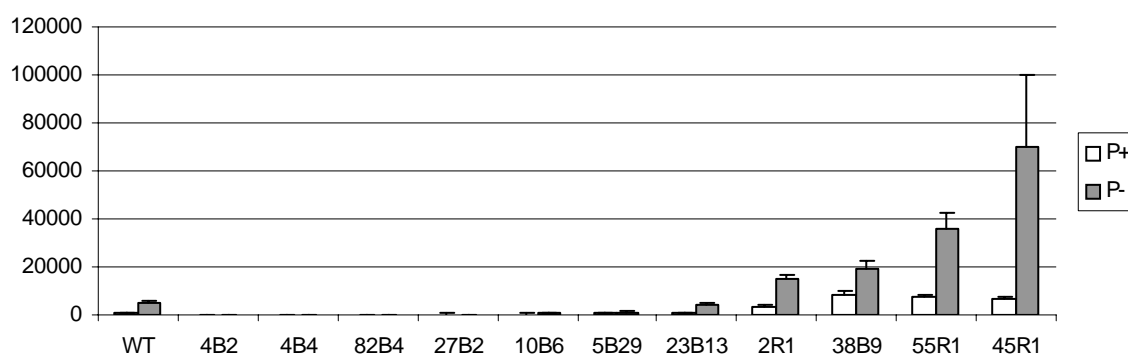
Figure 3-33. Phosphate starvation effect on *AVP-LUC* expression in putative JA-signaling
mutants. *AVP-LUC* was induced by phosphate starvation in wild type and over-regulation
mutants. Phosphate starvation response of most of the under-regulation mutants showed
similar JA response except for 38B9. Values of y-axis are luminescence intensities. Error
bar represents standard error.
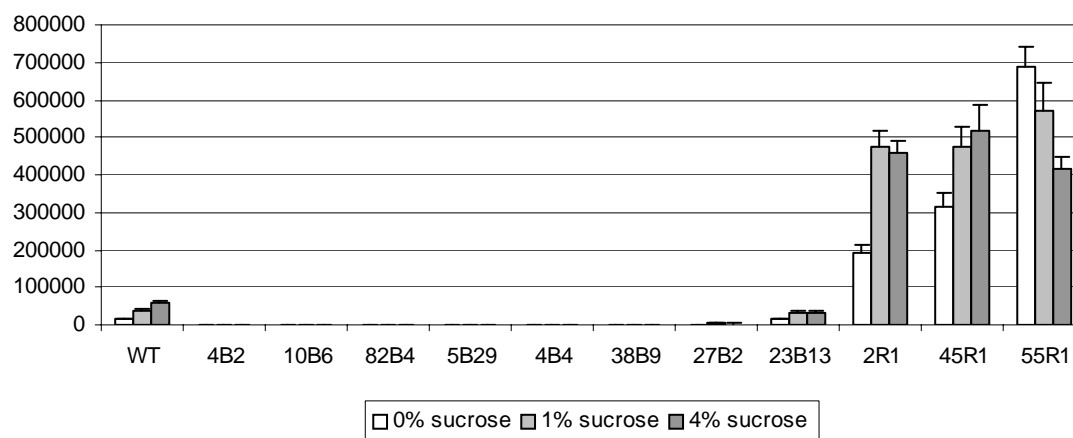
Figure 3-34. Sucrose concentration effect on *AVP-LUC* expression in wild type (WT) and putative JA-signaling mutants. *AVP-LUC* was induced by increasing sucrose concentration in wild type and one over-regulation mutant (45R1). Under-regulation mutants were little affected by increasing sucrose concentration. Four over-regulation mutants (49R1, 49R2, 49R3 and 55R1) showed that *AVP-LUC* expression was reduced by increasing sucrose concentration. Values of y-axis are luminescence intensities. Error bar represents standard error.

AVP-LUC expression in wild type plants was increased when the sucrose

concentration was increased from 0 to 4% (Fig.3-34). This was the same result reported

by Berger et al. (1995). Also, most of the putative mutants showed same pattern as the

wild type, that is the *AVP-LUC* expression level was increased by the increase of sucrose

concentration. *AVP-LUC* expression of under regulation mutants was lower than that of

wild types and *AVP-LUC* expression of over regulation mutants was higher than that of

wild types. In 2R1 and 55R1, 4% sucrose concentration inhibited *AVP-LUC* expression.

And in 55R1, even 1% sucrose caused the inhibition (Fig.3-34). These are interesting

because it might reflect a sugar response element or regulator was mutation in these plants.

2) Root growth inhibition by jasmonate

Similar root growth was observed for wild type and the mutants (Fig.3-35). Therefore, it is thought that root growth inhibition by JA in these mutants was not affected.
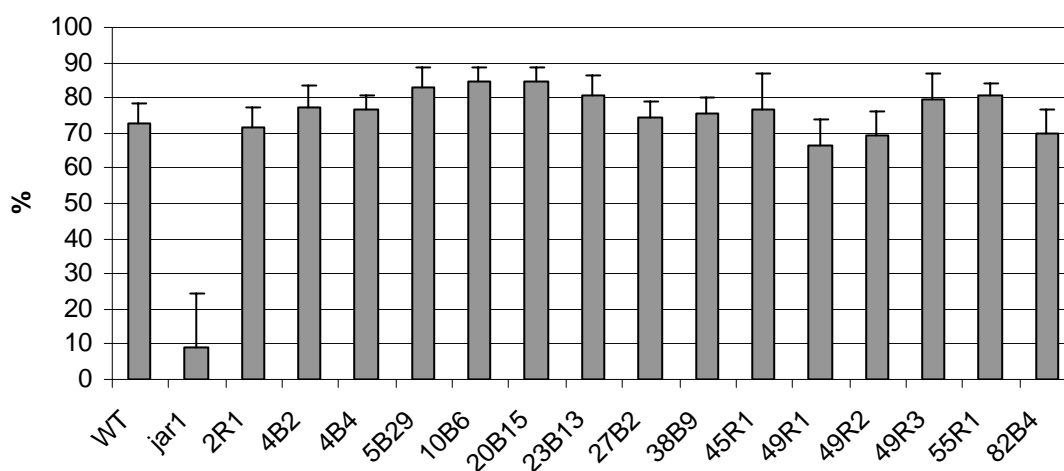
Figure 3-35. Root growth inhibition by JA. Percent reduction of root length of indicated seedlings grown on MS media containing JA (9 µM) comparing the root length of seedlings grown on MS media free of JA are shown time. The effect of JA on root growth was analyzed by one-way ANOVA and Dunnett multiple means comparisons tests. Error bars indicated standard deviation.

3) Phosphate starvation effect on root growth

82B4 also showed less sensitivity to phosphate starvation in root growth (Fig. 3-36). Conversely, 27B2 was more sensitive to phosphate starvation in root growth.
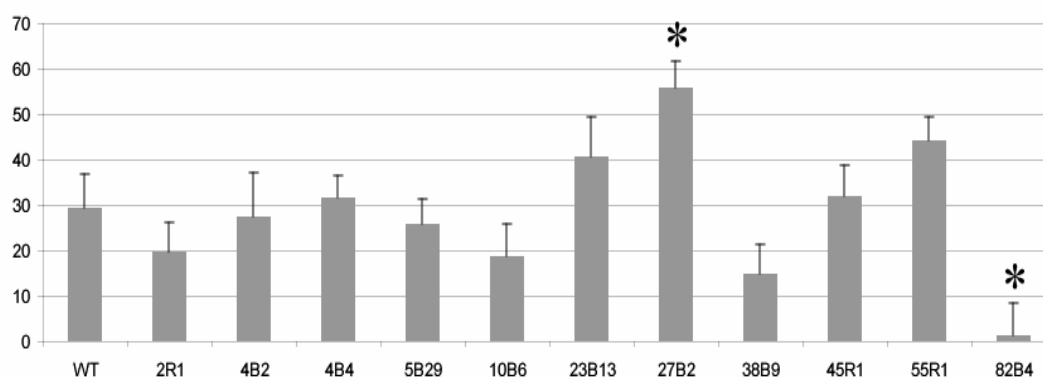
Figure 3-36. Root growth inhibition by phosphate starvation. Percent reduction of root length of indicated seedlings grown on MS media free of phosphate comparing the root length of seedlings grown on MS media containing phosphate (1.25mM) are shown. The effect of JA on root growth was analyzed by one-way ANOVA and Scheffe multiple means comparisons tests. Error bars indicated standard deviation. Asterisk shows the significantly difference from WT at p=0.05. 82B4 showed less sensitive than wild type to phosphate starvation in root growth. 27B2 was more sensitive than wild type to phosphate starvation in root growth. *Asterisk shows the significant difference from WT at p=0.05.

3.4. Discussion

I sought JA signaling mutants with under- and over-expressed *AVP-LUC* responses that would define positive and negative regulators of the JA signaling pathway, and isolated nine under-regulation mutants and six over-regulation mutants. Among

them, one under-regulation mutant (20B15) and three over-regulation mutants (49R1, 49R2 and 49R3) were further studied.

The T-DNA insertion in 20B15 was not linked to the mutant phenotype. Three lines of an over-regulation mutant, 49R1, 49R2 and 49R, had the same T-DNA insertion site (3' UTR region of At1g53540) and genetic analysis (backcrossing and analysis of the backcrossed line's progeny) suggested tight linkage between the mutant phenotype and the T-DNA insertion. At1g53540 is a 17.6 kDa small heat shock protein (sHSP). The sHSPs appear to represent a special adaptive mechanism of plants to high temperature. Some sHSPs have been demonstrated to act as molecular chaperones (Lee et al, 1995). sHSPs can protect other proteins from irreversible heat denaturation and facilitate reactivation of denatured protein. Malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase that are denatured and bound to sHSPs can then be refolded by other chaperones, such as HSP70 (Lee et al., 1997). sHSPs are also expressed during seed development and germination. Seed development is osmotically stressful: seeds are dried during maturation to less than 10% total water content and then rehydrate in a matter of hours during germination (Wehmeyer et al., 1996). sHSPs seem to play a role in late seed maturation processes, possibly involved in dormancy, desiccation tolerance and seed viability (Wehmeyer and Vierling, 2000). This sHSP (At1g53540) is developmentally regulated; it is first detected during mid-maturation, is most abundant in dry seeds, and declines rapidly during germination (Wehmeyer et al., 1996). At1g53540 is undetectable in the *abi3-6* mutant (Wehmeyer et al., 1996). The absence of At1g53540 in the *abi3* allele correlates with the lack of desiccation tolerance brought on by this allele and supports a role for At1g53540 in desiccation tolerance. These data indicate the expression

of At1g53540 in seeds is part of the normal developmental program of late seed maturation.

sHSPs contain the alpha-crystallin-type heat shock proteins domain. Alpha-crystallin-type heat shock proteins are a family of small stress induced proteins ranging from 12 -43 kDa, whose common feature is the alpha-crystallin domain. They are generally active as large oligomers consisting of multiple subunits. They are believed to be ATP-independent chaperones that prevent aggregation, and are important in refolding in combination with other Hsps (Lee et al., 1997, 2000). sHSPs are also expressed during specific stages of plant development. sHSP expression occurs during seed maturation and fruit ripening in strawberry (Medina-Escoba et al., 1998), during embryo development in alfalfa (Gyorgyey et al., 1991) and during osmotic stress in sunflower (Almoguera et al., 1992) as examples of non-heat shock stress regulation of this group. In Arabidopsis, there are examples of distinct developmental and stress regulation of HSP17.4 (At3g46230). HSP17.4 is necessary for desiccation tolerance and ABI3 is required for transcriptional activation of HSP17.4 in seed (Wehmeyer et al., 2000). Since the overall sequence of At1g53540 is very similar to At3g46230 (89.2% amino acid identity), their functions may be similar to each other.

In the 49R mutant, since At1g53540 is disrupted in the 3'UTR region, mRNA 3' end processing (cleavage, polyadenylation) could be affected. Early studies showed that the poly(A) signal on the transcript is in effect a termination signal for RNA polymerase II, since mutation of poly(A) signal leads both to the loss of mRNA through defective polyadenylation and to the continued nascent transcription of the 3' flanking regions of genes through defective termination (Whitelaw and Proudfoot, 1986; Connelly and

Manley, 1988). In addition to a requirement for the polyadenylation sequence to trigger

termination, induced pausing of the polymerase downstream of the polyadenylation site

encourages termination (Yonaha and Proudfoot, 1999). Since the cDNA was made by

using oligo-dT primer, *sHSP* cDNA of 49R mutant was made presumably by the cryptic

polyadenylation site in the T-DNA. Polyadenylation at a cryptic site in pBR322 has been

observed (Kessler et al., 1987). Cryptic polyadenylation also occurred on the 5' side of

the 3' UTR region in the transcripts of an RNA virus gene introduced into tobacco

(Kimura et al., 2005). But the efficiency of the polyadenylation signal and induced

pausing of RNA polymerase II in the 49R mutant should be lower than in wild type. The

transcription can affect the efficiency and specificity of mRNA processing. The reverse is

also true: transcription itself can be affected by mRNA processing. As with the

connections of mRNA 3' end processing and transcription, it is also becoming clear that

downstream events (subsequent export of mRNA from the nucleus to the cytoplasm) are

similarly connected. Efficient mRNA 3' end processing is required for successful release

of transcripts from transcription sites and the subsequent export of mRNA from the

nucleus to the cytoplasm (Proudfoot, 2004; Vinciguerra and Stutz, 2004). Hammell et

al., (2002) showed 3' end cleavage, polyadenylation, transcription termination and export

are tightly linked. The mRNA export adaptor Yra1p/REF and its partner, the

ATPase/RNA helicase associate with nascent mRNA during transcription (Strasser et al.,

2002). At a later stage, export adaptor recruits the export receptor Mex67p-Mtr2p which

promotes mRNP(messenger ribonucleoprotein complex) translocation by mediating

interactions with nucleoporins lining the pore (Stutz and Izaurrade, 2003; Reed and Hurt

2002). Cis-element (3' end cleavage and polyadenylation) and Trans-element (export

adaptor and export receptor) are requied for mRNA export. Both cis- and trans-acting 3'

end processing mutants block mRNA export (Hilleren et al., 2001; Brodsky and Silver,

2000; Hammell et al., 2002). mRNA 3' end processing is both necessary and sufficient

for export, and export factors can be recruited to mRNAs independently of RNA

polymerase II (Dower and Rosbash, 2002). The At1g53540 mRNA transcript of 49R

mutant, since it is most probably defective or less efficient than wild type in 3' end

processing, should have lower efficiency of export to the cytoplasm. Therefore, the actual

expression (translation) of At1g53540 in the 49R mutant should be much lower than in

wild type. Western blot analysis using sHSP-specific antibody will be able to determine

the sHSP protein expression levels in 49R mutant and wild type, pending the availability

of antibody.

One of the homologues, Hahsp17.6G1 is expressed during embryogenesis but

was not inducible by heat stress (Carranco et al., 1997). Two transcription factors

(drought-responsive element binding factor (HaDREB2) and heat stress factor A9

(HaHSFA9)) are involved in the regulation of the sHSP gene promoter (Diaz-Martin et

al., 2005). Analysis of the expression of sHSP genes in Arabidopsis mutants revealed that

additional transcriptional factors such as FUS3, LEC1, and ABI3 may cooperate to

regulate heat stress proteins. FUS3, LEC1, and ABI3 have broad effects on seed

maturation (Wehmeyer and Vierling, 2000). The At1g53540 sHSP promoter region was

analyzed using TFSEARCH: Searching Transcription Factor Binding Sites

(http://www.cbrc.jp/research/db/TFSEARCH.html). In addition to a heat shock

transcription binding site, many putative transcription factor binding sites were found

(Fig.2-26). Among them, an ADR1 (alcohol dehydrogenase regulator 1) binding site

existed (Fig.2-26). Alcohol dehydrogenase is a stress response gene and induced by osmotic stress (Xiong et al., 2002). Therefore, it is possible that this gene is induced by osmotic stress. Homeogene response elements and developmental transcription factor binding sites were also found with TFSEARCH (Fig.2-26). These developmental transcription factor binding sites may be responsible for the developmental regulation of sHSP. This sHSP is induced by heat shock and begins to accumulate when seeds enter mid-maturation stage and remains abundant through the late maturation stage and in dry seeds (Wehmeyer et al., 1996). The synthesis of sHSP during seed maturation suggests that At1g53540 might be important for protection of cellular components against desiccation.

In 20B15 mutant, an *AVP-LUC* under-regulation mutant, the *AVP-LUC* expression level was not expressed by the phosphate starvation and in all sugar concentrations (0 to 4% sucrose) and 20B15 showed lower sensitivity to phosphate starvation than wild type in root growth. 49R mutant, an *AVP-LUC* over-regulation mutant, showed higher expression of *AVP-LUC* expression in phosphate starvation and in all sugar concentration (0 to 4% sucrose) than wild type. 49R mutant plants also showed higher sensitivity to phosphate starvation than wild type in root growth inhibition. The other *AVP-LUC* under-regulation mutants (4B2, 4B4, 5B29, 10B6, 23B13, 27B2, 82B4) showed low expression of *AVP-LUC* than wild type during phosphate starvation and sucrose treatment (0 to 4% sucrose). The other *AVP-LUC* over-regulation mutants (2R1, 45R1, 55R1) showed higher expression of *AVP-LUC* than wild type during phosphate starvation and sucrose treatment (0 to 4% sucrose). But, in the case of 38B9, *AVP-LUC* expression level was higher than wild type in phosphate starvation, even though 38B9 is

an under-regulation mutant. 82B4 showed less sensitivity to phosphate starvation in root growth than wild type. Conversely, 27B2 was more sensitive to phosphate starvation in root growth than wild type.

The addition of sucrose in the presence of JA and phosphate starvation increases *VSP* expression in Arabidopsis (Berger et al., 1995). This result shows sugar can regulate JA responsive *VSP* expression. JA has also been found to induce gums in various specious such as plum, peach, cherry and apricot (Saniewskie et al., 1998, 2004). Gummosis is the process of accumulation and exudation of gums, which mainly consist of polysaccharides in some plant species. Gummosis has been recognized as a common response to various environmental stresses including biotic stresses such as pathogen infection or insect attack, and abiotic stresses such as wounding (Boothby 1983). JA is the essential factor inducing gummosis. JA substantially reduced the amount of glucose and sucrose in tulip stems and essential factor to induce gums in tulip shoots (Skrzypek et al., 2005). Composition of gum polysaccharides were quite different from that of matrix polysaccharides in tulip cell walls, suggesting that gum is not merely derived from degraded product of cell wall polysaccharides, but rather newly synthesized polysaccharides which shows JA can regulate sugar metabolism. One mechanism for use of carbon is to synthesize amino acids and protein when nitrogen is available (Champigny and Foyer, 1992). Plant cells can store excess carbon by synthesizing starch or sequestering sucrose in the vacuole (Preiss, 1984). Complex interactions are observed between C and N signaling (Scheible et al., 2004; Wang et al., 2003). Genome-wide patterns of carbon and nitrogen regulation of gene expression analysis suggest the existence of the combined carbon and nitrogen signaling in plants (Palenchar et al.,

2004). VSP can be synthesized by the mechanism for excess carbon is to be synthesized to protein (VSP) to balance carbon and nitrogen. Promoter studies in soybean *VSPB* demonstrated that *VSPB* promoter sequences between -585 and -535 mediated responses to JA, sequences between –536 and –484 were identified as important for phosphate responses, whereas the region between –486 and –427 mediated sugar responses (Tang et al., 2001). VSP expression is stimulated by sugars and repressed by phosphate (DeWald et al., 1994). Full induction of VSP mRNA accumulation in excised mature soybean leaves required 10 µM methyl jasmonate (MeJA) plus 0.2 M sucrose (Mason et al., 1992). The interaction between phosphate, sucrose and JA might regulate *VSP* expression. The -536 to -484 domain of soybean *VSP* promoter is able to activate transcription at low phosphate (Tang et al., 2001). Gel shift and DNase-I foot printing assays revealed the presence of two adjacent protein binding sites in phosphate responsive cis-element (Box II and Box III). *AVP* promoter has the ATTTTAAG which is similar to Box III sequence (ATTTTAGG). The potential binding site of the PHOSPHATE STARVATION RESPONSE 1 (PHR1) has the consensus sequence of GNATATNC (Franco-Zorrila et al., 2004). In *AVP* promoter, there is a similar sequence (GTTTATAC) which has one different base from consensus sequence. NIT-2 domain, TATCA(/T)A(/T), may be of significance in Pi starvation response because all of the nine Pi transporter promoters from Arabidopsis contain this element (Mukatira et al., 2001). *AVP* promoter has two NIT-2 domains (TATCAA). These elements (Box III, PHR1, and NIT-2) in *AVP* promoter might be responsible for phosphate starvation responses in *AVP-LUC* construct.

Trans factors can bind these elements to modify *VSP* expression. JA-responsive

expression of the *Str* (*Strictosidine synthase*) gene in *Catharanthus roseus* is regulated by

AP2/ERF-domain transcription factor ORCA3. ORCA3 is a master regulator of primary

and secondary metabolism in *Catharanthus roseus* (van der Fits and Memelink, 2000).

The JA-responsive element of the *Str* was shown to be a GCC-box-like element (Menke

et al., 1999). Promoter analysis of *PDF1.2* of Arabidopsis also showed a GCC box is

responsive to JA signaling (Brown et al., 2003). But analysis of the *VSP1* promoter of

Arabidopsis showed there is no GCC box (Guerineau et al., 2003). The JA responsive

element is an inverted repeat containing a G box like element (Guerineau et al., 2003).

The diversity of promoter elements identified so far as responsible for the activation of

transcription by JA is to be considered in connection with the various processes in which

JA is involved. A number of transcription factors activated by several signal transduction

pathways must contribute to these regulations. Promoters of nuclear-encoded

photosynthetic genes, particularly *RBCS* (encoding the small subunit of ribulose-1,5-

bisphosphate carboxylase), *CAB* (encoding chlorophyll a/b binding protein) have been

extensively studied to identify the cis-regulatory elements and the trans-acting factors

involved in their regulation. Three classes of conserved sequences, the G box, I box, and

GT box, are present in the promoter region of many light-regulated genes and have been

shown to be important in their light responsiveness (Donald and Cashmore, 1990; Puente

et al., 1996). A sugar-response sequence in the promoter of rice α-amylase gene also

contains an I-G combination, which suggests a role for these regulatory elements in

transcriptional regulation not only by light, but also by sugars (Lu et al., 1998). Studies of

functional characterization of the conserved modular arrangement 5 (CMA5), the shortest

native light-responsive element of a photosynthetic gene promoter characterized by

Martinez-Hernandez et al., (2002), showed that CMA5 is able to respond not only to light

but also to sugar signal (Acevedo-Hernandez et al., 2005). A JA-responsive element in

the Arabidopsis and soybean has G-box element (Guerineau et al., 2003; Tang et al.,

2001). Though the sugar response element of Arabidopsis *VSP* promoter was not yet

studied, analysis of soybean *VSP* promoter revealed *VSP* promoter has sugar responsive

element. In soybean *VSP* promoter study (Tang et al., 2001), the DNA region from -486

to -427 was found to mediate responses to sucrose, but not phosphate. DNase-I

footprinting assays revealed a protein binding region (Box IV) in the sugar response

domain. Box IV contains the sequence GAAATAAATTG that, like a sugar response

element (SURE) (Grierson et al., 1994; Sun et al., 2003), is AT rich (Smeekens and

Rook, 1997). *AVP* promoter has GAAATAAAACT which is similar to Box IV sequence

of soybean VSP promoter. In addition to the SURE, three different types of cis-elements

have been identified in sugar-regulated plant promoter: SP8 (Ishiguro and Nakamura,

1994), TGGACGG (Maeo et al., 2001), and B-box (Grierson et al., 1994). SP8,

TGGACGG, and B-box are not found in *AVP* promoter. Therefore the SURE could be

the sugar responsive element in *AVP* promoter.

VSP has been proposed as storage protein and might be related to photosynthesis.

All of the *AVP-LUC* over-regulation mutants showed higher *AVP-LUC* expression level

than wild type and all of the under-regulation mutants showed lower *AVP-LUC*

expression level than wild type in 0 to 4 % sucrose concentration. These show VSP

expression (and JA-signaling) could be related with sugar signaling. It is known that

sugars modulate hormone signaling at the transcriptional level. Best known case is that

glucose induces ABA and *ABI* gene expression (Arroyo et al., 2003; Cheng et al., 2002).

Interaction between sugar and ethylene signaling was found at the transcriptional level

(Price et al., 2004). Transcriptional regulation of other hormone signaling components by

sugars is also likely. Identification of the G box cis-element provides a link between

sugar effect and other environmental stress response. The G box motif (CACGTG) is

very similar to ABA responsive element (ABRE) (CCACGTGG). The ABRE-binding

factors ABF2, ABF3 and ABF4 have been implicated in sugar signaling (Kang et al.,

2002; Kim et al., 2004).Therefore analysis of the cis- and trans- factors of *AtVSP* will be

necessary to define biochemical pathways that link carbon metabolism, phosphate, *VSP*

expression and other signal transduction. In the case of the 49R mutant, *AVP-LUC*

expression in wild type plants was increased when the sucrose concentration was

increased from 0 to 4%. This is the same result reported by Berger et al. (1995). The 49R

mutant showed a similar pattern as the wild type, that is the *AVP-LUC* expression level

was increased by the increase of sucrose concentration. But in the 49R mutant, the basal

level of *AVP-LUC* expression was 15.8 fold higher than in wild type (Fig.2-28B). Since

sHSP was disrupted in the 49R mutant by T-DNA insertion, higher expression of *AVP-*

LUC under these phosphate starvation and sucrose treatments suggests that sHSP reduces

the sucrose response and phosphate starvation of *AVP-LUC*. In *AVP-LUC* over-regulation

mutants (2R1, 45R1, 49R and 55R1) and one under-regulation mutant (38B9) showed

high basal level of *AVP-LUC* expression in the phosphate rich condition. In these

mutants, positive phosphate starvation trans-factor might up-regulates the *AVP-LUC*

expression possibly because of the disorder of phosphate starvation signal. Disorder of

phosphate starvation signal could cause up-regulation of *AVP-LUC* expression in

phosphate rich condition. PHR1 is a master transcriptional activator of the phosphate

starvation response in Arabidopsis (Rubio et al., 2001). NIT-2 also is thought to be a

transcriptional activator of the phosphate starvation condition. Because PHR1 and NIT-2

responsive domain might be responsible for phosphate starvation responses in *AVP-LUC*

construct (described above), disturbance of the responses in these element can cause up-

regulation of *AVP-LUC* expression in the phosphate rich condition. High expression of

AVP-LUC can be come from the constitutive up-regulation of phosphate starvation

response. There is also a possibility that regardless of cis-element of phosphate

starvation, *AVP-LUC* expression level can be up-regulated by another master positive

trans-element as the case of the regulation of JA-responsive *Str* (*Strictosidine synthase*)

gene in *Catharanthus roseus* as described above. Over-regulation mutants showed high

expression of *AVP-LUC* in 0 % sucrose condition which also suggests a possibility that

up-regulation of positive sucrose cis-element in *AVP* promoter. But in this case,

activation of JA responsive element also can up-regulate *AVP-LUC* expression because

after sucrose treatment, JA was treated before the measurement of *AVP-LUC* expression

level. The higher expression of *AVP-LUC* in the condition of higher sucrose

concentration and phosphate starvation could be due to the activation of positive

responsive elements in *AVP* promoter. Since *AVP* promoter is thought to have phosphate

starvation response element and sucrose response element, the increased level of *AVP-*

LUC expression of 49R mutant in phosphate starvation and higher sucrose concentration

(Figure 2-28) means higher activation of phosphate starvation and sucrose responsive

elements in *AVP* promoter of 49R mutant. In addition to 49R mutant, *AVP-LUC*

expression levels of all over-regulation mutants (2R1, 45R1, and 55R1) in both phosphate

starvation in the absence of JA and 1 to 4 % sucrose concentration in the presence of JA condition were higher than wild type (Fig. 2-33, Fig. 2-34). This means all of the three responsive cis-elements (JA, sucrose and phosphate responsive elements) in the *AVP* promoter could be activated in the over-regulation mutants. In all under-regulation mutants except 38B9, both phosphate starvation in the absence of JA and 1 to 4 % sucrose concentration in the presence of JA condition could not induce *AVP-LUC* expression. Possibly, another master cis/trans regulator that can regulate these three responsive cis-elements (JA, sucrose and phosphate responsive elements) could be involved in *AtVSP* expression. In the case of 38B9, *AVP-LUC* expression level was higher than wild type in phosphate starvation, even though 38B9 is an under-regulation mutant (Fig.2-33). This result shows the signal from JA and phosphate are not always connected. In soybean *VSP* promoter study, Mason et al. (1993) demonstrated that a 50bp DNA region of *VSP* promoter (-585 to -535) could mediate responses to JA. The -536 to -484 domain without the JA responsive domain of *VSP* promoter stimulated transcription in low phosphate concentration (Tang et al., 2001). These data shows *VSP* promoter has JA-specific and phosphate-specific cis-element. Therefore the signal from JA or phosphate may not overlap.

Recently, it was shown that AtMYC2 interacts with light-responsive element of light-regulated promoters. AtMYC2 acts a negative regulator of blue light-mediated photomophogenic growth and blue and far-red-light regulated gene expression (Yadav et al., 2006). Potato MYC transcription factor, the JAMYC2 and JAMYC10 proteins recognize AAACGTG element (T/G-box motif) as the binding site for the JAMYC transcription factors (Boter et al., 2004). Noteworthy, G-box (CACGTG) sequence motifs

are preferential targets for MYC-like bHLH-ZIP DNA-binding proteins (Toledo-Ortiz et al. 2003). The Electrophoretic mobility shift assay studies of Boter et al. (2004) showed G-box element oligonucleotides are able to compete efficiently for JAMYC binding to the AAACGTG motif. This result suggests that there is MYC2 binding site in *VSP* promoter because soybean and Arabidopsis *VSP* promoter has G-box element. Analysis of the light-regulated gene expression in *atmyc2* mutants revealed that AtMYC2 represses the expression of *CAB*, *RBCS* which are important for photosynthesis (Yadav et al., 2006). The addition of sucrose causes an increase in hexose-phosphate and reduction in cytoplasmic phosphate level (Krapp et al., 1993). The reduction of cytoplasmic phosphate concentration stimulates the synthesis of sucrose by activating sucrose phosphate synthase (Huber and Huber, 1992). Synthesis of sucrose from triose-phosphates releases phosphate. Therefore sucrose production by photosynthesis and phosphate level could be inversely related. Since MYC2 is the repressor of photosynthesis, MYC2 expression by JA could decrease sucrose level and in turn, increase the level of phosphate. Therefore MYC2 and phosphate may counteract each other.

Biochemical responses to phosphate starvation are directed at maintaining a constant cytoplasmic phosphate concentration (Abel et al., 2002). Such metabolic adaptations involve enhanced phosphate uptake as well as synthesis and secretion of enzymes that increase phosphorus availability. Inducible acid phosphatases are an example of secreted enzymes that are proposed to release phosphate from organophosphates in the rhizosphere (Ticconi and Abel, 2004). VSPs are localized in

vacuoles in soybean leaves (Franceschi et al., 1983). Since VSP is not supposed as a secreted form of acid phosphatase, it may be involved in internal phosphate conservation.

Phosphate starvation also inhibits primary root growth and causes more generation of root hairs (Lenin et al., 2005; Williamson et al., 2001). JA also has a similar effect on root growth (Staswick et al., 1992). 20B15 (*AVP-LUC* under-regulation mutant) had no *AVP-LUC* expression under phosphate starvation or high sucrose concentration and showed less sensitivity to phosphate starvation than wild type in root growth. 49R (*AVP-LUC* over-regulation mutant) had higher *AVP-LUC* expression under phosphate starvation and high sucrose concentration than wild type, and showed more sensitivity to phosphate starvation than wild type in root growth. Ethylene and auxin are known to be involved in the regulation of root hair development (Kieber et al., 1993; Tanimoto et al., 1995; Masucci and Schiefelbein, 1994; Lincoln et al., 1990). JA has been known to inhibit root growth (Staswick et al., 1992). Recently JA has been shown to have a pronounced effect on promoting root hair formation (Zhu et al., 2006). AtMYC2 is a positive regulator for *AtVSP* expression (Boter et al., 2004) in JA signalingAtMYC2 also functions as a positive regulator of lateral root formation (Yadav et al., 2006). Since 20B15 is an under-regulation mutant of JA signaling, AtMYC2 expression level of 20B15 should be lower than wild type. Therefore, lateral root growth would be inhibited and primary root growth would be increased. Less inhibition of primary root growth in 20B15 can be explained by low expression of AtMYC2 in 20B15. On the contrary, 49R is an over-regulation mutant of JA-signaling, AtMYC2 expression level of 49R should be higher than wild type. More inhibition of primary root growth in 49R mutants can be explained by high expression of AtMYC2. In other JA-signaling mutants, the results were

various. 82B4 showed less inhibition of primary root growth which is same as 20B15.

But, in the case of 27B2, primary root growth under phosphate starvation was much more

inhibited than in wild type (Fig. 2-36). Another regulator of root growth could be

involved in the root growth phenotypes of JA signaling mutants. Possibly, the low

expression of *AVP-LUC* under phosphate starvation and high sucrose concentration in

20B15 resulted from the failure of phosphate and/or sucrose signaling. Conversely, the

high expression of *AVP-LUC* under phosphate starvation and high sucrose concentration

in 49R may result from the increase of phosphate and/or sucrose signaling. Further study

of interactions between phosphate signaling and JA signaling may reveal the reason for

root growth phenotypes of these mutants.

One of the regulators of root growth under phosphate starvation in Arabidopsis is

AtSIZ1 (Miura et al., 2005). AtSIZ1 is a plant small ubiquitin-like modifier (SUMO) E3

ligase and a focal controller of phosphate starvation-dependent responses (Miura et al.,

2005). The mutations in *AtSIZ1* enhance sensitivity of Arabidopsis plants to phosphate

starvation based on morphological responses, including reduction in primary root

elongation. That is, AtSIZ1 is negative regulator of primary root growth under phosphate

starvation. SUMO is a member of the superfamily of ubiquitin-like polypeptides that

become covalently attached to the various intracellular target proteins as a way to alter

their function, location, and/or half life (Kurepa et al., 2003). Sumolation is activated by

stress and some SUMO targets are the part of the stress response in animals (Melchior,

2000). Stress conditions including heat and H_2O_2 induced a dramatic increase in SUMO

conjugates (Kurepa, et al., 2003). Therefore, potential targets of SUMO include the HSF

(heat shock factor)-like proteins whose activation promotes the stress response (Nover et

al., 1996). The transcriptional regulatory protein HSF1 is the key mediator of induced heat shock protein gene expression in response to elevated temperature and other stress (Cotto and Morimoto, 1999). In response to stress, HSF1 acquires DNA binding ability and localizes to the nucleus (Jolly et al., 1999). HSF1 undergoes stress-induced modification at lysine 298 by the SUMO in animal cells (Hong et al., 2001). Heat shock-induced sumolation is reduced in *siz1* plants (Miura et al., 2005). Therefore, the heat shock protein could be involved in sumolation and root growth phenotype. The *sHSP* (At1g53540) promoter has four putative HSF binding regions (Fig.2-26). Since *sHSP* is disrupted in 49R mutants, *sHSP* expression is not responsive to sumolation which is induced by phosphate starvation. AtSIZ1-mediated primary root growth could involve *sHSP*. If this is the case, less sensitivity of primary root growth to phosphate starvation in 49R mutants can be explained by the disruption by *sHSP*.

sHSP can be considered an inhibitor of JA signaling because 49R mutants with have disrupted *sHSP* have higher *AVP-LUC* expression. In 49R mutants, disruption of *sHSP* in 49R mutants might cause the increase JA-signaling so that *AtVSP1* promoter is up-regulated. In addition, since *sHSP* is thought to be responsive to osmotic stress, a drought-responsive element may exist in the *sHSP* promoter. Transcription factor(s) (such as a drought-responsive element binding factor) may be involved in the regulation of the *sHSP* gene promoter. The target of sHSP should be related to JA-signaling. To reveal the relationship between small heat shock protein and JA-signaling, finding transcription factors and target proteins of sHSP will be needed.

3.5. Future work

Finding out the responsive gene to the mutant phenotype of 20B15

Since the T-DNA insertion is not linked to the mutant phenotype of 20B15, finding the responsible gene is important to further study of 20B15. Map-based cloning could be used for finding the responsible gene. With the sequencing of whole genomes of *Arabidopsis thaliana* and the development of saturating marker technologies, map-based cloning can now be performed efficiently (Peters et al., 2003).

Confirmation that sHSP is a repressor of JA-signal transduction

I propose sHSP may repress JA signal transduction (Fig. 3-37). To confirm that sHSP is the repressor of JA-signal transduction and to find a function of sHSP, finding out the target of sHSP is necessary. Finding sHSP-interacting proteins by the yeast two-hybrid method using sHSP protein as a bait to find out target protein that interacts with sHSP is a direct way to confirm sHSP is a repressor of JA signaling. sHSPs are important for osmotic stress (Almoguero et al., 1992; Almoguera et al., 1993; Coca et al., 1996; Sun et al., 2001). Osmotic stress induces *VSP* expression in soybean (Mason et al., 1990). *VSP* is one of the representative JA responsive genes. Since osmotic stress induces *VSP* expression, higher expression of VSP in 49R mutant in osmotic stress indicates another suggestion that sHSP is a repressor of *VSP* expression. If this is the case, this result will suggest sHSP is repressor of VSP expression.
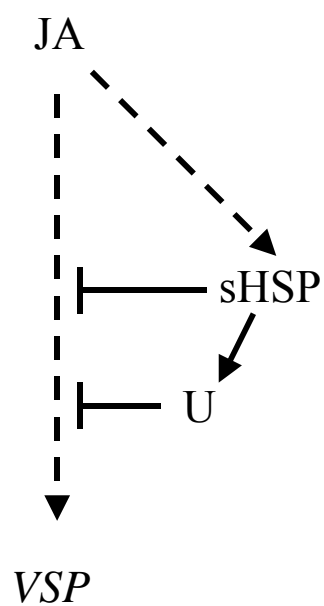
Figure 3-37. Model of JA signal transduction involved in sHSP. JA induces sHSP, then, the sHSP can repress JA-signaling directly or indirectly via an unknown component (U).

CHAPTER IV

CONCLUSIONS

The insect digestive system is the first line of defense against a broad spectrum of toxins and anti-nutritional factors in their diet. The insect alimentary tract not only breaks down complex food molecules into simple components, but plays a role in protecting the vulnerable cells and tissues of the insect body from various dietary toxins. Far more than providing a mere physical barrier, this protection takes the form of changes in gene expression and protein accumulation in the cells lining the digestive tract. These changes represent key processes enabling insects to adapt to the presence of specific plant toxins, such as protease inhibitors, in the diet. Further studies will be necessary to delineate functions of all responding genes in coping with dietary scN and regaining normal insect growth and development.

A screening strategy using T-DNA insertion mutagenesis and *AVP-LUC* as a reporter enabled discovery of JA-signal transduction mutants of *Arabidopsis thaliana*; nine under- regulation mutants and six over-regulation mutants. T-DNA insertion sites were found by TAIL-PCR analysis. The expression level of *VSP* and other JA responsive genes were measured by Northern blot, RT-PCR and quantitative real time PCR analysis. Other JA-related properties such as JA effect, phosphate starvation and sucrose concentration effect on *VSP* expression and root growth were studied. 20B15 (T-DNA insertion in At5g04500 which encodes glycosyl transferase family protein) showed reduced *VSP1*, *THI2.1* expression and increased *PDF1.2* expression compared to wild types when treated with JA, which are strongly suggestive of a JA signaling mutant. This

plant showed a stunted growth phenotype in the adult stage but normal growth in the seedling stage. The silique number of 20B15 was 1.5 fold higher than wild type in 18°C growth condition but 2.5 fold lower than wild type in final yield of seeds. The reason is that the silique size of 20B15 is smaller than wild type. This mutant plant also showed less sensitivity to phosphate starvation than wild types in root growth inhibition. Homozygous T-DNA insertion SALK line in the exon region of At5g04500 was tested as to whether this SALK line plant (SALK_002825) showed the mutant phenotypes that were shown in 20B15. SALK_002825 plants showed less sensitivity to phosphate starvation in root growth, but had normal growth phenotype and *VSP1* expression level compared with the wild type. SALK_002825 showed higher *VSP1* expression in control (without JA) state, so the *VSP1* induction fold by JA in SALK_002825 was much lower than the wild type but similar to 20B15. Back cross of 20B15 and analysis of next generation of back cross line showed the mutant phenotype was not linked to the T-DNA insertion. Therefore, the At5g04500 gene was not a JA signaling component, but this gene could be involved in *VSP1* expression regulation and phosphate starvation response in root growth. Genetic study showed there was only one T-DNA in 20B15, so the JA-signaling mutant phenotypes could be coming from point mutation in 20B15. In addition to 20B15, 82B4 also showed less sensitivity to phosphate starvation and more susceptibility to insect pathogen, thrips. 82B4 has a non-homozygous T-DNA insertion with a homogeneous mutant phenotype, meaning the mutant phenotype is not linked to the T-DNA insertion. A search for the gene responsible should be pursued. Conversely, 27B2 showed more sensitivity to root growth inhibition during phosphate starvation than

wild type. The T-DNA insertion sites of 27B2 could not be determined because there was no TAIL-PCR product in 27B2.

Among the mutants, 49R1, 49R2 and 49R3 plants showed about 10-fold higher *AVP-LUC* expression level than wild type when JA treated. 49R1, 49R2 and 49R3 had the same T-DNA insertion site (At1g53540). Diagnostic PCR showed these plants had homozygous T-DNA insertions. Backcross and following F1 and F2 analyses showed the mutations in these plants were recessive and demonstrated tight linkage between mutant phenotype and T-DNA insertion in At1g53540. Mutants 49R2 and 49R3 showed higher expression of *LUC* (*luciferase*) expression. These mutant plants also showed less sensitivity to phosphate starvation than wild types in root growth inhibition. Complementation and double strand RNA inhibition analysis of this gene could give evidence of At1g53540 involvement with mutant phenotypes.

JA initiates JA signal transduction and eventually induces VSP expression. Since I found *sHSP* was induced by JA and lower induction of *sHSP* was observed in 49R mutant (T-DNA inserted in the exon of *sHSP*), I propose that sHSP may repress JA signal transduction (Fig. 3-37). sHSP can repress JA-signaling directly or indirectly via an unknown component (U in Fig. 3-37).

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