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The Hormonal Characterization and Mapping of a New Jasmonate Hypersensitive Mutant (jah2) in Arabidopsis thaliana

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The Hormonal Characterization and Mapping of A New Jasmonate Hypersensitive Mutant (jah2) in Arabidopsis thaliana

By

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ABSTRACT

Jasmonic acid (JA) is a phytohormone that plays important roles in growth, development, and defense in plants. Although many functions and mechanisms of JA are known, some pathways are still unclear. One of the simplest approaches to understanding JA signal transduction is isolating mutants that respond abnormally to JA. From the defects of such mutants we can determine the regulatory functions of JA. *jasonate hypersensitive 2* (jah2) is a JA hypersensitive mutant. JA normally inhibits the root growth of plants and the jah2 mutant has an even shorter root compared with wild-type Col-0 (wild type) under the same JA concentration. Examination of the sensitivity of jah2 showed that jah2 and WT have a 100-fold JA-sensitivity difference. While jah2 was hypersensitive to JA, it was not hypersensitive to two other hormones, IAA and the precursor of ethylene, ACC. Two
types of PCR-based markers (CAPS and SSLP) were used to map jah2. The jah2 mutant (in Col-0) was crossed with Ler to generate recombinants with sequence polymorphisms between the two ecotypes. F2 mutants homozygous for the jah2 phenotype were selected from JA-containing agar media. Initially, bulk segregant analysis was used until a linked marker was found; afterwards individual F2 mutants were tested. The jah2 area was narrowed down to a region of about 177kb on Chromosome V. Illumina whole genome sequence analysis of jah2 was compared with a reference wild type sequence. The jah2 mutant had two G-A single nucleotide transitions within the mapped area. One was in an intergenic region and the other was in intron six of glutathione synthetase gene (GSH2). This suggests that GSH2 is the mutated gene in jah2, which is consistent with previous findings that JA signaling has some interactions with glutathione and redox signaling in plants.
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Chapter I: Introduction to Jasmonate Signaling

Like animals, plants have a variety of hormones, some of which are systemic, acting at a distance from the site of synthesis. Phytohormones include both peptide and endogenous small chemical molecules that function at low concentrations and act as signal transducers. They can have different effects depending on dose and tissue location. Growth, development, and cellular differentiation of plants are regulated and coordinated by hormones when plants receive the stimulations of environmental and developmental changes. To have complete plant development, hormones must have the proper concentrations in target tissues at each stage. The development of plants is controlled by genetic and environmental effects on gene expression related to cell growth (cell division/ enlargement/ differentiation) through the regulation of hormones. In addition, plant hormones play pivotal roles in signaling plant defense responses, generally through changes in gene expression. These responses help to protect plants from damage caused by pathogens, insects, and other herbivores, as well as abiotic environmental factors.

Many phytohormones have been identified including indole-3-acetic acid (IAA), ethylene, gibberellins, abscisic acid, cytokinins, brassinosteroids, salicylic acid, and jasmonates. Each hormone has its own specific functions; however, plant growth/
development are not the result of single hormones, but are due to combinations of several hormones [1]. Furthermore, ratios of different hormones must be considered since hormones are coordinated with each other by changing concentrations. For instance, the ratio of auxin and cytokinin controls embryogenesis [2]; gibberellins and abscisic acid are antagonistic in several developmental processes [1]; and jasmonic acid (JA), salicylic acid (SA), and ethylene modulate pathogen and insect resistance – sometimes in cooperation and other times in antagonistic ways [3].

Among these hormones, JA plays an essential role in development, growth, and defense in plants. In ancient times as well as now, the methyl ester form of JA (MeJA) was utilized as a perfume ingredient. MeJA was first extracted from flowers of *Jasminum grandiflorum* and *Rosmarinus officinalis* in 1962 [4]. In 1971, JA was identified in a fungus and began to be considered as a plant regulator (plant growth inhibitor) [5]. Until the 1980s, many inhibitory effects of JA on plants were observed in laboratories in Japan, and scientists in Germany detected differences between JA and abscisic acid [5]. These early studies supported the idea that JA is a hormone as it could control plant growth and had effects on gene expression [5]. However, it was not until later when details of the jasmonate signaling pathway began to emerge that its status as a phytohormone was generally accepted.
Jasmonate is now known to have a crucial role in the defense response in plants to biotic wounding induced by insect or pathogen attack and by abiotic stresses, including wounding, water deficiency and UV light. A defect of the JA active form results in Arabidopsis becoming more susceptible to pathogens. Jasmonate can also affect developmental mechanisms such as root growth, seed/pollen germination, and flower development, as well as tendril coiling, nyctinasty, and senescence [6].

“Jasmonates” refers to the JA-related oxylipins such as its methyl ester (MeJA) [5, 6, 7], various biosynthetic intermediates and metabolites of JA. This term is often used to denote several of these molecules as a group, or if there is ambiguity about which molecules are active. The structure of jasmonic acid consists of a cyclopentanone with a carboxylic acid and an aliphatic side chain of five carbons (see Figure 1). It is a volatile fatty acid-derived compound and α-linolenic acid (LA) [8], the precursor to JA synthesis, is released from chloroplast membranes [6, 9] when plant cells are damaged. After plant injury, many JA biosynthetic enzymes such as lipoxygenase (LOX) [10, 11], allene oxide synthase (AOS) [12, 13], and allene oxide cyclase (AOC) [9] are activated, leading to rapid synthesis of JA. LA is converted to 12-oxophytodienoic acid (OPDA) in chloroplasts by action of LOX, AOS, and AOC; then, OPDA is transferred to peroxisomes by an ATP-binding cassette transporter [5, 7]. After OPDA enters the peroxisomes, it undergoes a reduction catalyzed by
12-oxo-phytodienoic acid reductase and three rounds of β-oxidation, finally producing jasmonic acid [5, 7] (see Figure 2). Moreover, the resulting increase in JA can activate phospholipases and lead to more LA being released from membranes producing more JA [6].

In the JA chemical structure, there are two chiral centers at C-3 and C-7, and each of them contains two configurations. Therefore, JA can have 4 different stereoisomeric forms, but naturally occurring JA has the R configuration at C-3 with S or R configuration at C-7, (3R, 7S)- JA and (3R, 7R)-JA [14, 15]. The isomer synthesized by plants is (3R, 7S) with cis side chains, also called (+)-7-iso-JA, and it readily undergoes isomerization in vitro to form (3R, 7R) with trans side chains, also called (-)-JA (see Figure 1) [15].

Scientists now understand the basic mechanism of jasmonate signal transduction; however, some of the details of the mechanism are still unclear. Essential to determining the pathway of JA biosynthesis has been the identification and study of JA-related mutants with abnormal phenotypes or response to JA [16]. The following sections will discuss how some of these mutants have given us insights about how jasmonates function.

One of the surprising discoveries about jasmonate signaling is that JA itself is not
the active hormone. JA must be conjugated to an amino acid to produce the bioactive jasmonate. JA added to plants does produce physiological effects but this is because JA is metabolized *in vivo* to produce the conjugated form of JA. The activity of conjugated JA contrasts with other low molecular weight plant hormones, such as IAA, that are inactive when conjugated [17]. The mutant *jasmonate resistant 1* (*jar1*) was the first *Arabidopsis thaliana* mutant to be identified that showed altered response to JA and MeJA [5]. These jasmonates can inhibit root growth. When plants have no functional JAR1, there is reduced root inhibition when plants are grown on agar media containing JA. However, if the agar medium is supplemented specifically with JA-Ile, root inhibition will be observed to the same extent as in wild type plants [18]. JAR1 encodes an ATP-dependent JA-amido synthetase that catalyzes the conjugation of JA to isoleucine through an amide linkage [18]. The defect in JAR1 also makes Arabidopsis more susceptible to pathogens [19], ozone, and causes other defects in jasmonate response. JA biosynthesis is finished in the peroxisomes, while JAR1 is found in the cytosol [18, 20]; therefore, JAR1 likely works in the cytosol, not the peroxisomes. JA must be transferred to the cytosol for JA-Ile formation, and this transfer step may be one way of regulating JAR1 activity [18]. Interestingly, JAR1 may not be the only enzyme that can synthesize JA-Ile to trigger JA-involved genes expression. Other unknown enzymes or mechanisms appear to conjugate JA since
This text describes the effects of certain mutations on the JA-Ile signaling pathway in Arabidopsis and the role of the bacterial virulence factor coronatine (COR) in rescuing the jarl mutant. The natural formation of JA, (+)-7-iso-JA, can undergo isomerization to (-)-JA. Recently, (+)-7-iso-JA was shown to be the active precursor for JA-Ile formation because JAR1 prefers (+)-7-iso-JA as a substrate. Studies have also shown that (+)-7-iso-JA-Ile is the active hormone configuration, but (-)-JA-Ile has little or no activity to function with the JA-Ile hormone receptor (COI-JAZ complex). The jarl mutant can be rescued by the bacterial virulence factor coronatine (COR) — a structurally related molecular mimic of (+)-7-iso-JA-Ile, but not appreciably by (-)-JA-Ile. (+)-7-iso-JA-Ile converts easily to (-)-JA-Ile under elevated temperature and high-pH; however, (-)-JA-Ile does not readily convert back to (+)-7-iso-JA-Ile. Nevertheless, (+)-7-iso-JA-Ile is fairly stable under acidic or neutral pH status at room temperature. In tomato, (+)-7-iso-JA-Ile is the major isomer synthesized in response to wounding of leaves and it remains stable in leaves for up to 6 hr after wounding. So, epimerization does not appear to be a significant mechanism to inactivate the jasmonate signal. There is another regulatory strategy for the jasmonate signal in Arabidopsis. The hydroxylation of (+)-7-iso-JA-Ile to form inactive 12-OH-JA-Ile is a mechanism to remove JA-Ile from the active hormone pool [23].
The receptor for JA-Ile is CORONATINE-INSENSITIVE 1 (COI1), together with a jasmonate ZIM domain co-receptor that is a transcriptional repressor called JAZ [24, 25]. Binding of the active JA-Ile conjugate to this receptor complex promotes ubiquitination of JAZ followed by its degradation leading to activation of numerous jasmonate-responsive genes. So far, 12 JAZ repressors have been found (JAZ1-JAZ12) in the Arabidopsis JAZ family [21], and there is only one COI1. The Arabidopsis COI1-deficient mutant is insensitive to COR and JA-Ile. Thus, COR binding to the receptor of JA-Ile affects JA-induced gene expression in a similar manner as for JA-Ile [24]. Unsurprisingly, coi1 mutants do not have the normal jasmonate response after JA or JA-Ile treatment or following biotic stimulation. Their roots are not inhibited by either JA or JA-Ile. They are also male-sterile, which cannot be rescued by JA treatment, and they are susceptible to pathogens and insects that are known to stimulate to jasmonate signaling pathway for plant defense responses as well [26]. COI1 plays a critical role in most jasmonate- regulated mechanisms although a few jasmonate-dependent but COI1- independent responses have been identified [27]. The mechanism for this alternate signaling is not known.

The COI1 gene encodes a 67kDa protein that has 16 leucine-rich repeats and one F-box motif. Both sequences are required for regular COI1 function, and the COI1 protein is considered an F-box protein because of the F-box motif [24, 26, 28]. Some
F-box proteins act as receptors that bind to other hormones, such as IAA and gibberellic acid, and subsequently trigger ubiquitination and degradation of other target transcriptional repressor proteins. Other F-box proteins also function in a large number of signaling pathways that do not involve hormones. The COI1 F-box protein is part of the SCF complex, which combines with two other protein subunits Skp1 and Cullin to form the E3 ubiquitin ligase complex that effects ubiquitination.

Ubiquitination plays a key role in many hormone signal transduction pathways [29] and SCF complexes have different F-box proteins that act as the specificity factors for protein targeting. For instance, auxin has TIR1/AFB as the F-box protein, gibberellin has SLY/GID2, and jasmonate has COI1 [30]. Although COI1 is the F-box protein and binds with JA-Ile, it is necessary for COI1 to bind with the JAZ repressor to have high affinity for JA-Ile [25]. JA-Ile has low affinity for COI1 protein alone and displays no affinity for the JAZ repressor [25]. Moreover, JA-Ile is a trigger for the interaction of COI1 and JAZ. Importantly, JA and MeJA, as well as several other JA-amino acid conjugates, do not have this function and are not therefore considered as COI1-dependent jasmonate signals [7].

To understand the complete JA-Ile/COI1 signaling system, it is necessary to further mention the JAZ repressor and MYC2 transcription factors. JAZ repressors are essential regulators in JA-Ile signaling, they prevent MYC2 and other transcription
factors from activating jasmonate response genes. So the normal state of these genes in the absence of the JA-Ile signal is transcriptionally repressed. JAZ proteins are members of the ZIM-domain proteins that have two conserved motifs, a ZIM motif with conserved 28 amino acids and a Jas motif with 26 amino acids [7]. Both motifs are required for a normal ZIM-domain protein. In Arabidopsis, these proteins are expressed at different levels when plants encounter herbivore attack or mechanical wounding. These different JAZ repressor expression levels suggest that plants distinguish that the wounds from a biotic or an abiotic source allowing the response to differ according to the stimulus [21].

MYC2 is a nuclear-localized basic helix-loop-helix zipper transcription factor that is associated with jasmonate signaling [31, 32]. It can be an activator or repressor in JA-responsive gene expression in Arabidopsis [33]. Moreover, MYC2 protein can repress the defense response to pathogens and activate JA-related systemic responses to wounding [32, 33]. In fact, the MYC2 protein not only functions in JA defense or wound response systems, it is involved in many different mechanisms as well. For example, MYC2 is a positive regulator in abscisic acid-dependent drought responses, and is essential in oxidative stress tolerance [33]. From the coi1 mutant analysis, results show that expression of both JAZ family genes and MYC2 protein are COI1-dependent [21]. MYC2 is only one of the transcription factors interacting with
JAZ repressors, but others have not been well characterized.

In summary, when the basic JA defense responsive mechanism in plants is elicited, JAR1 converts JA to the active form of JA-Ile. JA-Ile from the cytosol enters the nucleus where MYC2 and JAZ repressor are located. JA-Ile promotes binding of COII F-box protein with JAZ proteins, allowing ubiquitination of JAZ proteins and their degradation by the 26S proteasome. This results in MYC2 and other transcription factors being released from the JAZ repressor, permitting JA-induced genes to turn on (see Figure 3). The products of these genes can then produce biochemical and physiological changes typical of a jasmonate response.

**Mutants in Jasmonate Signaling Analysis**

In studies of plant hormone signal transduction pathways, genetic screens have been utilized widely to help us identify and understand the interactions of different components [34]. So far, the JA-related mutants can generally be classified as two mutant types: biosynthetic mutants and JA-signaling mutants [16]. Some mutants were mentioned in the previous paragraphs and more details will be included here.

The biosynthesis mutants help the biosynthetic mechanisms to be understood. Some examples from Arabidopsis are fatty acid desaturation (*fad*), 12-oxophytodienoic acid reductase (*opr3*), and allene oxide synthase (*aos*). These
mutants are male sterile because they produce essentially no JA but fertility can be recovered by JA treatment of flower-buds [35, 36]. In these mutants treatment to add the missing downstream products can restore the normal phenotypes. After specific JA-precursors are tested, the order of the missing intermediates and effects on phenotypes can be determined. The jar1 mutant is also defective in jasmonate biosynthesis. It produces essentially normal levels of JA but fails to produce sufficient amounts of JA-Ile. In this case the defect is corrected only by adding the final product, JA-Ile.

The rest of this section will describe jasmonate signaling mutants. To identify these mutants in Arabidopsis, the simplest way has been to select seedlings growing on the JA-containing media and observing the root phenotype. Normally, JA and JA-Ile cause roots to grow slower. Mutagenized seeds can be compared with the wild-type on jasmonate containing medium, and those seedlings with the altered phenotype (e.g., longer root) may be caused by mutation of a gene essential for jasmonate signaling. Generally, the signaling mutants are of two types: jasmonate insensitivity or jasmonate hypersensitivity (shorter roots) and both types have been found. In contrast with biosynthesis mutants, adding the signal or downstream intermediate to the media does not correct the defect in signaling mutants.
JA insensitive mutants provide the opportunity to understand signaling mechanisms or essential features such as the active form of JA and the receptor, if the defective gene in the mutant can be identified and functionally characterized. Two examples from the previous paragraphs are mentioned here. *coil* has a defect in the COII receptor which results in suppressing the JA signaling and no expression of JA-induced genes. *myc2* is also JA-insensitive because it lacks one of the transcription factors necessary for control of jasmonate responsive genes.

Recently, two JA-hypersensitive mutants were found and they reveal more details about the jasmonate signaling mechanisms and interactions with other phytohormones. *partially suppressing coil (psc1)* is one mutant that has higher sensitivity than wild type under MeJA treatment [37]. Moreover, it has a dwarf phenotype, was identified from the mutagenized *coil-2* seeds, and was identified as having slightly greater JA-induced root inhibition as compared with *coil-2*. This mutation is an allele of DWF4 that affects an enzyme in brassinosteroid (BR) biosynthesis. Adding BR to seedlings corrects the defect in *psc1*, producing longer roots. This fact shows that BR antagonizes jasmonate inhibition of roots in wild type plants. Thus, BR is a negative regulator in JA-induced root inhibition. This result has revealed more details about the cross talk between JA and BR signaling in controlling root growth [37].
Another JA hypersensitive mutant, \textit{jasmonic acid-hypersensitive1 (jah1)}, is sensitive to JA in root inhibition as well [38]. However, it does not show JA hypersensitivity in other jasmonate responses. For example, JA-induced defense gene expression is actually reduced, it is more susceptible to the necrotrophic fungus \textit{Botrytis cinerea} which requires jasmonate signaled defenses in Arabidopsis, and it has less accumulation of JA-induced indole glucosinolates (IGs). After cloning via genetic mapping, this mutant was found to be deficient in P450 protein CYP82C2. This affects Trp accumulation which apparently decreases the IGs accumulation [38]. Decreased IGs may be partially responsible for the greater pathogen susceptibility, and altered regulation of other jasmonate-dependent defense genes may also be important. It is not clear how loss of CYP82C2 results in roots being more strongly inhibited by JA and the details of these mechanisms still need more research. New hypersensitive mutants may reveal additional details about the regulation of jasmonate signaled processes in plants. The rest of this thesis will describe the characterization and initial gene identification of a new jasmonate hypersensitive mutant.
Reference


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lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis.


Figure 1. The chemical structure of jasmonic acid and jasmonic acid-iso-leucine. Jasmonic acid consists of a cyclopentanone with a carboxylic acid and an aliphatic side chain of five carbons. JA-Ile can have 4 different stereoisomeric forms. Among these forms, the naturally occurring active form is (+)-7-iso-JA-Ile. (+)-7-iso-JA-Ile can undergo isomerization to (-)-JA-Ile that has little or no activity to function with the JA-Ile hormone receptor (COI-JAZ complex) [6, 15]. (+)-7-iso-JA-Ile converts easily to (-)-JA-Ile under elevated temperature and high-pH; however, (-)-JA-Ile only minimally converts back to (+)-7-iso-JA-Ile.
Figure 2. JA biosynthesis. \( \alpha \)-linolenic acid (LA) is the precursor for JA synthesis, released from chloroplast membranes of injured plant cells. After plant injury, many JA biosynthetic enzymes such as lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) are activated, leading to rapid synthesis of JA. LA is converted to 12-oxophytodienoic acid (OPDA) in chloroplasts by action of LOX, AOS, and AOC; then, OPDA is translocated to peroxisomes by an ATP-binding cassette transporter. After OPDA enters the peroxisomes, it undergoes a reductive step by 12-oxo-phytodienoic acid reductase and three rounds of \( \beta \)-oxidation, finally producing jasmonic acid [4, 6]. Moreover, the resulting increase in JA can activate phospholipases and lead to more LA being released from membranes producing more JA.

Figure 3. The mechanism of JA-Ile signaling transduction. JAR1 converts JA to the active form of JA-Ile. JA-Ile from the cytosol enters the nucleus where MYC2 and JAZ repressor are located. JA-Ile promotes binding of COI1 F-box protein with JAZ proteins, allowing ubiquitination of JAZ proteins and their degradation by the 26S proteasome. This results in MYC2 and other transcription factors being released from the JAZ repressor, permitting JA-induced genes to turn on [4, 7]. The products of these genes can then produce biochemical and physiological changes typical of a jasmonate response.
Chapter II: The Hormonal Characterization and Mapping of A New Jasmonate Hypersensitive Mutant (jah2) in Arabidopsis thaliana

Introduction

Mutants have been widely used to explore the biological and molecular mechanisms of plant hormone action. Useful mutants have been identified by screening for differences in phenotype from that of the wild type after a specific hormone treatment during germination or a few days after. Through mapping the mutant locus with molecular markers, the affected gene can then be identified. This approach has been widely used in Arabidopsis thaliana. In the study described here, Arabidopsis is used because of the short life cycle and comparatively small size of the genome and the availability of the complete genome sequence.

Jasmonate signaling is a critical component of plant responses to both biotic and abiotic stresses, as well as a regulator of growth and certain stages of development [1]. The basic mechanism of jasmonate signal transduction and the downstream responses emanating from it, are now understood [2], and a basic outline was presented in Chapter I. The mutant gene in this study causes hypersensitivity to jasmonates in root growth inhibition assays and has been called jasmonate hypersensitive 2 (jah2).

While mutants that are insensitive or resistant to jasmonates have been the most informative to date (see Chapter 1) mutants that are hypersensitive to jasmonates can
also provide further details about the regulation and function of jasmonate signaling.

Two such Arabidopsis mutants have been characterized by others, partially suppressing coil (psc1) [3] and jasmonic acid-hypersensitive 1 (jah1) [4]. Both of these show greater inhibition of root growth in the presence of JA than in wild-type. PSC1 encodes an enzyme of brassinosteroid (BR) biosynthesis, indicating that BR negatively regulates inhibition of root growth by jasmonates. JAH1 encodes a cytochrome P450 protein, CYP82C2. The mutant jah1 underproduced indole glucosinolates during pathogenesis and was more susceptible to Botrytis cinerea, suggesting that CYP82C2 influences Trp-derived secondary metabolites under elevated JA conditions that occur during pathogen infection. However, the mechanism of JAH1 action is still unknown.

In this study, sensitivity of jah2 to MeJA relative to wild type was determined and compared with sensitivity to indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC; the precursor of ethylene). Molecular markers were also used to map the JAH2 locus to narrow the chromosomal region. Finally, a candidate mutant gene was identified by whole genome sequencing.
Materials and Methods

Plant Materials and Growth Conditions

The jah2 mutant originated from a population of Arabidopsis thaliana M2 seeds mutagenized by ethyl-methanesulfonate (EMS) treated seed of ecotype Columbia wild-type, purchased from Lehle Seeds (Round Rock, Texas). Col-0 seeds were also utilized in the experiment. Seed sterilization was done with around 500 seeds in a 2-ml plastic microtube. Seeds were washed with 70% ethanol [5], and then with 50% fresh bleach (6% Sodium hypochlorite) for about 15 minutes after the 70% ethanol was removed. During the 15 minutes, the microtube was vortexed several times to mix the seeds and solution well. After centrifugation the solution was removed and seeds were washed around five times with sterile water to remove the residual ethanol and bleach. Seeds were then incubated at 4°C for two to three days for cold treatment.

In this study, 3 different phytohormones, IAA, ACC (the precursor of ethylene), and MeJA, were utilized. For IAA and ACC, the stock concentrations in ethanol were at 0.05, 0.1, 0.2, 0.5, and 1.0 mM, and MeJA was made to 0.05, 0.1, 0.2, 0.5, 1.0, 5.0, and 10.0 mM. Hormone stocks were stored at -20°C. Each hormone was added into sterile agar media before it solidified at a 1,000-fold dilution.

The agar media were made from MS Basal Salts (with the concentration of 4.33
g/2 L), MES buffer 0.5 g/1 L, 0.5% sucrose, 1% agar, adjusted to pH 6.0 with 1 M KOH. The solution was autoclaved for 20 min and 121°C.

After the agar media was autoclaved, it was cooled in a water bath at 55°C. Then 30 μl of the appropriate hormone was added to 30 ml of agar media solution. All these steps were done inside a laminar flow hood. The hormone was mixed with agar solution gently and poured into a Fisher brand sterile 100 mm x 15 mm Polystyrene Petri Dish. After the agar solidified a narrow strip of the agar was removed with a sterile spatula to create a slot in which the seeds can be placed on the agar bed. Seeds were distributed on the agar with a micropipeter [5]. After the seeds were planted, the plates were sealed with 3 M micropore tape. Media plates were placed on edge so that roots would grow down through the agar. Root length was measured after 7 days. Temperature and light were kept at 22°C and 8hr, respectively, with fluorescent illumination at about 100 μE • m⁻² • sec⁻¹ [5].

**DNA Extraction and Sequencing**

The DNA extraction buffer was prepared from 10.0 ml 1 M Tris-HCl (pH 7.4), 2.5 ml 5 M NaCl, 2.5 ml 0.5 M EDTA (pH 8.0), 2.5 ml 10% SDS, and 32.5 ml ddH₂O to make 50.0 ml of solution. 100 μl extraction buffer was placed in a microfuge tube, and about 50 mg of young fresh leaf tissue was added, followed by grinding with a
plastic micro pestle. The sample was then centrifuged at 21,000 RCF for two minutes.

80 μl of supernatant was removed to a clean tube and 80 μl isopropanol was added and mixed. The solution was incubated at room temperature for 10 minutes. After centrifugation again for two minutes, the supernatant was discarded and the remaining pellet was dried in air. About 30 μl TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) was added to the pellet without mixing, and the sample was stored in the 4°C refrigerator overnight.

Genomic DNA was also extracted for whole genomic sequencing by Illumina Solexa, sequencing and data analysis were done by the University of Nebraska Genomics and Bioinformatics core facility. The CTAB extraction buffer was prepared from 73.0 ml ddH₂O, 10.0 ml 1 M Tris (pH 7.5), 14.0 ml 5 M NaCl, 2.0 ml 0.5 M EDTA (pH 8.0), 1.0 g CTAB, and 1.0 ml 14 M BME.

The frozen leaves of jah2 mutant (about 2 to 5 g) were ground to powder by liquid nitrogen with a mortar and pestle. The powder was transferred to a 50 ml Falcon tube that contained 25 ml CTAB buffer, and incubated at 65°C for 20 minutes and mixed occasionally. 10 ml of chloroform was added and mixed well, then rotated for 20 minutes. The tube was centrifugal at 1,000 x g for 5 minutes separated the two phases. The aqueous phase (around 20 ml) was transferred to
a fresh 50 ml Falcon tube and an equal volume of isopropanol was added, mixed well, and the whole tube was put on ice for 10 minutes. It was then centrifuged at 1,000 x g for 10 minutes. Supernatant was transferred to a fresh tube, 16 ml 100% ethanol was added, and the tube was put on ice for 20 minutes. The tube was centrifugal at 1,000 x g for 5 minutes, supernatant was discarded and the inside of the tube was dried with paper towel. DNA was dissolved in 900 μl of TE, 8.0 buffer and 100 μl of 3M sodium acetate was added. The solution was separated to two equal volumes and put into a microfuge tubes. Then, an even volume of phenol (about 500 μl) was added to each tube and both tubes were mixed well. Tubes were centrifugal at 1,000 x g for 5 minutes to separate the two phases, and the aqueous phase was transferred to a clean microfuge tubes. An equal volume of phenol: chloroform (1:1) was added to each tube and tubes were mixed well. Tubes were centrifugal at 1,000 x g for 5 minutes to separate phases, and the aqueous phase was transferred to clean microfuge tubes. An equal volume of chloroform was added to each tube and tubes were mixed well. Tubes were centrifugal at 1,000 x g for 5 minutes to separate phases, and aqueous phase was transferred to clean microfuge tubes. A double volume of 100% ethanol was added to each tube and tubes can be incubated on ice for 5 minutes. Then, tubes were centrifugal at 5,000 x g for 5 minutes and supernatant was
discarded. 800 μl 70% ethanol was added to wash the pellet and tubes were centrifugal at 1,000 x g for 5 minutes. Supernatant was discarded and DNA pellet was dried about 15 minutes. DNA was dissolved in 100 to 400 μl TE, 8.0. Estimate the quality of DNA and quantity of RNA by agarose gel. If RNA is present, RNase A was added to remove those RNA.

**jah2 Mapping**

*jah2* was previously found to be a recessive mutation [Staswick, unpublished]. F2 seeds from the cross of *jah2* to Landsberg *erecta* (Ler), were planted on 1 μM MeJA to select for the JA-hypersensitive mutants, which have clearly distinguishable short roots [6]. These were transplanted to soil in pots with size of 8 cm x 8 cm x 8 cm. After the plants were growing stably, leaf tissue was collected for DNA extraction.

CAPS [7] and SSLP markers were utilized to map the mutant locus, and most of these markers were found on the TAIR website [8]. For more options, BAC clone data were utilized to produce more markers. From the BAC clone library, the DNA polymorphisms between ecotypes were identified and utilized to develop markers. Three markers flanking the *JAH2* locus were utilized in the end; they were CER444333 (from T21B4), CER428256 (from F21A20), and CER449302 (from
F15A18), respectively. CER444333 the left primer is 5’-
CGTGAGGAATGATGAGGAGG-3’, the right primer is 5’-CTGGATTCAGGCAGAAAATCCTCT-3’, and the restriction enzyme is Alu.
CER428256 the left primer is 5’-TCATGTTCTGAGGTAGGCAGC-3’, the right primer is 5’-CCTTGCTCCACATTTATGA-3’, and the restriction enzyme is BfaI.
CER449302 has 41bp deletion on Ler, and the left primer is 5’-AAGATGCTTGATTGTTGTG-3’, and the right primer is 5’-CAATTGAAAAATTCATCTAGG-3’. 
Results and Discussion

Hormonal Characterization The \textit{jah2} Mutant

Previous screening of seedlings from an ethylmethane sulfonate mutagenized population (Col-0 background) in the presence of the auxin inhibitor JA-Trp \cite{9} yielded a mutant that was inhibited in root growth compared to wild type. Further analysis showed the mutant was not sensitive to JA-Trp itself, but was hypersensitive to low levels of JA [Staswick, unpublished]. Apparently, the JA originated either as a minor contaminant in the JA-Trp preparation or was produced by conjugate hydrolysis after it was assimilated by seedlings.

To characterize the \textit{jah2} mutant, the response to different concentrations of MeJA was compared to the response of wild type seedling roots. We utilized MeJA because MeJA is readily demethylated by plants and has the same effects as JA, and it is easier to obtain. Figure 1 shows that the \textit{jah2} mutant has 50\% inhibition by MeJA at about 0.05 μM compared with 50\% inhibition of wild type on 5.0 μM. Thus, the \textit{jah2} mutant is about 100 times more sensitive than wild-type in this assay. Some hormone response mutants have altered response to multiple hormones. To test whether this is the case for \textit{jah2}, growth was evaluated in the presence of IAA and ACC, two other hormones that strongly inhibit root growth. The hypothesis tested was that \textit{jah2} is not only hypersensitive to JA but also hypersensitive to IAA and ACC.
In this experiment, the concentrations used were 0.05, 0.1, 0.2, 0.5, and 1.0 μM to test root inhibition from IAA and ACC. The other growth conditions were the same as the MeJA test. Figure 2 shows that for IAA, 50% inhibition occurred at around 0.2 to 0.5 μM. There was no evidence that jah2 was hypersensitive to IAA. Although in this experiment the mutant appeared slightly less sensitive at lower concentrations this effect was not seen in all replicate (see Appendix I) experiments. Thus, the hypothesis that jah2 was hypersensitive to IAA was found to be false. Similar results were obtained in ACC application. Figure 3 shows one of the ACC experiments. 50% root inhibition on jah2 was observed within 0.1 to 0.2 μM. In this experiment jah2 was slightly ACC-insensitive; and this was seen in the two additional experiments shown in Appendix I. However, the difference was small and there was no evidence for hypersensitivity to ACC. In conclusion, hypersensitivity of jah2 was specific to jasmonate signaling and did not occur for IAA or ethylene.
Figure 1. Root growth under MeJA treatment on agar media for wild-type and jah2 mutant. 20 seeds for each line (Col-0 and jah2) were tested on the MeJA-containing media for 5-day growth. The root inhibition percentage was calculated by the mean root length for each concentration divided by the untreated control. The error bars represent 95% confidence intervals calculated from Fieller's theorem (p value < 0.05).
Figure 2. Root growth under IAA treatment on agar media for wild-type and \textit{jah2} mutant. 20 seeds for each line (Col-0 and \textit{jah2}) were tested on the IAA-containing media for 5-day growth. The error bars represent 95% confidence intervals (p value < 0.05).

Figure 3. Root growth under ACC treatment on agar media for wild-type and \textit{jah2} mutant. 20 seeds for each line (Col-0 and \textit{jah2}) were tested on the ACC-containing media for 5-day growth. The error bars represent 95% confidence intervals (p value < 0.05).
Mapping The \textit{jah2} Mutant

In order to map the defective gene responsible for JA-hypersensitivity, the mutant \textit{jah2} in the Columbia (Col-0) background was crossed with Landsberg \textit{erecta} (Ler), which has numerous sequence polymorphisms relative to Col-0 throughout the genome. To detect recombinant chromosomes, homozygous \textit{jah2} seedlings from the F2 generation were identified by screening for hypersensitivity to MeJA. These homozygous mutants were then genotyped for markers throughout the genome using polymerase chain reaction (PCR) - based markers. Two types of markers were used, Cleaved Amplified Polymorphic Sequences (CAPS) and Simple Sequence Length Polymorphisms (SSLPs), originating from single nucleotide polymorphisms and small insertion-deletion polymorphisms, respectively. For each marker one expects three kinds of gel electrophoresis band patterns in the segregating generation: CC (both bands from Col-0 background), CL (one from Col-0 background and the other from Ler background), and LL (both bands from Ler background). If any marker is linked with the mutant locus, it should produce a disproportionate number of individual mutant seedlings with the CC pattern, indicating low recombination with the \textit{JAH2} locus.

Bulk segregant analysis is an efficient method for initially mapping the locus to a chromosome. Instead of genotyping numerous individual F2 samples with many
markers, this method mixes many samples as one DNA pool. Linkage for a given marker can then be seen as a distortion from the expected equality of the two allelic types, identified as intensity of ethidium bromide stained bands on a gel. Initially, 30 F2 $jah2$ recessive samples were mixed as a DNA pool. 16 CAPS markers distributed over the five Arabidopsis chromosomes were chosen initially (shown in Figure 4) for mapping. They were CAT3, UFO, GAPB, and ADH on chromosome I; PhyB and m429 on chromosome II; ALS, g4711, MS-3-1, and BGL1 on chromosome III; F10N7H, ch42, prha, and DHS1 on chromosome IV; DFR, and LFY3 on chromosome V. (Details for these markers are shown in Appendix II.) However, no marker showed linkage with the $jah2$ allele. This result suggested that more markers covering gaps on some chromosomes should be tried.

Chromosome I is the longest chromosome, and chromosome IV is the shortest. The length of chromosomes are approximately 34,964,571 bp (131 cM) on chromosome I, 22,037,565 bp (98 cM) on chromosome II, 25,499,034 bp (101 cM) on chromosome III, 20,862,711 bp (116 cM) on chromosome IV, and 31,270,811 bp (140 cM) on chromosome V, respectively. However, according to the centimorgan number on the 5 Arabidopsis chromosomes, the map shows that chromosome V may have higher recombination frequency. More markers were tested on chromosome V to cover the region on the left end previously not represented (PAI2, RCI1B, NIT4,
F13K20-T7, and PHYC.2). PAI2, RCI1B, and PHYC.2 were not linked to \textit{JAH2}. The fragments on gel analysis of NIT4 and F13K20 were unclear, so these markers were not used. Next, five additional markers were developed (T20D161 on chromosome II, MYB4 on chromosome III, GA1.1 on chromosome IV, ASA1, and PAT1 on chromosome V) and the DNA pool size was increased to 140 recessive \textit{jah2} individuals. T20D161 and MYB4 showed no linkage to the mutation site; however, PAT1 suggested a linkage pattern. GA1.1 and ASA1 were not used further (all the information on markers is given in Appendix II and IV).

PAT1.1 (5,956,164 bp on chr. V) marker on the DNA pool suggested a bias toward the Col-0 homozygous pattern on gel electrophoresis, although the Ler DNA apparently amplifies poorly relative to Col in the F1 (Figure 5). Figure 6 shows the pattern of two other markers on Chromosomes 4 and 5 that were not linked with the \textit{JAH2} locus. This result indicated the marker may be close to the mutation, but it did not show any information about which side of the mutation this marker is on. To do further mapping work, it was required to test individual plant DNA samples rather than the DNA pool. PAT1.2 (5,957,706 bp) is a SSLP marker at nearly the same site as PAT1.1, and was applied on all the 140 F2 recessive mutant samples. However, about 11\% of these samples were recombinant, indicating the \textit{JAH2} locus was still some distance away (Appendix III).
NGA151 (4,669,929 bp) was an additional marker near PAT1 that was used to analyze all 140 samples. The results showed that NGA151 is on the same side of *JAH2* as PAT1 since NGA151 had about 13% recombinant chromosomes and only one of the PAT1 recombinant samples did not appear in the NGA151 recombinant samples (Figure 7 and Appendix III). CIW8 (7,485,585 bp), NGA139 (8,428,133 bp), and NGA76 (10,418,610 bp) were the next round of markers applied onto the 140 F2 recessive samples. The results showed the mutation area was between NGA139 and NGA76 because the recombinant chromosomes for each marker were in different DNA samples, while all of the NGA139 recombinant DNA samples were also recombinant for CIW8 (Figure 7 and Appendix III). Next, N5 (8,821,668 bp) was developed as a marker. It was utilized on the 140 samples as well, and the possible mutant locus was determined to be closer to N5 than NGA139, between nucleotides 8,821,668 bp to 10,418,610 bp (Figure 7 and Appendix III). However, no additional CAPS and SSLP marker within this range were found in published papers or the TAIR genomics website. Sequence data from Bacterial Artificial Chromosomes (BAC) of Ler DNA was utilized for designing new markers. T1N24 (9,099,162 bp), F2P16 (9,445,156 bp), and F15A18 (9,775,141 bp) were SSLP markers developed based on sequence length differences between the two ecotypes. T1N24 produced about 2.5% recombinants from 140 F2 individuals, suggesting this marker was still considerable
distance from \textit{JAH2}. In order to further narrow the region the number of F2 mutant samples was increased to recover rarer recombinants. F2P16 and F15A18 were tested on 466 F2 recessive samples and the result showed that \textit{JAH2} locus was between these markers (9,445,156 bp to 9,775,141 bp) as there was no individual seedling recombinant for both markers (Figure 7 and Appendix III). CER430554 (9,505,081 bp), CER444333 (9,598,367 bp), and CER428256 (9,700,348 bp) then were tested. All 4 recombinant samples from CER444333 also appeared in the recombinant samples of CER430554, which indicated they were on the same side of \textit{JAH2}. CER428256 had no recombinant sample observed, and F15A18 had 3 recombinant samples, but they differed from the recombinant samples from CER444333. Hence, the closest flanking markers were determined to be CER444333 and F15A18 (Figure 7, Appendix III, and IV for all additional markers and their PCR conditions). This spans a distance of about 177kb on chromosome 5 and we can know that \textit{jah2} is a different mutant with \textit{psc1} and \textit{jah1} mutant. According to the information on the TAIR website, there are 48 possible protein coding genes within the 177k bp region [8].
Figure 4. The 26 CAPS markers were utilized on 5 chromosomes in Arabidopsis. Details for markers are shown in Appendix II and Appendix IV.
Figure 5. The gel image of the bulk segregant analysis by PAT1.1 CAPS marker (with SphI restriction enzyme). The left photo shows the PCR products, and the right photo shows the result after using the restriction enzyme. The pattern of Col-0 should have a 1.9 kb band, and Ler should have 1.3 kb and 0.6 kb bands together. From left to right, the first lane was markers (Lambda HindIII Ladder or 100 bp DNA Ladder), the second lane was DNA pool, and the third lane was Col x Ler F1 generation.
Figure 6. The example of markers not linked with the mutant locus. The digested DNA (the most right picture) included, from left to right, ch42 CAPS marker (with Cla I restriction enzyme) on chr. IV and DFR CAPS marker (with BsaAI restriction enzyme) on chr. V applied to three samples – DNA pool, Col x Ler F1 generation, and jah2 mutant, respectively, and the last lane was DNA marker. For the ch42 marker, the gel image showed 0.75, 0.65 kb bands on Col-0, and 1.4 kb band on Ler; and for DFR marker, the gel image showed 0.609, 0.534 kb bands on Col-0, and 0.609, 0.318, and 0.216 kb bands on Ler.
Figure 7. The 12 developed markers nearby the mutation locus on chromosome V in Arabidopsis. The total number of individual F2 mutant samples analyzed and the number showing recombination is shown. The relative position for the JAH2 location is based on whether recombinant individuals for one marker were also recombinant for an adjacent marker. Details for markers are shown in Appendix III and IV.
**JAH2 is Probably Glutathione Synthetase 2**

The mutation in *jah2* may be caused by the ethylmethane sulfonate mutagen, which usually causes single nucleotide substitutions. To identify the site of the mutation, the genomic DNA was sequenced by Illumina whole genome sequence analysis (the sequencing and data analysis were done by the University of Nebraska Genomics and Bioinformatics core facility) to compare the *jah2* mutant with the reference Col-0 wild-type sequence. The results showed two variant nucleotides within the mapped region on Chr. V and they were G to A nucleotide transitions at positions 9,669,462 and 9,747,219. Position 9,747,219 is in an intergenic region and the other occurs in intron six of the glutathione synthetase (GS or GSH2) gene [10]. Therefore, it is possible that the JA-hypersensitive trait results from abnormal GSH2 expression due to altered splicing of exons. A CAPS marker was designed for the G-A single nucleotide transition based on the fact that this creates an SspI restriction site that is not in wild-type Col-0. After PCR and DNA digest, gel electrophoresis analysis (see Figure 8), showed that the *jah2* mutant contained the nucleotide expected from sequence analysis, but Col-0 did not. The primer sequences were: 5’-GGGGATCAGATTGGCATAGAC-3’ and 5’-GAAATAAACCACTGCAGACTGC-3’.

Intron six of GSH2 has rare AT-AC ends rather than the regular GT-AG ends.
Most AT-AC introns are removed by a U12-dependent spliceosome rather than the U2 splicesome used for GT-AG introns [11]. In the jah2 mutant, the point mutation took place in the fifth nucleotide from the 5’ end of intron six. This replacement may influence the recognition of the spliceosome on a highly conserved U12-spliced intron sequence, 5’-ATATCCT, which is changed to 5’-ATATTCT in jah2. This may change the splicing efficiency of the GSH2 transcript, which might lead to lower accumulation of glutathione.

**Figure 8.** The GSH2 CAPS marker distinguished the Col-0 and jah2 mutant. The left image was the undigested PCR product for Col-0 and jah2 mutant, and the right image was Ssp1 restriction enzyme digested DNA from the same products. The first lane was 100-bp DNA ladder.
Glutathione is a tripeptide that is composed of Glutamate-Cysteine-Glycine and it plays a crucial role in being an antioxidant that can regulate the reduction-oxidation reaction in the cells of most organisms. Its biosynthesis is carried out by two enzymes, $\gamma$-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2). Generally, glutathione can have two forms, the reduced form (GSH) and the oxidized form (GSSG) with two glutathione molecules joined by a disulfide bond. Since glutathione has many functions in plants, a disruption of glutathione synthesis could cause many defects in plants. Glutathione is a redox buffer, it can detoxify reactive oxygen species, heavy metals, and xenobiotics, and it is used in glutathionylation, a posttranslational modification. Glutathione is associated with the G1/S transition of the cell cycle during postembryonic root development, seed maturation and germination, and pathogen resistance [10].

The gsh1 and gsh2 Arabidopsis mutants are defective in the GSH1 and GSH2 genes, respectively. The gsh1 null mutant is late embryonic-lethal and gsh2 null mutant is early seedling-lethal [12]. This indicates that glutathione synthesis is essential for seed maturation and germination. The jah2 mutant can still develop more or less normally indicating that the mutation does not eliminate glutathione synthesis. JA-hypersensitivity in jah2 may be caused by a lower, or perhaps higher, amount of glutathione biosynthesis, depending on the effect of the mutation on
splicing.

So far, it is unclear how glutathione regulates JA sensitivity in Arabidopsis.

Some research, however, indicates there is an interaction between glutathione and jasmonate signaling [13, 14, 15]. It is known that defective jasmonate signaling will disrupt the normal expression of GSH-involved genes, and abnormal functioning of glutathione signaling will affect the JA signaled gene expression. Both JA and glutathione are involved in pathogen resistance and antioxidation in plants. JA can induce some antioxidative genes that encode the enzymes of glutathione synthesis. When JA-deficiency mutants encounter ozone stress, the mutant cannot activate the antioxidant metabolism, including some glutathione-associated genes [13, 16]. This was examined in the opr3 mutant and jar1 by ozone-sensitivity characterization [16]. Furthermore, JA with other plant hormones can also trigger glutathione signaling pathways [10].

In the glutathione reductase1 (gr1) and catalase-deficient 2 (cat2) mutants, it was observed that the level of transcription of some JA synthesis and signaling genes was influenced by day-length [13]. This study established that not only day-length affects the JA response/synthesis, but also oxidative GSH status influences JA responses. It is also probable that glutathione status changes the level of other phytohormones (e.g. SA), and indirectly changes the JA signaling, since some
altered transcription of other hormone-associated genes was found. A 2011 paper reported that glutathione can repress JA signaling by an SA-dependent pathway [14]. This result suggests that if the jah2 mutant has lower glutathione levels, JA signaling may be strengthened, and would explain why the JA-hypersensitive phenomenon was observed in the jah2 mutant. In the previous section we have mentioned that the grl mutant had inhibition of some JA-induced genes, thus, if glutathione level is lower than normal in jah2, JA-promoted genes might not be expressed normally. Our understanding of the interaction between glutathione, SA signaling, and JA signaling under oxidative stress is limited. However, the jah2 mutant can be a good tool for further research on glutathione and JA signaling, providing more details about the role of JA with glutathione or other compounds involved with reactive oxygen species.
Conclusions and Future Research

jah2 is a new JA-hypersensitive mutant in Arabidopsis. It is different from the previously described JA-hypersensitive mutant, psc1 and jah1. In this research, jah2 was characterized to understand whether it has hypersensitive to different hormones as well. IAA and the precursor of ethylene, AAC, were used in this study and the results showed that jah2 is not hypersensitive to these two phytohormones. This result indicates that jah2 is not generally hypersensitive to multiple phytohormones, but specifically hypersensitive to JA.

To map jah2, about 40 CAPS and SSLP markers have been utilized. The final possible range of the mutant locus was narrowed down to 177kb on chromosome 5. Comparison of the sequence of jah2 gene generated by the Illumina whole genome sequence analysis with the wild-type reference genome found two sites with G-A single nucleotide transitions within the mapped interval. One of the G-A mutations took place in a conserved sequence of intron six of GSH2. The other nucleotide substitution occurred in the intergenic region.

The gsh2 null mutant is early seedling-lethal in Arabidopsis; therefore, we can speculate that GSH2 did not completely lose its function in jah2 and it may be important to determine the expression of GSH2 and glutathione reductase1, glutathione and ascorbate levels, and SA level [14] in jah2 to estimate how strong or
attenuated the production of glutathione is in jah2. Moreover, it is important to examine whether the JA-hypersensitivity in jah2 is SA-dependent [14]. To understand the effect of abnormal GSH2 on glutathione synthesis in plants, it may be important to measure levels of the glutathione precursor, γ-glutamylcysteine. The ratio of γ-glutamylcysteine to glutathione can indicate how much γ-glutamylcysteine is being converted to glutathione. Furthermore, it may also be informative to compare the differences between jah2 and wt in their glutathione concentration, GSH2 expression level, and transcription of other JA-responsive genes at distinct day-lengths, because it was shown that day length affects JA signaling and synthesis gene expression in glutathione associated mutants [13]. At least eight JA-responsive genes have been observed to be repressed in the grl mutant under LD, and similar results were found in the GSSG-accumulating cat2 mutant. If jah2 has abnormal glutathione levels, it may be possible that expression of those JA-responsive genes is altered in jah2.
Reference


8. TAIR website. **2009-2011** (http://www.arabidopsis.org/)


Appendix I.

**IAA treatment of wt and jah2 (i)**

![Graph showing root inhibition for wt and jah2 at different IAA concentrations.]

- **IAA concentration:** 0µM, 0.05µM, 0.1µM, 0.2µM, 0.5µM, 1.0µM
- **Root Inhibition (%):** 0%, 20%, 40%, 60%, 80%, 100%, 120%, 140%
- **Legend:**
  - wt
  - jah2

**IAA treatment of wt and jah2 (ii)**

![Graph showing root inhibition for wt and jah2 at different IAA concentrations.]

- **IAA concentration:** 0µM, 0.05µM, 0.1µM, 0.2µM, 0.5µM, 1.0µM
- **Root Inhibition (%):** 0%, 20%, 40%, 60%, 80%, 100%, 120%, 140%
- **Legend:**
  - wt
  - jah2
ACC treatment of wt and *jah2* (i)

ACC treatment of wt and *jah2* (ii)
### Appendix II.

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<th>Markers</th>
<th>Chr.</th>
<th>Name</th>
<th>Locus</th>
<th>Primer Sequences</th>
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<td>R 5' - ACCAAATCTTTCGTGGGGCTCAGCAG - 3'</td>
<td></td>
<td>Ler: 1000, 500</td>
<td></td>
</tr>
</tbody>
</table>
| CAPS | III | ALS | 18001597 bp | F 5’- GGCAACACATGTTCTTGGTG - 3’  
R 5’- ATCACAGGACAAGTCCCTCG - 3’ | Hae III | Col-0: 952, 420  
Ler: 952, 220, 200 |
|------|-----|-----|-------------|---------------------------------|---------|-----------------|
| CAPS | III | MS_3_1 | 18487392 bp | F 5’- GAGAGTAAACTTGACAATTACAAGAGA - 3’  
R 5’- TTCCCAATTTTTTTCAAAGTTTTTAGGG - 3’ | HindII | Col-0: 840, 360, 240  
Ler: 455, 385, 360, 240 |
| CAPS | III | BGL1 | 75.23 cM | F 5’- TCTTCTCGGTCTATATTCTCG - 3’  
R 5’- TTATCACCATAACGCTTCCC - 3’ | Rsa I | Col-0: 785, 340, 105  
Ler: 785, 485 |
| CAPS | IV | GA1.1 | 1242594 bp | F 5’- CCGGAGAATCGTGACGTGACGT - 3’  
R 5’- AAGCTTGAACTCAAGGTTTC - 3’ | BsaB I | Col-0: 707, 527  
Ler: 1196 |
| CAPS | IV | ch42 | 10201954 bp | F 5’- CATCTTCTTCTGCAATTAGGA - 3’  
R 5’- CAGTGGAATCTTTCTTCAGACG - 3’ | Cla I | Col-0: 750, 650  
Ler: 1400 |
| CAPS | IV | Prha | 14650868 bp | F 5’- CTTGTTTCGCACTGCTCCACC - 3’  
R 5’- GCGGAAGAAGAACTCTGTTCG - 3’ | Dde I | Col-0: 778, 530, 348, 30  
Ler: 778, 530, 30, 50, 30 |
| CAPS | IV | F10N7H | 15498955 bp | F 5’- CCTGCCAATATGCCAAGGC - 3’  
R 5’- GTGTATACTGCGTGTCAGC - 3’ | Hae II | Col-0: 1000, 200  
Ler: 1200 |
| CAPS | IV | DHS1 | 18537948 bp | F 5’- AGAGAGATGAGAATGAGAGG - 3’  
R 5’- CAGTGGACTTTTTCTCTGCAGGC - 3’ | Dde I | Col-0: 1491, 129, 48  
Ler: 1620, 48 |
| CAPS | V | PAI2 | 1667313 bp | F 5’- GTTGAGAATTCATTTCTTGGTG - 3’  
R 5’- CAGTAAATGGAACAAGCTTTGTC - 3’ | AFLIII | Col-0: 594, 50  
Ler: 644 |
| CAPS | V | ASA1 | 1720605 bp | F 5’- CCTCTAGGCTGAATAACAGAAAC - 3’  
R 5’- CTTACTCTGTTCTTGGTTCAG - 3’ | BciI | Col-0: 1042, 686  
Ler: 686, 553, 489 |
| CAPS | V | RC11B | 3284396 bp | F 5’- ATCGATTTGGTGCGAGAAC - 3’  
R 5’- CAGCTCGTTACAGGGCGCTAC - 3’ | MBOI | Col-0: 900, 350, 260, 150  
Ler: 800, 350, 260, 150 |
<table>
<thead>
<tr>
<th>CAPS</th>
<th>V</th>
<th>Gene</th>
<th>Length (bp)</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Restriction Enzyme</th>
<th>Col-0</th>
<th>Ler</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>PAT1</td>
<td>5956164 bp</td>
<td>F 5' - GTATGAGAACATAGTAAACCCCATG - 3'</td>
<td>R 5' - GTCGACGTGGTGCGGTGGTGTTG - 3'</td>
<td>Sph I</td>
<td>1000, 600, 300</td>
<td>1000, 900</td>
</tr>
<tr>
<td></td>
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<td>NIT4</td>
<td>7377397 bp</td>
<td>F 5' - CAACCTCCACATCCGTCGCGG - 3'</td>
<td>R 5' - CGTTTTCTTGTGCAATGGACATGAGAG - 3'</td>
<td>MBOII</td>
<td>Col-0: 1.9</td>
<td>six fragments</td>
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<tr>
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<td></td>
<td>F13K20-T7</td>
<td>11021947 bp</td>
<td>F 5' - TTTGTGCAATTTATTAGGGTAGTTAGGCAGGTTCATGGACAGCTG - 3'</td>
<td>R 5' - ATTTGCAGAAGTTGAAGTTGGTC - 3'</td>
<td>MSEI</td>
<td>Col-0: ~260, etc.</td>
<td>~180, etc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PHYC.2</td>
<td>14007935 bp</td>
<td>F 5' - CTACAGAATCCGCCTCCTCAACG - 3'</td>
<td>R 5' - CCTAATGGAGAATCATTCGG - 3'</td>
<td>PSTI</td>
<td>Col-0: 1700, etc.</td>
<td>900, 800, etc.</td>
</tr>
<tr>
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<td>DFR</td>
<td>17164364 bp</td>
<td>F 5' - AGATCCTGAGGTGAGTTTTTC - 3'</td>
<td>R 5' - TGTTACATGGCTTCACTACCA - 3'</td>
<td>BsaAI</td>
<td>Col-0: 609, 534</td>
<td>609, 318, 216</td>
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<td>LFY3</td>
<td>24843357 bp</td>
<td>F 5' - TAACTTATCGGGCTTCTGC - 3'</td>
<td>R 5' - GACGGCGTCTAGAAGATTCC - 3'</td>
<td>Rsa I</td>
<td>Col-0: 708, 236, 147, 126, 78</td>
<td>855, 236, 126, 78</td>
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</tbody>
</table>
## Appendix III.

### Markers near the mutation region (on Chr. V)

<table>
<thead>
<tr>
<th>Markers</th>
<th>Name</th>
<th>Loci</th>
<th>Primer Sequences</th>
<th>Restriction Enzyme</th>
<th>Fragment size (bp)</th>
<th>Recombinant rate/ Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPS</td>
<td>N5</td>
<td>8821668 bp</td>
<td>F 5’- TAACTTATCGGGCTTCTGC - 3’&lt;br&gt;R 5’- GACGGCGTCTAGAAGATTC - 3’</td>
<td>BclI</td>
<td>Col-0: 371, 100&lt;br&gt;Ler: 471</td>
<td>3.2% / 140</td>
</tr>
<tr>
<td>SSLP</td>
<td>PAT1.2</td>
<td>5957706 bp</td>
<td>F 5’- CATGCTTCATCATGCCC - 3’&lt;br&gt;R 5’- AGCTGAAGCTCTGCCACC - 3’</td>
<td></td>
<td>Col-0: 706&lt;br&gt;Ler: 606</td>
<td>11% / 140</td>
</tr>
<tr>
<td>SSLP</td>
<td>NGA151</td>
<td>4669929 bp</td>
<td>F 5’- CAGTCTAAAAGCGAGTAGTAGTG - 3’&lt;br&gt;R 5’- GTTTTGGGAGTTTGCTGG - 3’</td>
<td></td>
<td>Col-0: 150&lt;br&gt;Ler: 120</td>
<td>13% / 140</td>
</tr>
<tr>
<td>SSLP</td>
<td>CIW8</td>
<td>7485585 bp</td>
<td>F 5’- TAGTGAAACCTTCTCAGAT - 3’&lt;br&gt;R 5’- TTATGTTTTCTTAATCAGTT - 3’</td>
<td></td>
<td>Col-0: 100&lt;br&gt;Ler: 135</td>
<td>7% / 139</td>
</tr>
<tr>
<td>SSLP</td>
<td>NGA139</td>
<td>8428133 bp</td>
<td>F 5’- GGGTTTCGTTTCATCATCCAGG - 3’&lt;br&gt;R 5’- AGAGCTACCAGATCCGGATG - 3’</td>
<td></td>
<td>Col-0: 174&lt;br&gt;Ler: 132</td>
<td>5.36% / 140</td>
</tr>
<tr>
<td>SSLP</td>
<td>NGA76</td>
<td>10418610 bp</td>
<td>F 5’- AGGCATGGGAGAGCATATTACG - 3’&lt;br&gt;R 5’- GGAGAAATGTCACTCCTCCACC - 3’</td>
<td></td>
<td>Col-0: 220&lt;br&gt;Ler: 300</td>
<td>9% / 139</td>
</tr>
<tr>
<td>SSLP</td>
<td>T1N24</td>
<td>9099162 bp</td>
<td>F 5’- TGGATGAAAATGAGACAATCA - 3’&lt;br&gt;R 5’- TTAGCTTCTTTCTTCTGTTCTTT - 3’</td>
<td></td>
<td>Col-0: 316&lt;br&gt;Ler: 289</td>
<td>2.5% / 140</td>
</tr>
<tr>
<td>SSLP</td>
<td>F2P16</td>
<td>9445156bp</td>
<td>F 5’- TGGATAACTTCGAAGCCACTTACG - 3’&lt;br&gt;R 5’- TCCTGTCTTGTGCAGAATGC - 3’</td>
<td></td>
<td>Col-0: 149&lt;br&gt;Ler: 116</td>
<td>0.97% / 466</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>F 5’- AAGATGCTTGGATTGTTGTGC - 3’</td>
<td>R 5’- CAAATTGAATTATGCACATCTAGG - 3’</td>
<td>Col-0: 427</td>
<td>Ler: 386</td>
</tr>
<tr>
<td>-----</td>
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<tr>
<td>SSLP</td>
<td>F15A18</td>
<td>9775141 bp</td>
<td>F 5’- TCAGGTGAAGGTAGCCATT - 3’</td>
<td>MboI</td>
<td>Col-0: 396, 145</td>
<td>Ler: 541</td>
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<tr>
<td>CAPS</td>
<td>CER430554</td>
<td>9505081 bp</td>
<td>R 5’- TCAGAGCGACAATGAATCAA - 3’</td>
<td>AluI</td>
<td>Col-0: 460, 160</td>
<td>Ler: 620</td>
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<tr>
<td>CAPS</td>
<td>CER444333</td>
<td>9598367 bp</td>
<td>R 5’- CTGGATCAGGCAAATCTCT - 3’</td>
<td>BfaI</td>
<td>Col-0: 521</td>
<td>Ler: 431, 90</td>
</tr>
<tr>
<td>CAPS</td>
<td>CER428256</td>
<td>9700348 bp</td>
<td>F 5’- TCATGTTCCTGAGGTGAGC - 3’</td>
<td>BfaI</td>
<td>Col-0: 521</td>
<td>Ler: 431, 90</td>
</tr>
</tbody>
</table>

### Appendix IV.

**PCR condition of different CAPS/ SSLP markers**

<table>
<thead>
<tr>
<th>Annealing temperature (°C)</th>
<th>The markers of MgCl₂ concentration (μM)</th>
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<tbody>
<tr>
<td></td>
<td>1.5</td>
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<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>F10N7H, PA12, T1N24</td>
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<tr>
<td></td>
<td>BGL1, PHYC.2</td>
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<tr>
<td>52</td>
<td>MS_3_1, prha, DFR, LFY3, NGA139, NGA76</td>
</tr>
<tr>
<td></td>
<td>NGA151</td>
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<tr>
<td>55</td>
<td>CAT3, UFO, GAPB, T20D161, ALS, g4711, ch42, DHS1, RCI1B, PAT1.1, NIT4, F13K20-T7, CER430554, CER444333, CER428256</td>
</tr>
<tr>
<td></td>
<td>ADH , PhyB, m429, GA1.1, ASA1, CIW8, F2P16</td>
</tr>
<tr>
<td>57-58</td>
<td>F15A18, PAT1.2</td>
</tr>
<tr>
<td></td>
<td>MYB4, N5</td>
</tr>
</tbody>
</table>
