ABSTRACT

ROBERTSON-HOYT, JOYCE MARIE. Identification of Two Auxin Biosynthesis Mutants Which Result in Root-Specific Ethylene Insensitivity. (Under the direction of José Alonso.)

The objective of this research was to identify genes involved in ethylene-auxin crosstalk that are required to maintain wild-type ethylene signaling and response in the roots but not hypocotyls of Arabidopsis seedlings. Using this root-specific phenotype, two genes related to Indole-3-Acetic Acid (IAA) biosynthesis were identified that are required for proper ethylene response. WEAK ETHYLENE INSENSITIVE7 (WEI7) codes for the beta subunit of anthranilate synthase, which catalyzes the first committed step in the tryptophan biosynthesis pathway, the conversion of chorismate to anthranilate, a tryptophan, therefore, IAA precursor. WEAK ETHYLENE INSENSITIVE8 (WEI8) codes for an aminotransferase, which is believed to convert tryptophan to indole-3-pyruvic acid (IPA) in the IAA biosynthesis pathway.

WEI7 works in conjunction with *WEI2* (the anthranilate synthase α-subunit protein) and both are up-regulated by ethylene and responsible, at least in part, for the ethylene-mediated stimulation of auxin biosynthesis. This increase in IAA then is required for a normal response to ethylene in the root. Since tryptophan treatments do not induce auxin phenotypes of elongated hypocotyls and epinastic cotyledons in Arabidopsis, this regulatory scenario is likely to exist for other genes of the tryptophan and IAA biosynthesis pathway, such as *WEI8*.

wei8, a loss of function mutant of an auxin biosynthetic gene, results in root-specific ethylene insensitivity. WEI8 has aminotransferase activity *in vitro*, can use tryptophan as a substrate, and therefore, it could be responsible for the *in vivo* conversion of tryptophan into

IPA. Double mutant studies support this model. When *wel2,* a mutant of the *WEI8* homolog, *WEL2*, is used to generate *wei8 wel2* double mutants, auxin defects such as agravitropism and loss of apical dominance were seen. Double mutant seedling roots were agravitropic, and adult plants had severe loss of apical dominance as well as reduction in fertility due to defects in flower anatomy. Additionally, analysis of the auxin reporter DR5-GUS expression suggests that WEI8 is required for maintaining normal levels of auxin in the roots of Arabidopsis.

WEI7 and *WEI8* regulate IAA production and are unexpected interaction points between ethylene response and IAA biosynthesis. Identification of these genes will enable further research on the role of specific pathways of IAA biosynthesis in different tissues and during specific developmental programs. Moreover, it will contribute to a better understanding of hormonal crosstalk in plants.

IDENTIFICATION OF TWO AUXIN BIOSYNTHESIS MUTANTS WHICH RESULT IN ROOT-SPECIFIC ETHYLENE INSENSITIVITY

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

GENETICS

Raleigh, NC

2006

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ACKNOWLEDGEMENTS

From beginning to end, this work has been influenced by a great many people. I would like to first thank José Alonso for providing scientific direction for four years. Through patient discussion he has provided me with an enhanced molecular skill set with which I can make a contribution to science and society. In addition, the members of the Alonso Laboratory, as well as those in the laboratory of Dr. Robert Franks, provided great insight and avenue for scientific discussion that helped to form many of the ideas put forth in this work.

I would also like to thank my committee, Dr. Steven Spiker (Co-Advisor), Dr. Steven Clouse, and Dr. Ralph Dewey for direction and guidance to my research. Additionally, I am very grateful to Dr. Robert Bruck for being so gracious to serve as my Graduate School Representative at short notice. Without his help, scheduling my dissertation defense in a timely fashion would have not been possible.

Finally, the most critical contributors to my successes have been my family and friends. Leilani, my partner of nine years, and our 14 month old daughter Sarah, are the joys of my life. They put my life into perspective and give me love, support, and happiness beyond expectation, and for this I am truly blessed. Also, I am fortunate to have the support and love of the rest of my family (Hoyt's and Robertson's), as well as an excellent group of friends with which to share my joys and tribulations.

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ABBREVIATIONS AND EXPLANATION OF TERMS

TERMS

Auxin mutants	*aux1*, *axr1*, *eir1, pin*
Auxin defects	agravitropic roots, loss of apical dominance, flower defects

ABBREVIATIONS

ACS	1-aminocyclopropane-1-carboxylic acid synthase
ACC	1-aminocyclopropane-1-carboxylic acid
AGD2	ABERRANT GROWTH AND DEATH2
AP2	APETALA2
ARE	Auxin Response Element
ARF2	AUXIN RESPONSE FACTOR2
ASA	ANTHRANILATE SYNTHASE alpha subunit
ASB	ANTHRANILATE SYNTHASE beta subunit
ASK1	*Arabidopsis* SKp1-like 1
AUX1	AUXIN RESISTANT1, an auxin influx carrier protein
AXR1	AUXIN RESISTANT1, a ubiquitin-like activating enzyme
Col-0	Columbia-0, an *Arabidopsis thaliana* ecotype
CORI3	CORONATINE INDUCED1
CTR1	CONSTITUTIVE TRIPLE RESPONSE1
CUL	CULLIN
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EBF1	EIN3-BINDING F-BOX PROTEIN1
EBF2	EIN3-BINDING F-BOX PROTEIN2
EGF	Epidermal Growth Factor
EIL	EIN3-Like
ETR2	ETHYLENE RESISTANT2
EIN2	ETHYLENE INSENSITIVE2
EIN3	ETHYLENE INSENSITIVE3
EIN4	ETHYLENE INSENSITIVE
EIR1	ETHYLENE INSENSITIVE ROOT1
EMS	EthylMethaneSulfonate
EREBP	ETHYLENE RESPONSE BINDING PROTEIN
ERF1	ETHYLENE RESPONSE FACTOR1
ERS1	ETHYLENE RESPONSE1
ERS2	ETHYLENE RESPONSE2
ETO	ETHYLENE-OVERPRODUCER
ETR1	ETHYLENE RESISTANT1
HLS1	HOOKLESS1

IAA	Indole-3-Acetic Acid
IAN	Indole-3-Acetoitrile
IAOx	Indole-3-Acetaldoxime
IPA	Indole-3-Pyruvic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAX1	Like-AUX1
Ler	Landsberg erecta, an *Arabidopsis thaliana* ecotype
MPK6	MITOGEN-ACTIVATED PHOSPHORYLATION KINASE6
MS	Murashige and Skoog
NPH4	NON-PHOTOTROPHIC HYPOCOTYL4
NIT1	NITRILASE1
PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIN	PIN-FORMED
PLP	pyridoxal 5'phosphate
RAN1	RESPONSIVE-TO-ANTAGONIST1
RBX	RING-BOX protein
RTE1	REVERSION-TO-ETHYLENE SENSITIVITY1
RTY1	ROOTY1 (A.K.A. SUPERROOT1)
SAM	S-adenosyl-methionine
SKP1	SUPPRESSOR OF KINETOCHORE protein 1
SSLP	simple sequence length polymorphism
SUR1	SUPERROOT1
SUR2	SUPERROOT2
TF	transcription factor
TIR1	TRANSPORT INHIBITOR RESISTANT1
TRP	Tryptophan
UBC	UBIQUITIN-CONJUGATING
WEI1	WEAK ETHYLENE INSENSITIVE1
WEI7	WEAK ETHYLENE INSENSITIVE7
WEI8	WEAK ETHYLENE INSENSITIVE8
WEL	WEI8-like
5-MT	5-Methyltryptophan

Chapter I. Ethylene and Auxin—Biosynthesis, Signaling, and Crosstalk

LITERATURE REVIEW

Ethylene Biosynthesis

Ethylene is a small molecule composed of two carbon and four hydrogen atoms with a single covalent double bond. Despite its molecular simplicity, ethylene is a powerful plant hormone and is involved in the regulation of a great number of physiological processes. The roles of ethylene include, but are not limited to, the regulation of seed germination, cell elongation, cell division, cell fate in the root epidermis, root nodulation, programmed cell death, fruit ripening, organ senescence, organ abscission, and pathogen defense (Abeles et al., 1992; Mattoo and Suttle, 1991; O'Donnell et al., 1996; Penmetsa and Cook, 1997; Penninckx et al., 1996; and Tanimoto et al., 1995).

Ethylene biosynthesis has been well studied (Wang et al., 2002). Studies involving 1-aminocyclopropane-1-carboxylic acid synthase (ACS), which catalyzes the first committed step in the ethylene biosynthetic pathway, the conversion of S-adenosyl-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Fig.1), show ACS to be expressed in numerous plant organs and cell types. ACS family members share a similar expression pattern, and it has been shown that IAA can induce *ACS* expression in a gene-specific, cell type-dependent manner in the root tip (Tsuchisaka and Theologis, 2004). Ethylene itself has also been found to induce *ACS* transcription. Using northern analysis, it was found that

ethylene induces *ACS6* transcription (Arteca and Arteca, 1999). ACC synthases are also regulated at the post-transcriptional level. *In planta*, ethylene-overproducer (*eto*) mutants were found to have wild-type levels of ACS2, ACS4, and ACS5 mRNA, but increased levels of ACS activity, suggesting post-transcriptional regulation resulting in increased stability of ACS protein or perhaps an increase in translational efficiency (Woeste et al., 1999). More recent studies by Wang et al. (2004) show direct interaction of ETO1 with CUL3 (a constituent of an E3 ubiquitin ligase complex) and ACS5 using *in vitro* pull-down assays. These findings suggest that ETO1 may act as a substrate recognition molecule for ubiquitin-mediated proteolysis of specific ACS family members resulting in decreased levels of ethylene production.

In the last step of ethylene biosynthesis, ACC is oxidized by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Fig. 1). Although in most cases ACO activity is not limiting, there are examples that suggest a critical role of this enzyme in the control of ethylene biosynthesis. Transcription of *ACO* has been shown to be hormonally regulated. Microarray analysis has shown that ethylene can induce transcription of *ACO2*, hence ethylene biosynthesis is under a positive feedback regulation (Zhong and Burns, 2003). Once produced, this gaseous hormone is then disseminated throughout the plant by simple diffusion.

Ethylene Signaling

In contrast to the biosynthetic pathway, the ethylene signaling and response pathway remains much less understood. The triple response assay (Guzman and Ecker, 1990), which is described as exaggeration of the apical hook, elongation and radial swelling of the

hypocotyl, and decreased root length, has been utilized extensively to identify mutants in the ethylene signaling and response pathway (Fig. 2) (Chen, et al., 2005). Signaling is initiated by the perception of ethylene by a family of membrane-bound receptors, ETHYLENE RESISTANT1 (ETR1), ETHYLENE RESISTANT2 (ETR2), ETHYLENE INSENSITIVE4 (EIN4), ETHYLENE RESPONSE1 (ERS1), and ETHYLENE RESPONSE2 (ERS2) which have sequence similarities to the two component histidine kinases of bacteria (Moussatche and Klee, 2004). Interaction between ethylene and ethylene receptors is mediated by copper, a metal cofactor which is transported to the receptors by RESPONSIVE-TO-ANTAGONIST1 (RAN1), a copper transporter whose function is required for receptor activity (Hirayama et al., 1999). In addition to RAN1, additional elements affecting the activity of the receptor are likely to exist. In search for such elements, genetic screens directed at the identification of mutants that suppress the ethylene insensitivity of the *etr1* receptor mutant have been carried out. Recently, Resnick et al. (2006) have characterized one of such mutants named REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1), a negative regulator of ethylene response of unknown biochemical function that most likely acts at the receptor level.

At the next step of ethylene response, binding of ethylene to the receptor results in the inactivation of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a Raf-like kinase which physically interacts with ethylene receptors and negatively regulates ethylene response (Keiber et al., 1993; Hua and Meyerowitz, 1998; Clark et al., 1998). Signal transduction from CTR1 to ETHYLENE INSENSITIVE2 (EIN2), is believed to proceed through a mitogen activated phosphorylation kinase (MAPK) pathway. Ouaked et al., (2003) proposed a model in Arabidopsis whereby MITOGEN-ACTIVATED PHOSPHORYLATION

KINASE6 (MPK6) and other MPK proteins are involved in ethylene signal transduction from CTR1 to EIN2, however, arguments (such as the need for MAPKKK repression of MAPKK, inability to replicate data, and finding that reducing *MPK6* expression does not effect ethylene response) have been proposed which refute this claim (Chen et al., 2005). It does remain possible, however, that the ethylene signal is transduced from CTR1 to EIN2 via an unidentified MAPK cascade. Inactivation of the negative regulator, CTR1, allows the signal to be transduced to EIN2, a positive regulator of the ethylene pathway. Alonso et al. (1999) cloned *EIN2* and determined that it encodes an integral membrane protein. Once the signal is received, EIN2, through a series of unknown intermediates, propagates the signal to the transcription factor ETHYLENE INSENSITIVE3 (EIN3). The EIN3 transcription factor family proteins, EIN3 and EIN3-Like (EIL), promote the transcription of *ETHYLENE RESPONSE FACTOR1* (*ERF1*), an ETHYLENE RESPONSE ELEMENT BINDING PROTEIN (EREBP), by dimerization and binding to the *ERF1* promoter. ERF1, an APETALA2 (AP2)-domain-containing transcription factor, then binds to downstream targets of ethylene response (Lorenzo et al., 2003).

Significant ethylene signaling regulation occurs by the accumulation or degradation alternatively, of EIN3 and EIL proteins dependent on the presence or absence of ethylene, respectively. In the absence of ethylene, EIN3 is specifically targeted by EIN3-BINDING F-BOX (EBF) proteins, EBF1 and EBF2, for ubiquitin-mediated proteolysis resulting in a reduction in EIN3 levels. However, in the presence of ethylene, EIN3 becomes more stable which results in an increase in its protein levels in the nucleus (Potuschack et al., 2003; Guo et al., 2003). However, despite a relatively well understood signaling pathway, there remain many intermediates yet to be discovered. Among the most notable are the unknown

components of the ethylene signaling pathway between CTR1 and EIN2, transduction of the ethylene signal from EIN2 to EIN3, as well as the identification of putative points of interactions between this and other signaling/response pathways.

As mentioned above, ethylene has many, and in some cases opposite effects, opening the question of how tissue and developmental stage specific responses are achieved. The identification of mutants that affect particular branches of the ethylene responses suggests that the interaction with other signaling pathways plays a critical role in this information integration process. For example, *ERF1* transcription is induced by ethylene and results in the initiation of transcription of defense related genes in a jasmonate-dependent manner (Lorenzo et al., 2003). In another example, differential growth in the hypocotyl is achieved through the ethylene-mediated regulation of AUXIN RESPONSE FACTOR2 (ARF2) protein levels by HOOKLESS1 (HLS1), an N-acetyltransferase protein (Li et al., 2004) in a light-dependent manner.

Auxin Biosynthesis

The plant auxin, indole-3-acetic acid (IAA), is important for many physiological aspects of plant development. Among them are stem and coleoptile elongation, apical dominance, and tropic response to gravity and light (Blilou et al., 2005; Benkova et al., 2003; Blancaflor and Masson, 2003). IAA is synthesized primarily in young tissues (leaves, shoot tips, young fruits, and immature seeds), and its biosynthesis can be either tryptophan-dependent or tryptophan-independent (Cohen et al., 2003). However, regardless of the utilization of tryptophan, IAA biosynthesis is accomplished through a complex and redundant pathway (Fig. 3), in which many of the genes coding for the enzymes remain

unknown (Woodward and Bartel, 2005). In tryptophan-dependent IAA biosynthesis, tryptophan can be converted to IAA via many biosynthetic pathways. IAA can be synthesized from the conversion of tryptophan to IAA via the indole-3-pyruvic acid (IPA) pathway. Alternatively tryptophan can be directly converted into indole-3-acetaldoxime (IAOx) or indirectly converted IAOx via the tryptamine pathway, and IAA is then generated through the IAOx pathway.

Many of the intermediates in the IAA biosynthesis pathway are known, however the specific proteins responsible for catalyzing each reaction are known in only a few of these steps. Three auxin biosynthetic genes have been identified through mutagenesis screens. The first is gain of function mutation in the *YUCCA* gene which encodes a flavin monooxygenase–like enzyme (Zhao et al., 2001). Second are gain of function mutant forms of the cytochrome P450 proteins, *CYP79B2* and *CYP79B3*, which catalyze the conversion of tryptophan to indole-3-acetaldoxime (IAOx) (Hull et al. 2000). Finally is the loss of function mutation in *NITRILASE1* (Normanly et al., 1997), which converts indole-3-acetonitrile (IAN) to IAA. In the case of nit1, IAA levels were not found to be different from wildtype. The function of proteins involved in glucosinolate biosynthesis, a sink for the IAA biosynthesis intermediate IAOx, such as CYP83B1 (Bak and Feyereisen, 2001) (a.k.a. SUR2) and the CS-lyase, SUR1 (a.k.a. RTY1), can effect the level of IAA generated by a plant. Mikkelsen et al. (2004) showed through *in-vivo* labeling experiments that *cyp83b1(sur2)* and *sur1* loss of function mutants reduce or perhaps block IAOx-derived glucosinolate production potentially resulting in increased levels of IAOx and ultimately IAA.

Once produced, IAA is transported from biosynthetically active tissues in a basipetal direction to other regions of the plant using an active transport system. IAA can enter the

cells by diffusion or through influx carriers, AUX1 or Like AUX1 (LAX1 to 3) (Parry et al., 2001), however it must exit through various PIN-FORMED (PIN1 to 8) efflux carrier proteins (reviewed in Paponov et al., 2005). PIN1, PIN2 (a.k.a. ETHYLENE INSENSITIVE ROOT1 (EIR1)), and PIN3 are known to respond normally to gravity in the root and have the ability to relocalize to the region of the plasma membrane which orients with the gravity vector (Friml et al., 2002; Friml et al., 2004). Relocation of these efflux carrier proteins mediates the gravitropic response. This control of IAA transport provides a means for the plant to respond to various stimuli that affect differential growth.

Ubiquitin-mediated Proteolysis

The auxin response in plants can be achieved via a ubiquitin-mediated degradation process beginning with the activation of ubiquitin, a 76 amino acid peptide that acts as a recognition tag for protein-specific proteolysis. The ATP-dependent activation reaction is catalyzed by E1, the ubiquitin-activating enzyme. A transthioesterification reaction transfers the ubiquitin molecule to E2, a ubiquitin-conjugating enzyme. E2 enzymes are of the *UBIQUITIN-CONJUGATING* (*UBC*) gene family and are known to be involved in stress response and the regulation of transcription factors (TFs) (Scheffner et al., 1988). E3 ubiquitin ligase then transfers ubiquitin to the protein that is to undergo targeted degradation (for further review see Ingvardsen and Beierskov, 2001). There exist over 1200 E3 components which combine into various multiprotein complexes. These fall into one of four E3 complex families (HECT, SCF, APC, and RING/U-box). This diversity within the E3 ligase family allows for a high level of substrate-specific proteolysis (Zeng et al., 2006). Ubiquitin acts as a recognition molecule and has specific affinity to the 26S proteosome. The

tertiary structure of the substrate-ubiquitin complex allows recognition by the 26S proteosome.

The Role of Ubiquitin in the Auxin Response Pathway

The presence of IAA causes degradation of repressors of IAA response. These repressors are known as Aux/IAA proteins and control auxin response by binding to and inactivating AUXIN RESPONSE FACTORS (ARFs), which are considered to be key transcriptional regulators of the auxin responses (Fig. 4). Ubiquitin-mediated degradation of regulatory proteins involved in auxin response has been the focus of many studies. Auxin responses begin with the degradation of Aux/IAA proteins, and there is a direct relationship between auxin concentration and the rate of Aux/IAA degradation (Zenser et al., 2001).

The general mechanism by which IAA removes repression is understood. Dharmasiri et al., (2003) used cell-free extracts to investigate the interaction between Aux/IAA proteins and the E3 ligase, SCF^{TIR1}. The SCF^{TIR1} is a multi-subunit complex consisting of ASK1 (Skp1), CULLIN (CUL), RBX, and TRANSPORT INHIBITOR RESISTANT1 (TIR1). Application of auxin to the extract resulted in the binding of Aux/IAA proteins to SCF^{TIR1}. From this finding they were able to conclude that a soluble receptor is involved in the auxin response mechanism. Recent studies have identified TIR1 as an IAA receptor (Dharmasiri et al., 2005, Kepinski and Leyser, 2005). The binding of IAA to the TIR1 receptors results in an increase in the affinity between TIR1 and AUX/IAA proteins and a consequent activation of ubiquitin-mediated proteolysis of the AUX/IAA proteins by the 26S proteosome. Degradation of AUX/IAAs results in the derepression of AUXIN RESPONSE FACTORS

(ARFs) which regulate transcription by binding to the auxin response elements (AREs) in the promoter region of target genes.

Ethylene – Auxin crosstalk

The study of crosstalk between ethylene and auxin has greatly advanced in the last decade. Mutants that accumulate auxin, such as *sur1* have been used to better understand the roles ethylene and auxin play in plant hormone response. Suppression of leaf epinasty and hypocotyls elongation, both high auxin phenotypes of *sur1*, occurred when this mutation was introduced into the ethylene-insensitive background of *etr1-1*. Additionally, when *sur1* was in the auxin-resistant background of *axr1-3,* leaf epinasty and cell elongation were partially suppressed (Boerjan et al., 1995; King et al., 1995).

Response in other tissues has also been investigated, for instance root growth is also regulated by the interaction of ethylene and auxin. Initial experimental screens identified mutants that had auxin defects (Swarup et al., 2000; Dharmasiri and Estelle, 2002; Liscum and Reed, 2002). However the hormonal defects of these mutants were not limited to auxin; many had ethylene defects as well (Alonso et al, 2003b; Swarup et al., 2002; Larsen and Cancel, 2003). Furthermore, investigation of ethylene defects by Estelle (1996) resulted in the conclusion that high levels of auxin were required for root growth regulation by ethylene in Arabidopsis. This conclusion was refined by Rahman et al. (2001), who determined that high levels of auxin were not required for ethylene response in *aux1* or *eir1* mutants, however auxin appears to sensitize the plant to ethylene when it is present at a critical level in the cell. This dependent relationship does not hold true for auxin response as it has been determined that ethylene is not required for auxin response (Collett et al., 2000).

Transcription factors also play a vital role in hormonal crosstalk. The *HOOKLESS1* mutant, *hls1*, is insensitive to ethylene in the apical hook (Lehman et al., 1996). In a later study by Li et al. (2004) HLS1 was shown to be a critical integrator of hormone signaling pathways in the hypocotyl. In the presence of ethylene, HLS1 was found to be required for ARF2 accumulation. Additionally, *nph4*, a mutant of the auxin-regulated transcription factor, ARF7, has provided additional insight into the interaction of ethylene and auxin at the molecular level. Light and auxin defects of *nph4* (Stowe-Evans et al., 1998) are rescued by treatment with ethylene, hence ethylene may be responsible for regulating an activity equivalent to that performed by NPH4 (a.k.a. ARF7) (Harper et al., 2000). Mutants, *arf7* and *arf19* were both found to have root-specific ethylene insensitivity, and *ARF19* was induced by ethylene (Li et al., 2006). Additionally, a genomic fragment of *ARF19* was shown to be able to complement *arf7* mutations, suggesting that the activation of ARF19 by ethylene could be responsible for the ethylene-mediated rescue of the *nph4* defects. Together these findings illustrate the significance of the interconnection between these two hormones.

Ethylene and IAA are vital to maintaining proper plant development. Understanding their individual biosynthetic and response pathways have been a challenge and are yet to be fully elucidated. A greater challenge is determining the mechanism by which these hormones interact to provide specific responses to a variety of biotic and abiotic stimuli. In the following chapters I will describe research which resulted in the identification of two mutants with tissue-specific ethylene insensitivity that are involved in crosstalk between ethylene and auxin.

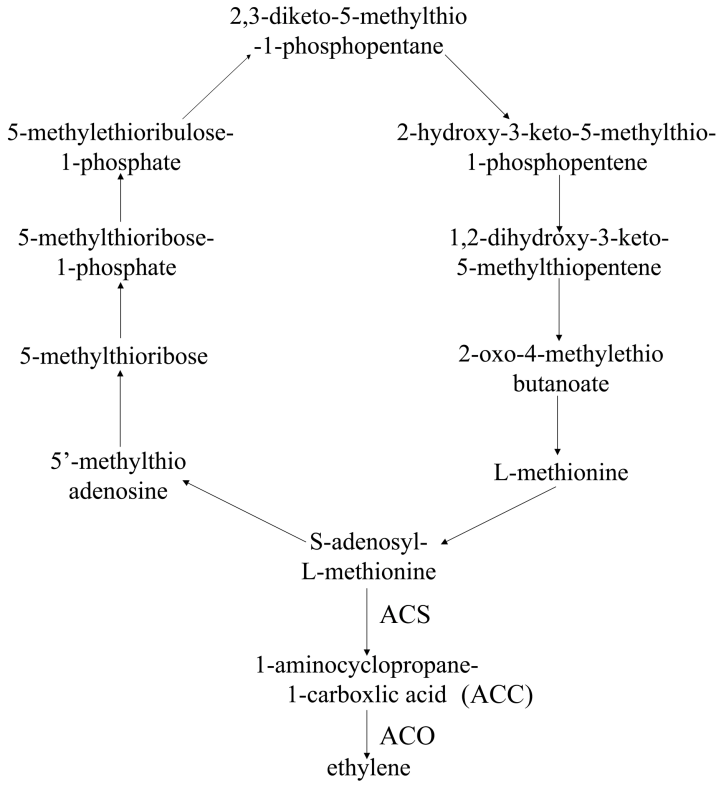


Fig. 1. The ethylene biosynthesis pathway. S-adenosyl-methionine is provided by the Yang Cycle. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is generated from the conversion of S-adenosyl-methionine by ACC synthase (ACS). ACC oxidase (ACO) then converts ACC to ethylene.

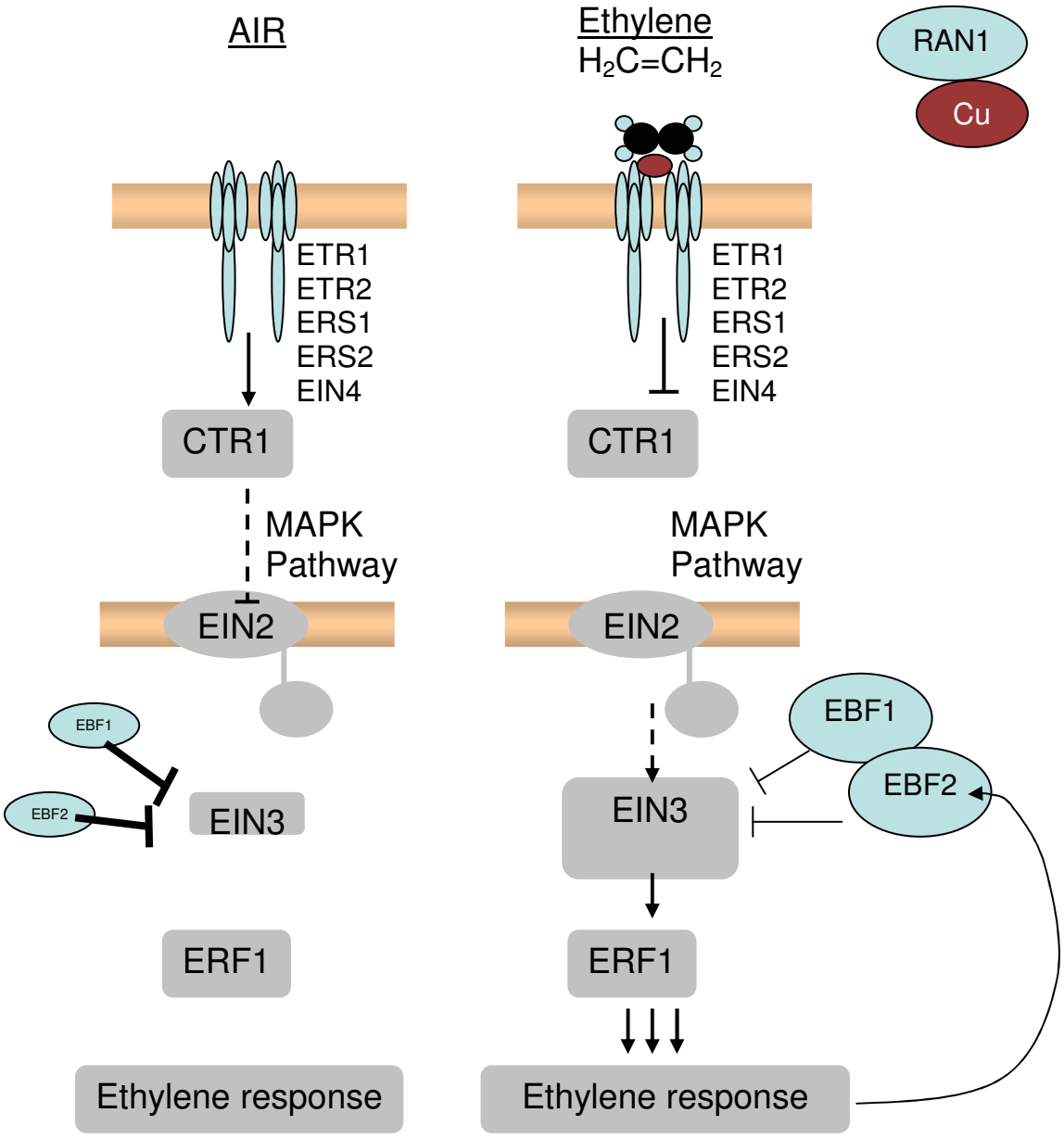


Fig. 2. Schematic representation of the ethylene signal transduction pathway. In the absence of ethylene (depicted as "AIR"), the endoplasmic reticulum-localized receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) negatively regulate the signaling pathway through interaction with CTR1. Ethylene, when present, binds to these receptors and CTR1-mediated negative regulation is removed and through an unknown series of intermediate steps thought to be controlled by map kinases, EIN2, a positive regulator of the pathway is activated. Another series of unknown signaling reactions occur, and the nuclear-localized family of EIN3 transcription factors are stabilized and accumulate resulting in transcription of Ethylene Response Factors (*ERF*s). However, in the absence of ethylene, the F-box proteins, Ethylene Binding Factor1 and 2 (EBF1 and EBF2), target EIN3 for proteolysis.

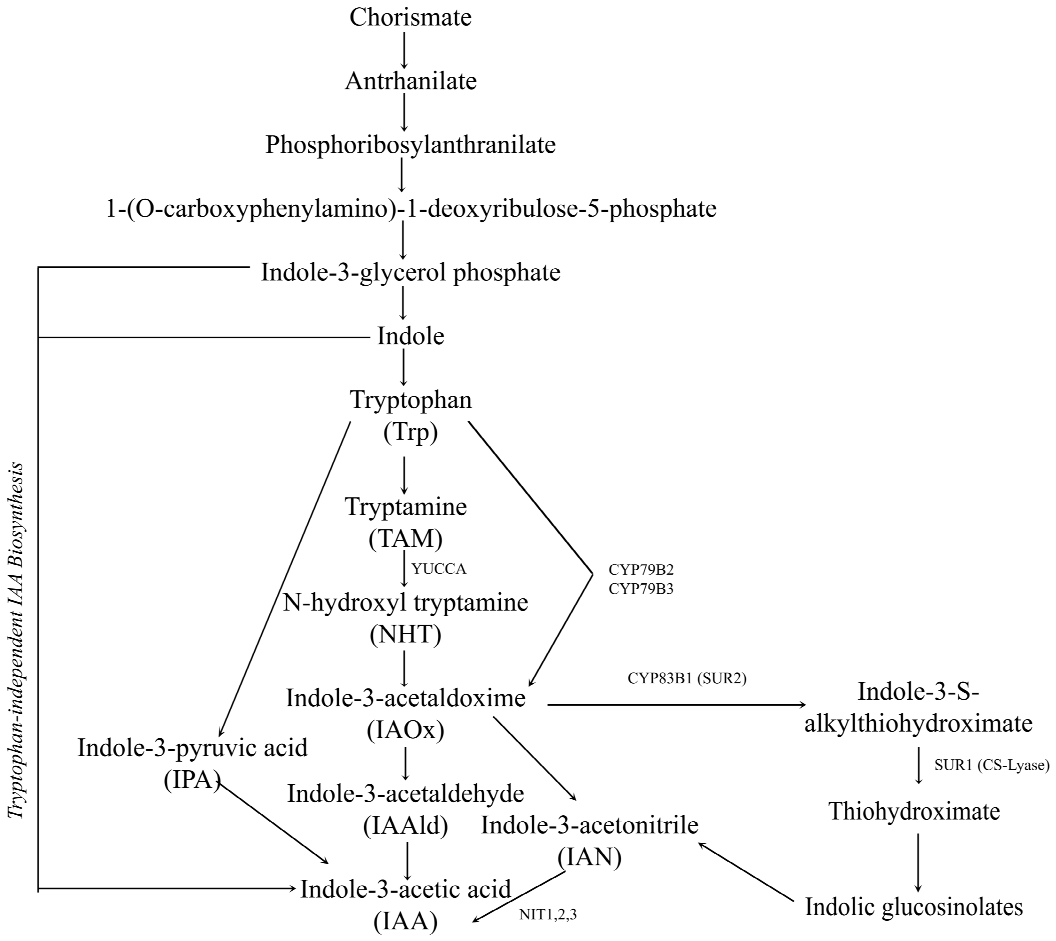


Fig. 3. Tryptophan and IAA Biosynthesis Pathway. The biosynthesis of tryptophan begins with the rate limiting conversion of chorismate to anthranilate via anthranilate synthase. IAA biosynthesis may be achieved in either a tryptophan dependent or independent manner. Additionally, tryptophan-dependent biosynthesis may occur via the indole-3-pyruvic acid (IPA), tryptamine (TAM), or indole-3-acetaldoxime (IAOx) pathways. IAOx may also be converted into indolic glucosinolates.

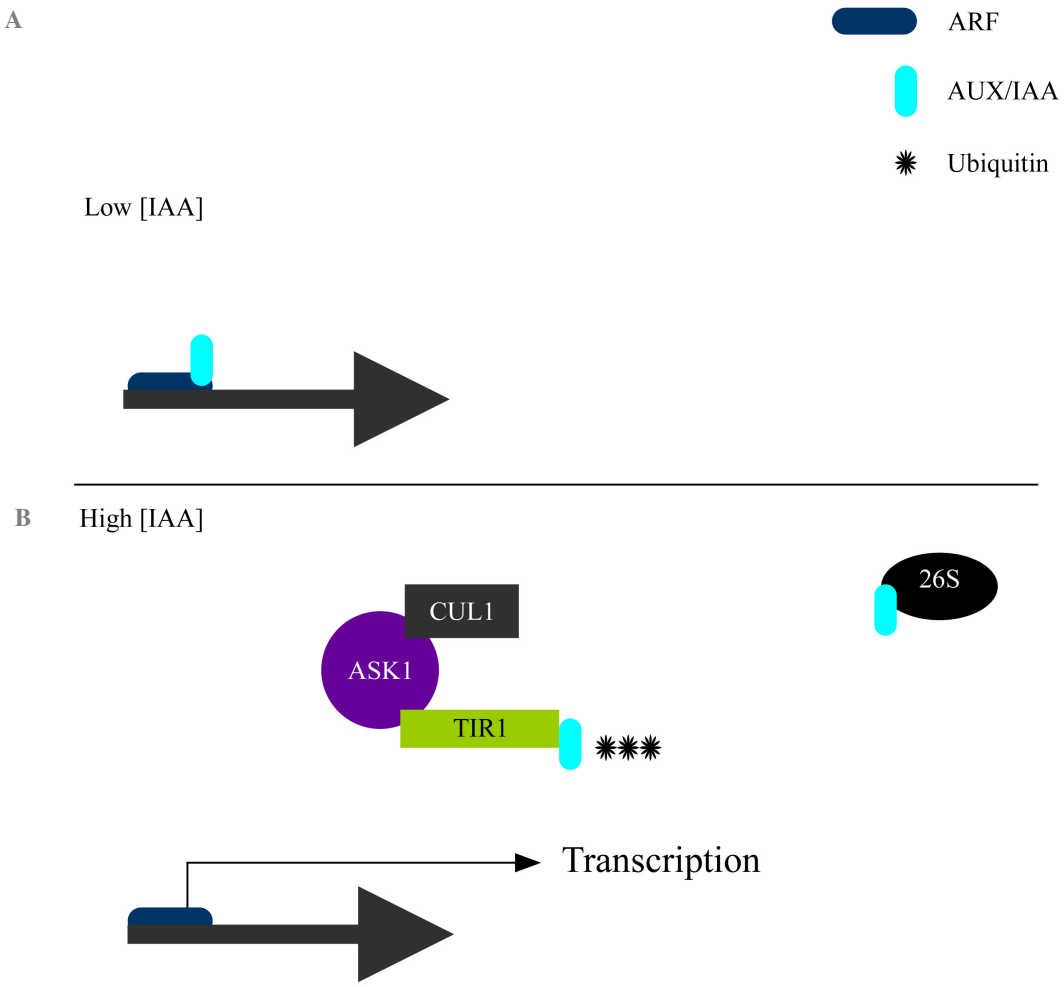


Fig. 4. Auxin Response in Arabidopsis. **(A)** In a low IAA concentration, AUX/IAA proteins bind to ARF transcription factors and cause repression of transcription. **(B)** As IAA concentrations increase, AUX/IAA proteins are targeted for proteolysis by the ASK1/CUL1/TIR1 complex. AUX/IAAs are ubiquitinated and subsequently degraded by the 26S proteosome.

Chapter II. Mapping of *WEAK ETHYLENE INSENSITIVE7* (*WEI7*) in *A. thaliana*

Work presented in the "Results" section of this chapter was contributed by Joyce M. Robertson-Hoyt. The remaining portion of this research was conducted by other scientists. This work has been previously published (*Stepanova, A.N., Hoyt, J.M., Hamilton, A.A., and Alonso, J.M. (2005). A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in Arabidopsis. Plant Cell.* ***17****, 2230-2242*).

ABSTRACT

WEAK ETHYLENE INSENSITIVE7 (*WEI7*) encodes the beta subunit of ANTHRANILATE SYNTHASE (ASB), an enzyme which catalyzes a rate-limiting step of the tryptophan biosynthesis pathway, converting chorismate to anthranilate. Tryptophan and its biosynthetic intermediates serve as the precursors for auxin biosynthesis. *wei7* produces an ethylene insensitive phenotype only in the root tissue. This tissue-specific ethylene insensitivity is a characteristic of auxin mutants. However, unlike auxin mutants, *wei7* shows a normal gravitropic response, and normal response to auxin treatment. The *wei7* phenotypes are, therefore, not exclusive of either the ethylene or auxin responses, but are inclusive of some aspects of each. Thus, WEI7 may be involved in crosstalk between ethylene and auxin signaling and response pathways.

INTRODUCTION

In previous experiments conducted by Anna Stepanova (North Carolina State University), over thirty root-specific ethylene insensitive mutants were obtained by screening seed pools of activation-tagged lines (Weigel et al., 2000). Complementation analysis was performed and indicated that eight of these mutants (*T1A2-3* (*wei7-1*), *T3D1-3* (*wei7-2*), *T3A2-1* (*wei7-2*), *T3A2-2* (*wei7-2*), *T3A2-3* (*wei7-2*), *T2B2-1* (*wei7-3*), *T2B2-2* (*wei7-3*), and *T4C1-2* (*wei7-4*)), which came from four independent pools, appeared to represent four *wei7* alleles. However, *wei7-2* and *wei7-3* were found to contain the same sequence mutation (details shown later), therefore only three mutant alleles exist (*wei7-1*, *wei7-2* and *wei7-4*). Allelisem between these three mutants was confirmed in the F2 generation of reciprocal crosses (Fig. 1). Conversely, several known ethylene mutants (i.e. *wei2* and *eir1*) fully complemented the *wei7-2* and *wei7-4* mutant phenotypes, suggesting that the *wei7* mutants may be novel. Epistatic analysis with *ctr1-1* determined WEI7 to be acting downstream of CTR1 in the signaling pathway. Additionally, all of the *wei7* mutant alleles showed normal sensitivity to auxin and were complemented by application of exogenous tryptophan (Stepanova et al., 2005).

Since WEI7 appears to function in the tryptophan biosynthesis pathway, and tryptophan is a precursor to IAA biosynthesis, DR5-GUS (Ulmasov et al., 1997) was used to infer the local levels of auxin in *wei7*. DR5-GUS is a synthetic auxin reporter composed of seven tandem repeats of an 11bp sequence which contains the auxin-responsive element, TGTCTC. This synthetic promoter is fused to *GUS*. DR5-GUS expressed in the *wei7* background was evaluated in both air and ethylene. In air, GUS expression in *wei7* was less than GUS expression in the *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). Importantly,

ethylene strongly stimulates DR5-GUS expression in the roots of wild-type plants while it had only a very small effect in the *wei7* mutant. This indicates that WEI7 is likely required for an ethylene-mediated increase in DR5-GUS and suggests that this protein may help to regulate auxin biosynthesis in response to ethylene (Stepanova et al., 2005).

To further investigate the role of WEI7 in the regulation of auxin biosynthesis, the effect of the *wei7* mutations on several mutants with increased levels of IAA (*cyp79b2ox*, *yucca*, *sur2*, and *rty1*) was investigated (Zhao et al., 2002) Analysis of the double mutant lines showed *wei7* was able to suppress the auxin overexpression phenotypes of elongated hypocotyls and epinastic cotyledons in the overexpression mutants. This implies that *WEI7* activity is required for maintaining high auxin biosynthetic rates in the plant (Stepanova et al., 2005).

Using the *wei7* activation-tagged ethylene–insensitive mutants identified by A. Stepanova, I conducted a mapping project. Cloning experiments show that *WEI7*, located on chromosome one, encodes a β-subunit of ANTHRANILATE SYNTHASE (ASB1), a key enzyme in the tryptophan biosynthesis pathway.

RESULTS

Determining the presence of a T-DNA element in *wei7* mutant lines

Initial cloning efforts were directed to determine whether or not the *wei7* alleles were tagged with a T-DNA. *wei7* plants were screened for resistance to Basta conferred from *BAR* in the T-DNA of the pSKI015 activation tag vector. Presence of Basta resistance would indicate the presence of a T-DNA tag in the mutant lines and plasmid rescue could be attempted. Two segregating populations derived from the mutants *wei7-2* and *wei7-4*

crossed with Ler were examined. Twenty-nine F2 families were examined for *wei7-2* and *wei7-4*. None of the 29 *wei7-2* F2 families showed herbicide resistance, implying that this mutant may not be tagged. Conversely, mutant *wei7-4* families known to be homozygous for *wei7* based upon ethylene insensitivity were found to be 100% resistant to Basta, whereas heterozygous mutant families segregated both Basta sensitive and resistant individuals. Based on these results, the *wei7-2* mutant allele is unlikely to harbor a T-DNA insertion, in contrast, *wei7-4* is likely tagged. Our data indicated, from complete concordance of the Basta resistance and ethylene insensitivity assays, that the *BAR* gene is tightly linked to the mutant gene in *wei7-4*. The evidence of cosegregation, along with the recessive nature of the *wei7* mutant, suggests that the ethylene insensitivity of *wei7-4* may be due to gene disruption by the T-DNA. However, in the case of *wei7-2*, ethylene insensitivity could be caused by a variety of factors including, but not limited to, the presence of an indel or single nucleotide mutation, insertions or rearrangements which effect transcription of *WEI7*, or perhaps insensitivity is caused by the T-DNA insertion, however *BAR* may be silenced. Despite multiple attempts to identify *wei7-4* sequence adjacent to the T-DNA insertion, we were not able to generate sequence-ready PCR products. Map-based cloning was next pursued to identify the location of *WEI7*.

WEI7 **maps to chromosome one**

Using a population of a total of 129 individual F2 plants obtained from crosses of the three *wei7* alleles to Ler, we initiated the mapping of the *WEI7* gene. No linkage between *WEI7* and known ethylene-related genes was observed, suggesting that these mutants may represent new components of the ethylene signaling or response pathway. The approximate

location of *WEI7* in the *wei7-2* x Ler population was identified by genotyping 23 individuals at eleven SSLP loci distributed across the *A. thaliana* genome. Linkage was detected at marker nga248 located at 9.887 Mb on chromosome one. Similar linkage with nga248 was identified in the *wei7-4* x Ler population.

Cloning of *WEI7*

WEI7 was mapped to a 2.6-Mb region between the SSLP markers F9H16-1 (7.245 Mb, 18 recombinants) and nga248 (9.887 Mb, 7 recombinants) on the top arm of chromosome one (Fig. 2A). Based on the approximate location of *WEI7* in combination with current knowledge of genes in this region, and the fact that the previously cloned ethylene insensitive gene, *WEI2*, encodes ASA1, it was deduced that the *wei7* mutation may be caused by a defect in one of the putative five *ASB1* (*ANTHRANILATE SYNTHASE*, beta subunit) genes located between these two markers. ASB1 is a partner of ASA1, both of which are required for the conversion of chorismate to anthranilate in the tryptophan biosynthesis pathway. As mentioned earlier, tryptophan, and several intermediates of tryptophan biosynthesis are biosynthetic precursors to the plant auxin, indole-3-acetic acid (IAA).

This region contains five putative *ASB* subunit genes (*At1g24807*, *At1g24909*, *At1g25083*, *At1g25155*, and *At1g25220*) (Fig. 2B) whose protein products are thought to function as heterodimers with ASA subunits in the biosynthesis of tryptophan (Radwanski and Last, 1995). Of the five *ASB* genes in the region, four are identical (with sequence identity expanding beyond the coding regions) and are likely to be functionally redundant. Therefore, we decided to first focus on the most divergent *ASB* gene in the region *ASB1,* (*At1g25220*). Because the *wei7* mutants are derived from an activation-tagged T-DNA

collection, we attempted to amplify T-DNA junction sequences from the border-specific primers. In the process of testing gene-specific primers, we discovered that *wei7-1* lacks ~1.6 kb of the 5' end of the *ASB1* gene, including the first 156 bp of the open reading frame, whereas in *wei7-4*, the entire chromosomal region that contains *ASB1* in wild-type plants is deleted (Fig. 2C). Upon sequencing of the remaining *wei7-2* allele, a G-to-A transition at position 1569 in the sixth exon that results in a Gly-176-to-Glu amino acid substitution was found. Glycine, a very small amino acid with no charge or polarity, is often associated with structural areas in a molecule where hairpin-like turns are found (Kwasigroch et al., 1996). Glutamic acid is a substantially larger amino acid (147.13 (Glu) vs 75.07 (Gly)) with acidic properties and is negatively charged, thus this substitution may likely have a dramatic effect on protein structure, as well as function. Gly-176 is highly conserved not only in all six ASB isoforms of Arabidopsis, but also in each of the ASB proteins examined across different genera, including in the bacterial orthologs (Fig. 3). Based on these results, we conclude that *wei7* is a loss-of-function mutant of *ASB1* that encodes the β-subunit of anthranilate synthase, which controls a rate-limiting step in Trp biosynthesis.

DISCUSSION

Through the cloning and characterization of *wei7*, an ethylene response mutant in the tryptophan biosynthesis pathway has been discovered and is found to be a link between the ethylene and auxin pathways. *WEI7* as well as *WEI2* (Stepanova et al., 2005) reveal a mechanism by which ethylene can regulate the levels of auxin in the plant. *WEI2* and *WEI7* encode the alpha and beta subunits of anthranilate synthase, respectively. The products of these two genes work together to catalyze a rate-limiting step in the tryptophan biosynthesis

pathway, the conversion of chorismate to anthranilate. The ethylene-mediated regulation of IAA levels (via *DR5-GUS* expression), in conjunction with data showing the ability of ethylene to induce transcription of *WEI2* and *WEI7* using transcriptional fusions (*ASA1-GUS* and *ASB1-GUS*) (Stepanova et al., 2005), indicates that ethylene plays a role in the regulation of IAA biosynthesis, a process that is required for normal ethylene response.

The mutant *wei7* was identified as having root-specific ethylene insensitivity and normal responses to auxin. Cloning revealed *WEI7* encoded anthranilate synthase1 β-subunit (ASB1). *WEI2*, which was previously cloned (Stepanova et al, 2005), codes for the corresponding alpha subunit (ASA1) and these proteins work together to catalyze the conversion of chorismate to anthranilate, the first committed step in the tryptophan biosynthesis pathway. The biosynthesis of the plant hormone IAA uses tryptophan as a precursor, hence the ethylene defect seen in *wei7* may be the result of having reduced levels of IAA. Complementation of ethylene insensitivity in *wei7* by IAA has been proven, and this supports work by Rahman et al. (2001), which showed that mutants, *aux1* and *eir1* show ethylene defects and can respond normally to ethylene following addition of exogenous auxins.

Mutation in *WEI7*, would logically result a reduction in IAA levels and manifest in the form of auxin defects, such as agravitropism and loss of apical dominance, in *wei7*. However, neither *wei2* nor *wei7* show auxin or tryptophan defects. Interestingly, strong tryptophan biosynthesis mutants *trp2* and *trp3*, show no decrease in IAA levels although each had strong tryptophan-related phenotypes (Last et al, 1991; Normanly et al., 1993; Radwanski et al., 1996). Therefore, instead of an overall decrease in the level of IAA being the cause of ethylene insensitivity in *wei2* and *wei7*, insensitivity may be due to only specific

pools of tryptophan being reduced in these mutants. Moreover, since application of exogenous tryptophan can complement ethylene insensitivity, it seems unlikely that *wei2* and *wei7* ethylene insensitivity could be due to a defect in the tryptophan-independent auxin biosynthetic pathway.

The hypothesis that ethylene is able to regulate tryptophan, and hence IAA, levels in the plant through WEI2 and WEI7 was supported through use of the IAA reporter construct *DR5-GUS* (Ulmasov et al., 1997). The *DR5* element is an auxin reporter that contains a synthetic promoter of seven repeated auxin-responsive elements. This promoter is fused to *GUS* and in the presence of IAA drives the transcription of the *GUS* reporter gene. This construct is used to determine relative levels of local IAA *in planta* and has been found to be highly correlated to IAA levels in the root (Casimiro et al., 2001). Based upon GUS activity of transcriptional reporters *ASA1-GUS* and *ASB1-GUS* (Stepanova et al., 2005), *WEI2* and *WEI7* expression is induced by ethylene. In the presence of ethylene, *DR5-GUS* expression in *wei7* and *wei2* is lower compared to Col-0. This implies that *WEI7* and *WEI2* are regulated by ethylene and required for an ethylene-mediated activation of the auxin reporter *DR5-GUS*. Additionally, *ASA1-GUS*, *ASB1-GUS*, and *DR5-GUS* are each expressed in the root tissues (a site of auxin biosynthesis). This indicates that in the root tip, ethylene induces *ASA1* and *ASB1* transcription and suggests that this induction is required for an ethylene-mediated increase of auxin levels.

In summary, *WEI7* encodes the β-subunit of anthranilate synthase and is involved in the conversion of chorismate to anthranilate (Fig. 4) and along with *WEI2* (the α-subunit protein), *WEI7* is up-regulated by ethylene and responsible, at least in part, for the ethylene-mediated stimulation of auxin biosynthesis. This increase in IAA then is required for a

normal response to ethylene in the root. Since tryptophan treatments do not induce the auxin phenotypes of elongated hypocotyls and epinastic cotyledons in Arabidopsis, this regulatory scenario is likely to exist for other enzymes of the tryptophan and IAA biosynthesis pathway, such as WEI8 (discussed in the next chapter). This work has provided an example of the interconnection of ethylene and IAA in controlling plant development.

MATERIALS AND METHODS

Arabidopsis strains and growth conditions

Seeds were surface sterilized in 50% bleach solution containing 0.005% Triton X-100 for 10 minutes, followed by three rinses in autoclaved dH2O. Sterile seeds were planted on agar medium pH 6.0 (1X Murashige and Skoog (MS) medium, 1% (w/v) sucrose, and 0.8% (w/v) agar) and placed at 4 degrees for 3-5 days in the dark. Plates were then moved to the light for two hours to promote germination, followed by 72 hours of growth in the dark at 22ºC. Seedlings transplanted to soil [1:1 mixture of MetroMix-200 (Scotts; Marysville, OH.) and Fafard Germinating Mix (Conrad Fafard; Agawam, MA.)] were grown under 16 hour light cycles at 22ºC or constant light at 22C.

Determination of presence of activation tag in mutant lines

The expression of a selectable marker can be used to indicate presence of T-DNA insertion in transformed lines. The vector construct used to generate the *wei7* mutants consisted of the plasmid pSKI015, which contains the phosphonothricin resistance gene (*BAR*) in the T-DNA. Expression of *BAR* confers resistance to the herbicide Basta. If co-segregation of Basta resistance with ethylene insensitivity exists, this indicates that a T-DNA

insertion is likely the cause of insensitivity or is at least closely linked to the gene responsible for insensitivity, and plasmid rescue may be attempted to identify the location of the gene. F3 families from each population were screened on media supplemented with either 10uM ACC or the herbicide Basta to determine if a correlation exists between ethylene insensitivity and Basta resistance.

WEI7 mapping and Sequence Analysis

A map-based approach was use to clone *WEI7*. The mutants, *wei7-1*, *wei7-2* and *wei7-4*, all in Col-0 background, represented three mutant alleles and were crossed to *L. erecta* (Ler). A total of 129 F2 ethylene-insensitive individuals were selected across the populations and were retested in the F3 generation. DNA was isolated from the selected lines according to Doyle and Doyle (1987) and subsequently genotyped using genome-wide simple sequence-length polymorphic (SSLP) markers (Bell and Ecker, 1994) polymorphic between Col-0 and Ler. Linkage was detected with markers SO392 (F1 5'-GTTGATCGCAGCTTGATAAGC-3'; R1 5'TTTGGAGTTAGACACGGATCTG-3'), NGA248 (F1 5'-TCTGTATCTCGGTGAATTCTCC-3'; R1 5'-TACCGAACCAAAACACAAAGG-3'), and F9H16 (F1 5'-GGTGAGATACTGAGATTATCCTTG-3'; R1 5'-GATTCTATTTTGCTTGGCGTATGTG-3') (Table 1). Nucleotide fragments were amplified using polymerase chain reaction (PCR) for 40 cycles, where one cycle consisted of 15 seconds at 94ºC, 40 seconds at 52ºC, and 1 minute at 72ºC. Electrophoresis on 3% agarose gel, containing ethidium bromide was used to separate amplified PCR products. Fragments were visualized under ultraviolet light.

ASB1 tandem repeat analysis

In an attempt to determine if the mutant lines contain mutations within the *ASB1* gene, six primers were designed which were specific for *ASB1* (*At1g25220*) but which will not work in a PCR assay with any of the other *ASB1*-like genes. *At1g25220* was chosen for primer design since the remaining four *ASB1* genes (*At1g24807*, *At1g24909*, *At1g25083*, *At1g25155*) contain very high levels of homology (*WEI7-like* tandem repeats are identical and vary from *WEI7* at only a handful of single residues, even in non-coding and promoter regions, (data not shown) hence functional redundancy is extremely likely. Their redundant nature makes them very unlikely candidates for detection in an ethylene insensitivity screen. However, the few instances of novel sequence of *At1g25220* compared with the *WEI7-like* genes, provides the opportunity for novel and selectable mutation in this gene. Four unique forward and reverse primers were synthesized to amplify genomic regions of *At1g25220* (Table 2). These are:

ASB1-F1 (5'-GAATCTGAGCTCCTCAGACAATGGCGGCTTC-3')

ASB1-F2 (5'-GTATCTTTTTGAAGTGCCCGAG-3')

ASB1-F3 (5'-CTTCACATACAATCTCTGCCAGG-3')

ASB1-F4 (5'-TCTAATATAGTAAATCATAAGTCTG-3')

ASB1-R1 (5'-GGAACAAGTGGTCCGAGTTCC-3')

ASB1-R2 (5'-CCATAGCTTTCTGCTTTCCACTT-3')

DNA from each mutant was amplified using varying combinations of forward and reverse primers, followed by purification and unidirectional sequencing BigDye Terminator v3.1 kit (ABI Prism; PE-Applied Biosystems, Sunnyvale, CA). Complete sequences between the

ASB1-F1 and *ASB1*-R2 primers were obtained for *wei7-2* and *wei7-3*. The recovered sequences were analyzed in Sequencher™ (Gene Codes Corporation, version 4.1).

Table 1. Primer sequences for three markers used in the fine mapping of *WEI7* in three populations

Marker	Chromosome	Position Mb	Primer sequence	
			Forward	Reverse
F9H16	1	7.245	GGTGAGATACTGAGATTATCCTTG	GATTCTATTTTGCTTGGCGTATGTG
nga248	1	9.945	TCTGTATCTCGGTGAATTCTCC	TACCGAACCAAAACACAAAGG
SO392	1	10.825	GTTGATCGCAGCTTGATAAGC	TTTGGAGTTAGACACGGATCTG

Table 2. *WEI7* sequencing primers designed for the identification of intragenic mutation in *wei7* mutants

Marker	Primer sequence
ASB1-F1	GAATCTGAGCTCCTCAGACAATGGCGGCTTC
ASB1-F2	GTATCTTTTTGAAGTGCCCGAG
ASB1-F3	CTTCACATACAATCTCTGCCAGG
ASB1-F4	TCTAATATAGTAAATCATAAGTCTG
ASB1-R1	GGAACAAGTGGTCCGAGTTCC
ASB1-R2	CCATAGCTTTCTGCTTTCCACTT

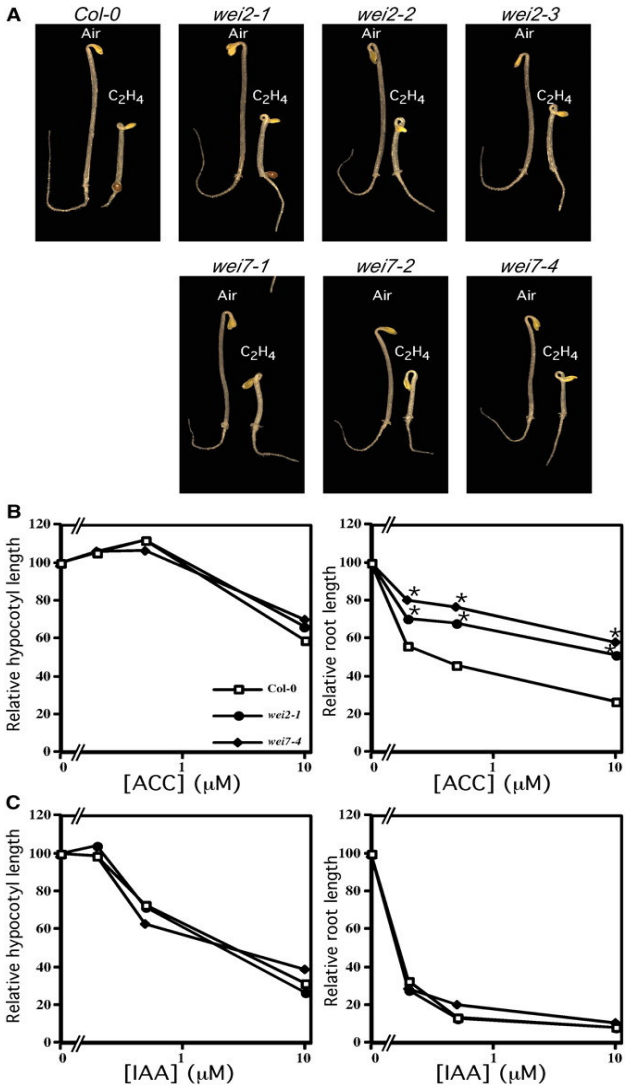


Fig. 1. *wei2* and *wei7* Are Root-Specific Ethylene-Insensitive Mutants
(A) Phenotypes of 3-d-old etiolated Col-0, *wei2-1*, *wei2-2*, *wei2-3*, *wei7-1*, *wei7-2*, and *wei7-4* seedlings grown on AT plates in the presence of hydrocarbon-free air or air supplemented with 10 ppm ethylene.
(B) and **(C)** Dose-response curves of hypocotyls (left) and roots (right) of 3-d-old etiolated Col-0, *wei2-1*, and *wei7-4* seedlings grown in AT medium.

This figure is appears in Plant Cell (17:2230-2242) and is reproduced with permission.

Stepanova, A.N., J.M. Hoyt, A.A. Hamilton, and J.M. Alonso. 2005. A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in Arabidopsis*. The Plant Cell. 17:2230-2242.*

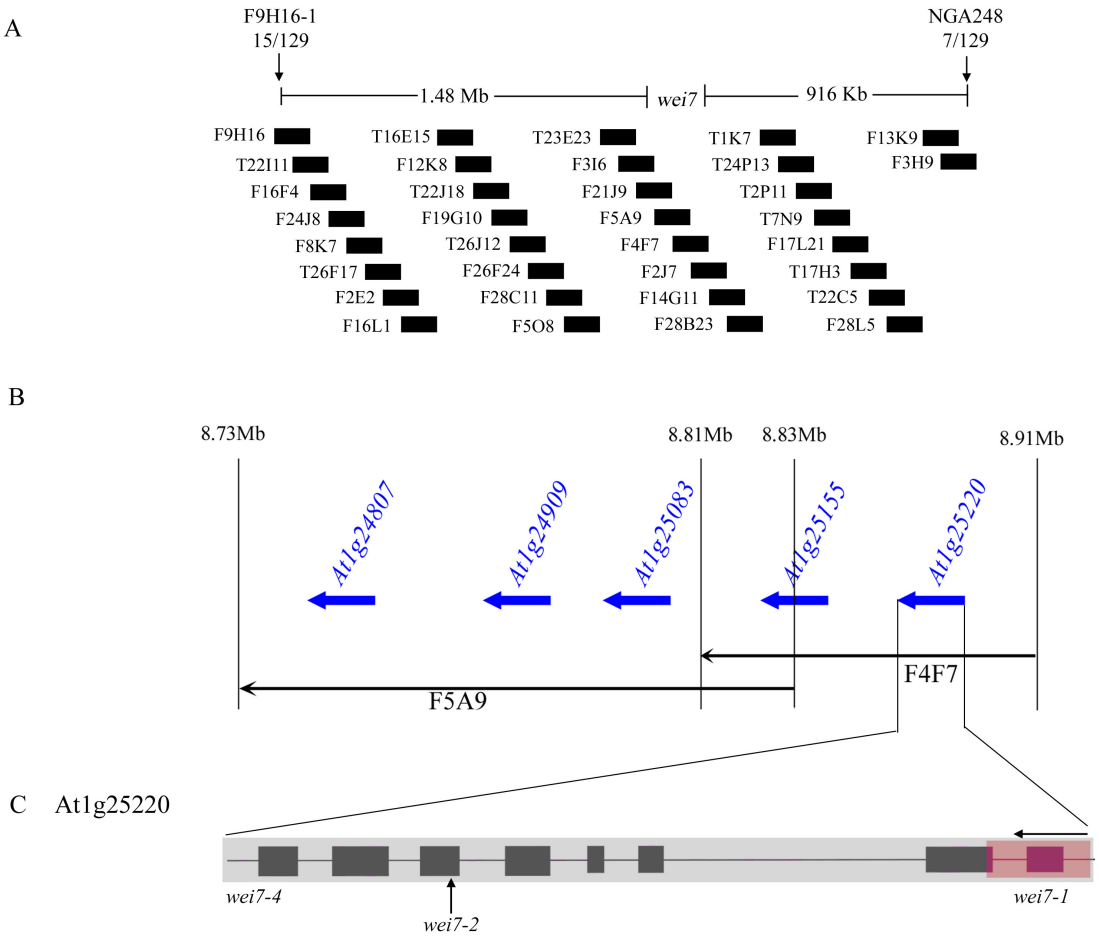


Fig. 2. *WEI7* Gene Map. **(A)** 129 F2 individuals from a population segregating for ethylene insensitivity were genotyped revealing an approximate location for *WEI7*. Fifteen and seven recombinants were identified at markers F9H16-1 and nga248, respectively. Solid bars represent BACs. **(B)** *WEI7* (At1g25220) and *WEI7* homologs in tandem orientation on chromosome one. **(C)** At1g25220 was sequenced revealing *wei7* mutations. *wei7-1* contains a 5' deletion, *wei7-2* contains a single nucleotide polymorphism (SNP), and *wei7-4* is the result of a whole gene deletion.

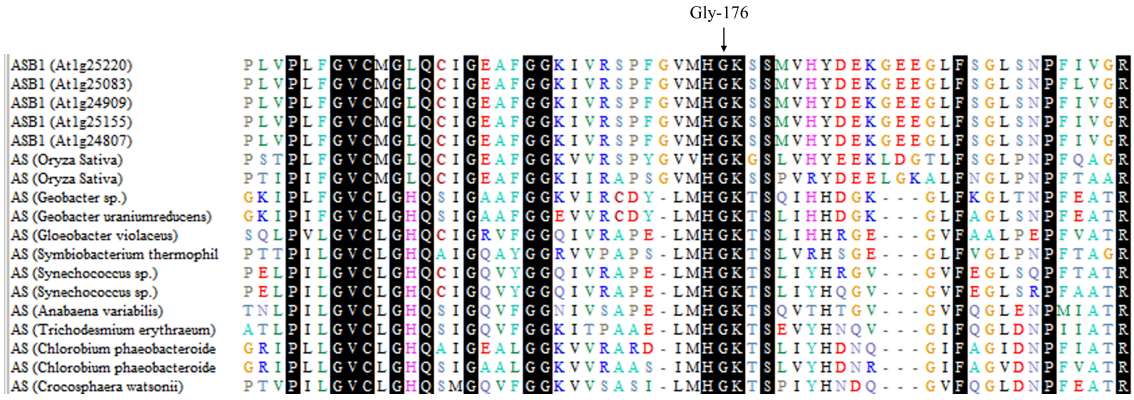


Fig. 3. Sequence Conservation of WEI7. Amino acid sequence analysis of WEI7 compared with plant and bacterial homologs. Gly-176 is mutated to Glutamic acid in *wei7-2*. Glycine is highly conserved throughout plant and bacteria Anthranilate Synthase beta subunit (ASB1) peptides. Amino acids are shown in color to aid in visual differentiation. Residues that are 100% identical at a single position are shown with a black background.

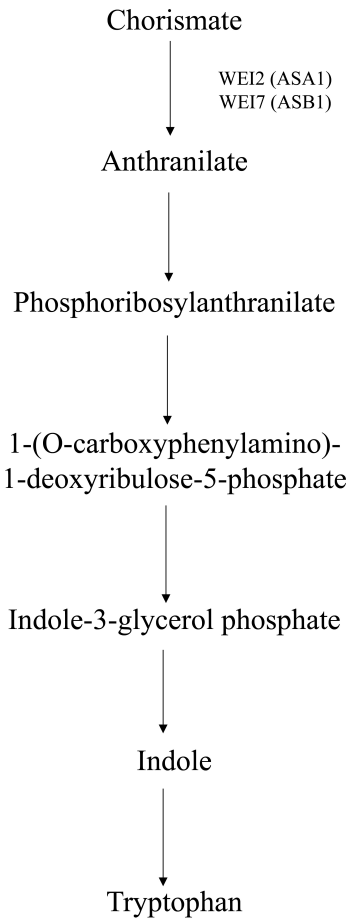


Fig. 4. Position of WEI7 in tryptophan biosynthesis. WEI7 codes for the beta subunit of Anthranilate Synthase1 (ASB1). This enzyme which works with ASA1 (WEI2) to convert chorismate to Anthranilate, is the first committed step in the tryptophan biosynthesis pathway.

Chapter III. Identification of *weak ethylene-insensitive8* (*wei8*) reveals a link between auxin and ethylene

ABSTRACT

Interaction between the plant hormones ethylene and indole-3-acetic acid (IAA) is of increasing interest in the plant biology community. The novel IAA knock-down biosynthetic mutant of *WEAK ETHYLENE-INSENSITIVE8*, *wei8*, results in root-specific ethylene insensitivity. Double mutant lines generated by combining *wei8* with *wel2*, a *WEI8* homolog, show increased ethylene insensitivity, a loss of apical dominance in adult plants, greatly reduced fertility, and a reduction in the expression levels of the auxin reporter *DR5-GUS*. In vitro, WEI8 can act as an aminotransferase that can use tryptophan, an IAA precursor, as a substrate. Overexpression of *WEI8* and *WEL2,* in the *wei8-1* mutant background can restore normal ethylene sensitivity to *wei8* mutant plants. Additionally, like the overexpression mutant of the cytochrome P-450 *CYP79B2*, or flavin monooxygenase *YUCCA,* overexpression of WEI8 can confer resistance to the toxic tryptophan analog, 5-methyltryptophan (5-MT). *wei8*, as an auxin biosynthesis mutant and a root-specific ethylene response mutant, provides additional insight to the potential for crosstalk between ethylene and auxin, and allows further study of the auxin biosynthetic pathway using a tissue specific ethylene insensitive phenotype.

INTRODUCTION

Recently, two genes, *WEI2* and *WEI7*, which are weakly insensitive to ethylene were identified (Stepanova et al., 2005). *WEI2* and *WEI7* encode the alpha and beta subunits of anthranilate synthase, an enzyme that catalyzes a rate-limiting step of the tryptophan biosynthesis pathway. Tryptophan, and its biosynthetic intermediates serve as the precursors for auxin biosynthesis. Remarkably, *wei2* and *wei7* result in ethylene insensitive phenotypes only in the root tissue, a characteristic of auxin mutants such as *aux1* and *axr1* (Lincoln et al., 1990; Yamamoto and Yamamoto, 1998). However, unlike auxin mutants, they showed a normal gravitropic response, and normal response to auxin treatment. These phenotypic observations are not exclusive of either the ethylene or auxin responses, but are inclusive of some aspects of each, suggesting that these proteins may function in an interaction point between ethylene and auxin signaling and response pathways. This is consistent with the molecular identity of *wei2 and wei7*, and the fact that ethylene gas induces the expression of *WEI2* and *WEI7* in the root tip as well as an increase in the auxin levels in these tissues.

The mutant *wei8* also shows the same ethylene insensitivity and normal gravitropism as *wei2* and *wei7*. However, whereas *wei2* and *wei7* can be complemented by tryptophan and IAA, only IAA can restore ethylene sensitivity in *wei8*. Based upon this, we expect *wei8* mutants to contain lesser amounts of IAA. However, determining if WEI8 affects IAA production is quite complex, since IAA can be generated via many different pathways and partially blocking one of these pathways may not dramatically reduce the total levels of IAA. One of the branches in the auxin biosynthetic pathway which is not well characterized at the genetic level is that indole-3-pyruvic acid (IPA), where IAA is believed to be synthesized from the conversion of tryptophan to IPA by an unidentified aminotransferase (Chapter I,

Fig. 3). Based upon a query of the TAIR database (www.arabidopsis.org) there are 56 aminotransferases (known and putative) in the Arabidopsis genome (Rhee et al., 2003). Similarly, Leipman and Olsen (2004) identified 44 aminotransferases as well as a number of uncharacterized aminotransferase-like genes. This number of potential aminotransferases makes the identification of the specific aminotransferase that catalyzes tryptophan to IPA reaction difficult. Annotating solely on the basis of sequence (genomic or amino acid) remains challenging due to the sequence similarity between aminotransferases, alliinases and carbon-sulfur (CS)-lyases. Thus, for example, genes originally annotated as aminotransferases, such as SUR1 and CORI3, were latter shown to function as CS-lyases (Mikkelsen et al., 2004: Jones et al., 2003). Therefore it is possible that many of these 56 genes do not function as aminotransferases while other genes thought to be CS-lyases could well be functioning as aminotransferases.

Structural studies have examined the three-dimensional structure of garlic (*Allium sativa*) alliinase, a class of CS-lyases. Alliinase has a unique N-terminal epidermal growth factor (EGF) domain, a pyridoxal 5'phosphate domain, and a C-terminal domain (Kuettner et al., 2002), and it is proposed that this EGF domain of alliinase may act as a binding site for interacting proteins or a hypothetical alliinase receptor. Comparisons of alliinase to CS-lyases and aromatic aminotransferases reveal a very high degree of similarity among the three proteins. Aminotransferases and CS-lyases are similar in that they both require a pyridoxal 5'-phosphate cofactor, however CS-lyases cleave cysteine-containing compounds by cleaving the β-carbon-sulfur link of L-cysteine (Ramirez and Whitaker, 1998), whereas aminotransferases are responsible for the relocation of NH_2 groups between molecules. As mentioned above, one of the challenges in studying the function of putative alliinases, CS-

lyases, or aminotransferases is the difficulty in discerning the true function of a protein of this class based exclusively on its sequence, and experimental confirmation is essential as has been shown in recent studies mentioned above (Jones et al., 2003).

WEI8 is annotated as an alliinase-related aminotransferase. Alliinase is an enzyme of onion (*Allium cepa*) and garlic (*Allium sativa L.*) of the CS-lyase family which converts allin to allicine and in the process emits the characteristic onion and garlic odor. Allinase is also thought to be involved in the mechanism of plant defense due to the antimicrobial qualities of allicine (Block, 1992). Binding specificity was shown in a study by Manabe et al. (1998) whereby alliinase isolated from Chinese chive (*Allium tuberosum*) revealed a pyridoxal 5'-phosphate cofactor binding site, Lys280, which was conserved across Allium CS-lyases. Functional studies of allium protein from onion reveal both alliinase and more general CS-lyase activities (Lancaster et al., 2000).

Based upon experiments identifying the tryptophan biosynthetic enzymes WEI2 and WEI7 as necessary components for ethylene response, we are convinced that screening for tissue specific ethylene insensitivity is a powerful approach that can lead to the identification of genes involved in ethylene-auxin crosstalk. In the current study, *wei8* was identified using a root-specific screen for ethylene insensitivity. Genetic and functional assays reveal *WEI8* encodes an alliinase-related aminotransferase which is proposed to be an essential protein to maintain wild-type levels of IAA. A high degree of gene redundancy has been suggested as the main reason for the difficulty in identifying the genetic elements of the auxin biosynthetic pathway. Consistent with this, most of the known genes in this pathway were identified as gain of function alleles (*yucca*, *cyp79b2*, *cyp83b1*, (Bak et al., 2001, Zhao et al., 2001)). Although *WEI8* is also a member of a multigenic family, loss of function *wei8* alleles were

identified due to their tissue-specific ethylene insensitivity, indicating the utility of this new screen in the study of auxin biosynthesis. This also suggests that the redundancy in the auxin biosynthetic pathway can be overcome not only by using gain of function approaches, but also, by using screens based on very specific stimulus, in this case ethylene, that affect only some of the members of a gene family.

RESULTS

Phenotypic and genetic characterization of *wei8*

Phenotypic deviations from the ethylene-induced triple response assay were used to identify two new root-specific ethylene-insensitive mutants, *wei8-1* (provided by Anna Stepanova, North Carolina State University) and *wei8-2* (provided by Larissa Benevente, North Carolina State University) (Fig. 1). *wei8-1* was identified from the screening of activation-tagged lines, and *wei8-2* from an EMS mutagenesis screen. Unlike *wei2* and *wei7*, *wei8* ethylene insensitivity mutants are not complemented by exogenous tryptophan, but can be complemented by the exogenous auxin, IAA (Fig. 2).

To determine the dominance or recessiveness of the *wei8* mutant alleles, a population derived from a cross of *wei8-1* and Col-0 was generated. In the F1 generation all plants showed normal ethylene response and in the F2 the segregation ratio of the ACC-insensitive phenotype indicated the recessive nature of *wei8*. Additionally, analysis of the *wei8-1 ctr1-1* double mutant (performed by A. Stepanova) revealed a *wei8*-like phenotype. Based upon this phenotype it can be concluded that WEI8 works downstream of CTR1 in the ethylene signaling/response pathway.

Mapping and sequence analysis

WEI8 was cloned by map-based positional cloning methods using a genome-wide scan of SSLP markers which were known to be polymorphic between Col-0 and *Landsberg erecta* (Ler), followed by a reverse genetic approach. The mapping population consisted of 487 F2 lines derived from a cross of *wei8-1* (Col-0 background) to Ler. Linkage was identified on chromosome one. Six recombinants were identified with a marker designed in BAC F20P5 (26.3 Mb), there were two recombinants in F5A18-1 (26.5 Mb), and four recombinants were genotyped in F23N20 (26.7 Mb) (Fig. 3A) (Table 1). Once a rough map position was determined, we attempted to identify the gene required for ethylene sensitivity by screening T-DNA knockouts of candidate genes in the recombinant region. Salk T-DNA lines (Alonso et al., 2003) corresponding to the genes in the region were screened for ACC insensitivity (Table 2) and only knockouts in *At1g70560* (*Salk_127890* (*wei8-3*), *Salk_22743* (*wei8-4*), and *Salk_48897* (*wei8-5*)) were found to show potential insensitivity (Fig. 3B) (Table 2), but all the experiments described here were performed with the better characterized *wei8-1* and *wei8-2* alleles.

Sequence analysis revealed *wei8-1* contained a T-DNA insertion in the 3' region of exon three in the alliinase-related CS-lyase, aminotransferase (*At1g70560*) (Fig. 3C). This insertion is located at nucleotide position 1716 (669 downstream of the ATG of the cDNA). The *wei8-2* mutation is a C-T base change at nucleic acid position 1544, resulting in a missense mutation of Pro166 to Ser166. Proline is an intermediate sized, hydrophobic residue, whereas serine is a small residue which forms side chains with hydrogen bonds or salt bridges. This proline is highly conserved among queried plant and bacterial aminotransferases. Since the amino acid sequence SPNNP is conserved at a high level, it

may be an essential structure which must be maintained for proper function (*wei8-2* affects the first proline of this sequence), and the phenotype of *wei8-1* and *wei8-2* are indistinguishable, we speculate that these are loss of function alleles or at least strong hypomorphs.

WEI8 is a member of a multigenic family in *Arabidopsis*

In order to identify the existence of proteins homologous to WEI8, a query was performed. Four genes were found to have a high level of amino acid sequence similarity with WEI8. The level of similarity ranges from 68% identical (82% similar) to 37% identical (58% similar) in the WEI8 homologs (Fig. 7). These homologous proteins are annotated as alliinase-related aminotransferases, however they also contain epidermal growth factor (EGF) and alliinase-like C-terminal domains (Fig. 7). These homologs will be referred to as WEI8-Like (WEL) 1 to 4.

WEI8 is required for ethylene response

As shown in figure 1, *wei8* mutants do not have normal sensitivity to ethylene, resulting in a root-specific insensitive phenotype. To further demonstrate that mutation in *At1g70560* (for now on called *WEI8*) is responsible for this phenotype and to determine if sensitivity could be restored by the expression of *WEI8*, *wei8-1* plants were transformed using an agro-mediated floral dip technique with overexpression constructs containing *WEI8* or, the *WEI8* homolog, *WEL2*. The constructs *35S-GFP-WEI8* and *35S-MYC9-WEL2* were generated. *Agrobacterium*-mediated transformation of *wei8* was performed and T2 seedlings were examined on 10uM ACC. This screen revealed lines in the *wei8-1* background which

segregated ethylene-sensitive and insensitive plants (Fig. 4). This restoration of ACC sensitivity of *wei8* mutants by the expression of *At1g70560* or *At4g24670* further supports that the molecular identity of WEI8 had been uncovered, and that the GFP-WEI8 and GFP-WEL2 fusion proteins were functional and could restore normal ethylene response to *wei8* mutants.

From previous experiments we have shown that WEI8 is required for ethylene sensitivity, but can we determine where this protein is localized? We attempted to visualize the subcellular localization of the N-terminal GFP-tagged WEI8 in the roots of transformed *wei8-1* plants. Two independently transformed lines were used and it was found that GFP was visible in the roots of the lines with restored ACC sensitivity, whereas segregating progeny which remained ACC insensitive did not show any visible GFP fluorescence (Fig. 5B,C) and was similar to the untransformed control (Fig. 5A). Subcellular localization of WEI8 was examined using confocal imaging. Using this approach it should be possible to determine if WEI8 accumulates in specific organelles. Analysis of multiple root cells was consistent with WEI8 being localized in the cytoplasm of the cell, and showed no evidence of organelle-specific accumulation (Fig. 6). This is consistent with work from Sandberg et al. (2004) which showed conversion of tryptophan to IAA in crude cytoplasmic fraction isolated from protoplasts in barley.

Expression of WEI8 and WEI8-like (WEL2) proteins

Since similar protein sequence is one indicator of proposed similar function, we may expect redundant gene family members to have a similar pattern of expression. The expression profile of *WEL1*, *WEL2*, and *WEL3* (no expression data was available for *WEL4*)

were compared with that of *WEI8* using AtGenExpress (Schmid et al., 2005). It was found that *WEL2* is expressed at similar levels as *WEI8* in different tissues and developmental stages (Fig. 8) (Table 3). *WEI8* and *WEL2* appear to be coexpressed in the root, apical meristem and floral organs. However, *WEL3* expression profiles did not overlap substantially with those of *WEI8* or *WEL2*. *WEL1* expression remained at a consistent level throughout the various tissues and stages and was considerably less than that of *WEI8*, *WEL2*, or *WEL3*. From this analysis we hypothesize that *WEL2* and *WEI8* may be the most functionally related members of this gene family.

WEI8 and WEL2 are functionally redundant and are required for a normal ethylene response and auxin homeostasis in *Arabidopsis*

If WEL2 is a functional homolog of WEI8, then we would expect mutants in *WEL2* to show ethylene insensitivity. In order to determine if the identified sequence homologs were involved in maintaining a proper response to ethylene in plants, Salk T-DNA lines available in *WEL2* were assayed for ethylene insensitivity. Although consistent ethylene insensitivity was not observed in any of the examined lines, two *wel2* alleles *Salk_021258* (*wel2-1*) and *Salk_137800* (*wel2-2*) did show some root-specific ethylene defects (Fig. 9). These were used for double mutant studies and to further characterize the role of these proteins in ethylene response.

Double mutants were generated and assayed for ethylene insensitivity to further evaluate the role of the *WEI8* gene family in ethylene response. After three days of growth under dark conditions *wei8 wel2* seedlings showed increased ethylene insensitivity compared to *wei8* single mutants when grown in the presence of in 10uM ACC (Fig. 10). Furthermore,

homozygous *wei8 wel2* seedlings had an open hook phenotype as well as agravitropic roots which can be caused by auxin-related defects.

No auxin defects, such as loss of apical dominance or morphological floral defects were seen in the adult single mutants, however a multitude of auxin-related alterations are present in the double mutant plants. Double mutants showed reduced apical dominance, increased branching, and reduced fertility while single mutants displayed wild-type morphology (Fig. 11). *wei8-1 wel2-1* double mutant lines showed extreme loss of apical dominance, an auxin defect. In addition, other tissues were also affected by the *wei8 wel2* mutations. Flower development appeared to arrest in early stages, never forming mature organs. For this reason, homozygous *wei8-1 wel2-1* plants did not yield any seed. *wei8-1 wel2-2* double mutant flowers had missing valves as well as enlarged stigma and curved pistils (Fig. 11). The apical dominance defect of homozygous *wei8-1 wel2-2* was also less pronounced than in the *wei8-1 wel2-1* double mutants. The adult phenotype differences between *wei8-1 wel2-1* and *wei8-1 wel2-2* double mutants were likely the result of a partial loss of function in the *wel2-2* mutant. This is in agreement with the fact that the *wel2-2* insertion is in an intron and could be spliced out. Lines which were homozygous for *wei8* and heterozygous for *wel2* have adult phenotypes which were indistinguishable from wild-type, however these mutant plants displayed a range of ethylene insensitivity in the root from weak to very strong, similar to that of the double mutant. Double mutants generated with the *wei8-2* allele and each of the *wel2* alleles have similar seedling, flower, and adult phenotypes compared to the corresponding double mutants generated with *wei8-1* (Fig. 11) further confirming that the phenotypes described above are indeed caused by the mutations in the *WEI8* and *WEL2* genes.

***wei8 wel2* double mutant shows altered gravity response**

The loss of apical dominance, as well as the presence of floral defects, are often seen in auxin mutants (Lincoln et al., 1990; Yamamoto and Yamamoto, 1998). Another hallmark of auxin mutants is their altered response to gravitational stimuli, since auxin is essential to maintaining proper gravity response. To determine if WEI8 is involved in gravity response, a standard 90º vertical plate rotation assay was performed (Rashotte et al., 2000). Using Col-0 as a control we grew *wei8-1* and a segregating double mutant population of *wei8-1/wei8-1 WEL2-1/wel2-1* on vertical plates for five days in the dark. The plates were then rotated 90º and the seedlings were allowed to grow for an additional two days. The mean gravitational response of *wei8* was compared to Col-0 using a Student's t-test and found to not be significant (P=0.2306). However, profound gravitropism defects were seen in the segregating *wei8-1*/*wei8-1 WEL2-1*/*wel2-1* population (Fig. 12). Lines within this population in some cases grew directly against the gravity vector or showed a spiral growth pattern which appeared to be random in nature. These results suggest that WEI8 and WEL2 are required for proper gravitational response.

***WEI8* and *WEL2* function is required downstream of tryptophan in the auxin biosynthetic pathway**

The single mutants, *wei8-1* and *wei8-2*, are insensitive to ethylene, however since their insensitivity increases substantially in combination with *wel2-1*, a segregating population of *wei8-1*/*wei8-1 WEL2-1*/*wel2-1* was used to evaluate the ability of tryptophan or IAA to complement this ethylene response defect. A concentration of 10mM tryptophan,

which is sufficient to complement the ethylene insensitivity of tryptophan biosynthetic mutants *wei2* and *wei7* (Stepanova et al., 2005), and 10nM IAA, which complements the ethylene insensitivity of the auxin efflux carrier, *eir1* (Rahman et al., 2001), were used. Ethylene insensitivity of the *WEL2* segregating population revealed a bimodal distribution where approximately one quarter of the seedlings showed increased insensitivity compared to the rest of the population (3.5mm compared to 1.75mm) (Fig. 13). In the presence of 10uM ACC and 10nM IAA ethylene sensitivity was restored and the population seedlings had a mean length of 0.41 ($\sigma = 0.13$) which is comparable to the root length of Col-0 (mean = 0.39, $\sigma = 0.09$ under the same experimental conditions). No sensitivity was restored upon application of exogenous tryptophan. These findings further support the functional redundancy between WEI8 and WEL2 and their role in the auxin biosynthetic pathway. These results together with the predicted molecular nature of WEI8 and WEL2 support the hypothesis that these proteins code for the aminotransferase responsible for the conversion of tryptophan to IPA.

wei8 wel2 double mutants have reduced *DR5-GUS* expression

As shown previously, *wei8 wel2* double mutants display many phenotypes in seedling and adult stages which are associated with reduced auxin levels or responses and are similar to known auxin defect mutants such as *axr1* and *aux1* (Lincoln et al., 1990; Yamamoto and Yamamoto, 1998). Data presented above also indicate that these genes are likely to participate in the control of auxin biosynthesis. To gain further evidence for the role of these genes in the regulation of auxin biosynthesis, the expression of the auxin reporter *DR5-GUS* was examined. The *DR5* element is a synthetic promoter composed of seven repeats of an

11bp sequence which contains the auxin-responsive element, TGTCTC. The synthetic DR5 promoter is fused to the GUS gene. In the presence of IAA, DR5 drives the transcription of the *GUS* reporter gene. This construct can be used to estimate relative levels of local IAA *in planta* and has been found to be highly correlated to IAA levels in the root (Casimiro et al., 2001). Using the *DR5-GUS* construct, we were able to visually analyze the level of IAA response in Col-0, *wei8-1*, and double mutants *wei8-1 wel2-1* and *wei8-1 wel2-2*. Our hypothesis was that if WEI8 and WEL2 function in the auxin biosynthesis pathway, levels of *DR5-GUS* expression would be reduced in the double mutants. To test this, *DR5-GUS*/*wei8*/*wel2* lines were generated such that *DR5-GUS* and *wei8* were homozygous, and *wel2* was heterozygous. Multiple independent F3 lines with seedling phenotypes known to be consistent with lines homozygous for *wei8* and *wel2* (no apical hook with a long, agravitropic root) showed an obvious reduction in the level of *DR5-GUS* expression when visually compared with *wei8* and Col-0 (Fig. 14). This suggests reduced levels of auxin in the double mutant and, together with the complementation and functional data, provides further support for the role of WEI8 and WEL2 in IAA biosynthesis.

IAA quantification

The most direct way to determine whether or not WEI8 and WEL2 are involved in auxin biosynthesis, is to measure the IAA levels in the *wei8 wel2* double mutant. To evaluate the effect of *wei8* on IAA levels *in planta*, an experiment was designed to directly measure IAA using HPLC-MS (collaboration with Dr. J. Normanly, University of Massachusetts, Amherst) in wild-type and *wei8-1 wel2-1* double mutants. Homozygous *wei8-1 wel2-1* etiolated seedlings can be identified among the progeny of a *wei8/wei8 wel2/WEL2* plants,

even in the absence of ethylene, by their abnormal apical hook and long, agravitropic root (Fig. 10). Using these phenotypic characteristics we were able to select true double mutants with approximately 90% certainty. To test the efficacy of this selection method, 63 selected seedlings were verified at the adult stage where the double mutants have obvious loss of apical dominance phenotypes and altered flower morphologies, additionally homozygosity was confirmed at the genetic level by genotyping for each allele.

IAA can be measured in many ways. One technique is by determining the level of newly synthesized IAA after exposing selected seedlings to labeled IAA biosynthesis precursors, $^{2}H_{5}$-tryptophan and ^{15}N-anthranilate. However, instead of measuring IAA generated by the plant, the level of total IAA can be determined in the double mutant vs. wild-type seedlings. Although the experimental design to measure IAA was worked out for this collaborative effort, and multiple attempts to quantify *in planta* were made, no conclusive IAA measurement data has been generated to evaluate if the homozygous *wei8-1 wel2-1* double mutant lines have reduced levels of IAA. However, based upon complementation analysis of the mutant, the identification of gravitational defects and extreme auxin-like defects in the adult double mutant lines, as well as molecular evidence of reduced *DR5-GUS* expression in mutant lines, a role for WEI8 and WEL2 in IAA biosynthesis is suggested. The most likely role for WEI8 within this pathway would be the catalysis of the conversion of tryptophan to IPA since the enzyme predicted at this step is an aminotransferase, and WEI8 and WEL2 have been annotated as alliinase-related aminotransferases.

Mutant protein structure and motifs

Similarity of protein structure often translates to similarity of function. So we decided to investigate similarity of WEI8 sequence and motifs to those of known aminotransferases in the *A. thaliana* genome. *WEI8* is homologous to alliinase-related CS-lyases or aminotransferases based on nucleic acid sequence. This, in combination with the identification of an aminotransferase step converting tryptophan to IPA in auxin biosynthesis, justifies comparing WEI8 sequence with protein sequence from characterized aminotransferases. At the protein level, WEI8 contains an N-terminal epidermal growth factor (EGF) domain, a C-terminal alliinase domain, and a large aminotransferase domain that overlaps with the alliinase domain (Fig. 15). Since WEI8 is homologous to alliinase-related proteins containing multiple domains, we analyzed in greater depth the degree of similarity of the domains with the WEI8 protein sequence using clustalW. A known alliinase protein (GI|27573774) which has been characterized using crystallographic methods and three characterized aromatic aminotransferases, ABERRANT GROWTH AND DEATH2 (AGD2) (GI|3068977), an aspartate aminotransferase (GI|531555), and a tyrosine aminotransferase (GI|15076853), were used in this sequence analysis. Based on multiple sequence alignment of the amino acid sequence within the predicted aminotransferase domain, WEI8 shares a greater level of sequence homology with alliinase compared to the known aminotransferases used in this study (Fig. 15). Additionally, the highly conserved SPNNP motif was present in only one out of the three characterized aminotransferases. Comparison of global sequence identity between WEI8 and alliinase using the BLOSUM62 scoring matrix was 28.72%, whereas the WEI8 to aminotransferase similarity was markedly less (ranging from 9.83 to 11.76%. WEI8 shared 13.82% identical sequence with SUR1

(Table 4). These findings indicate that based upon identical matching of sequence WEI8 is more related to SUR1 than to the aminotransferases. However, analysis of residue similarity indicated that WEI8 is slightly more similar to the aromatic aspartate/tyrosine aminotransferase (25.57%) than WEI8 is to SUR1 (25.00%). The phylogenetic analysis reveals relationships which support the similarity and identity scores calculated from the BLOSUM62 matrix. WEI8 and the WEL homologs more tightly cluster with alliinase (GI|27573774) than with the known aminotransferases or SUR1 (Fig. 16). SUR1 and the aromatic aspartate/tyrosine aminotransferase group together in one clade and their relationship with WEI8 appears to be equidistant. Based upon our belief that WEI8 acts as an aminotransferase (since SUR1 catalyzes a step which has been shown to not require any redundant proteins, and the conversion of tryptophan to IPA is the only predicted aminotransferase-driven reaction in the IAA biosynthesis pathway) we hypothesized WEI8 to function as an aminotransferase. Therefore the fact that it does not tightly cluster with this group was unexpected. However, sequence similarity is not always a reliable predictor of enzyme function. An example of this fact is SUR1, which was initially annotated as an aminotransferase but was later instead found to possess CS-lyase activity (Jones et al., 2003). For this reason a functional assay is essential to more clearly characterize this protein.

WEI8 has aminotransferase activity

Alliinases are a subclass of CS-lyase, and are responsible for cleaving C-S bonds in allin in its conversion to allicine (Nock and Mazelis, 1986). CS-lyase and aminotransferase activity have been difficult to discern from one another when sequence was used as the only criterion. For this reason we used a functional in vitro assay to determine whether or not

WEI8 had aminotransferase activity. There is evidence for both CS-lyase and aminotransferase function at two different conversion steps in the auxin biosynthesis pathway. CS-lyase activity has been reported in the glucosinolate biosynthesis pathway. *SUPERROOT1* (*SUR1*) encodes this CS-lyase and was found to be non-redundant (Mikkelsen et al., 2004). However, at a different location in the auxin biosynthesis pathway, aminotransferase activity is responsible for the conversion of tryptophan to indole-3-pyruvic acid (IPA).

Despite the relaxed clustering seen in figure 16, the experimental examination of the possibility of WEI8 acting as an aminotransferase seemed appropriate. To test this hypothesis, an assay described by Baca et al. (1994) and Pedraza et al. (2004), was performed on protein extracts isolated from *E. coli* expressing *WEI8* to determine presence of aminotransferase activity using tryptophan as a substrate.

E. coli DH5α strain transformed with WEI8 overexpression vectors were grown and total protein was extracted and analyzed using polyacrylamide gel electrophoresis (PAGE). Denaturing gels were stained with coomassie blue to visualize WEI8 in the absence of secondary structure. Constructs, *pHB1::MYC9* and *pHB1::GST* (controls) and *pHB1::MYC9-WEI8 and pHB1::GST-WEI8*, were first studied in *E. coli* strain DH5α. Coomassie blue staining and showed that WEI8 protein was produced in *E. coli* that was transformed with MYC9-WEI8 and GST-WEI8 (Fig. 17A). Additionally, WEI8 was able to use tryptophan as a substrate in an *in vitro* aminotransferase activity assay (Fig. 17B). However, the size of the WEI8 protein was only 40kDa which is smaller than the predicted mass of 44.801 kDa and suggests a proteolytic cleavage reaction may have taken place. For

this reason, a less proteolytic strain of *E. coli* was transformed with newly designed constructs.

The *E. coli* strain, BL21 (pLys) is known to have reduced levels of proteolysis. Using this strain we attempted to examine WEI8 activity following induction of the *T7* promoter using IPTG. BL21(pLys) competent cells were transformed with three additional constructs, *pT7::GST-EAD* (as a *GST* control), *pT7::WEI8*, *pT7::GST-WEI8*, and *pT7::WEI8-GST*. WEI8 was seen in the *pHB1::MYC9-WEI8* transformed cultures regardless of presence of IPTG. This result is as expected since IPTG does not induce transcription of genes under the control of the *pHB1* promoter (Fig. 17C). When a T7 IPTG inducible promoter was used we saw WEI8 expression in only the *pT7::GST-WEI8* culture. Although aminotransferase activity was seen in both of the MYC9-WEI8, as well as in the GST-WEI8 protein isolations only the *pT7::GST-WEI8* seems to produce the expected complete fusion protein (Fig. 17D).

One of the advantages of using a GST-tagged construct is the potential for protein purification. Purification of GST-WEI8 was performed using a glass bead-mediated isolation technique. Again, coomassie blue-stained gels were used to visualize presence of denatured protein (Fig. 17E) and aminotransferase activity was performed on native protein (Fig. 17F). GST-WEI8 protein was successfully purified and revealed activity.

Expression of *pT7::WEI8-GST* did not result in observable purified protein on a denaturing gel, nor aminotransferase activity when separated on native gel, suggesting fusion of GST in the C-terminus may have strong effects on the stability or function of WEI8 expressed in *E. coli*. Overall, the presence of *E. coli*-expressed WEI8 showing aminotransferase activity provides further support that WEI8 can use Trp as a substrate in an

aminotransferase reaction, hence may be involved in auxin biosynthesis catalyzing the conversion of tryptophan to IPA.

WEI8 overexpressors are 5-MT tolerant

To further examine the role of WEI8 *in planta* we examined the effects of 5-methyltryptophan (5-MT) on plants which are expressing high levels of WEI8. Theoretically, resistance to the toxic Trp analog, 5-MT can be used as a diagnostic tool for the involvement of a gene in a Trp-dependent auxin biosynthesis pathway. For example, the overexpression of a gene can increase the metabolism of TRP conversion to IAA. In this case, 5-MT is incorporated at a higher rate into IAA instead of protein, thus reducing 5-MT toxicity in the cell. Reduced sensitivity of lines to 5-MT has been used in previous studies to analyze overexpression mutants in the auxin biosynthetic pathway (Zhao et al., 2001; Zhao, et al., 2002).

To determine if WEI8 acts in the auxin biosynthesis pathway, we analyzed multiple families of two independently transformed *wei8* lines overexpressing *GFP-WEI8* for tolerance to 5-MT. These families were selected based on their complementation phenotype (restoration of normal ethylene response) in previous experiments. Seedlings which are ACC insensitive and do not express GFP in the root, were found to not be tolerant of the toxic 5-MT and these seedlings had very little root growth after eight days of growth in the light in the presence of the tryptophan analog. However, ACC sensitive, GFP-WEI8 expressing lines had long roots when grown on 50uM 5-MT, hence were highly tolerant to this toxic analog (Fig. 18). Col-0, and lines overexpressing CYP79B2 and YUCCA were used as controls. 5-MT treated Col-0 seedlings were generally stunted showing no significant root growth.

However, tolerant control seedlings, such as those overexpressing the auxin biosynthesis proteins CYP79B2 and YUCCA, have hypocotyls and cotyledons which are similar to Col-0 grown in the absence of 5-MT, and their roots are long and branched. Our findings of reduced toxic effects of 5-MT in WEI8 overexpression lines are consistent with the proposed role of WEI8 in the IAA biosynthesis pathway.

DISCUSSION

There is growing evidence for the interaction between hormones and in recent years several genes involved in ethylene-auxin crosstalk have been identified (Alonso et al, 2003b; Swarup et al., 2002; Larsen and Cancel, 2003; Lehman et al., 1996). WEI2 and WEI7 are just two examples of proteins involved in crosstalk in Arabidopsis. These proteins are auxin biosynthesis-related proteins that are necessary to produce a complete ethylene response in the Arabidopsis seedling. WEI8 is also involved in ethylene-auxin crosstalk. Here we describe the cloning and functional characterization of *WEI8* through experiments with *wei8* and its nearest homolog, *wel2*. Root-specific ethylene insensitivity of the *wei8* single and *wei8 wel2* double mutants is not complemented by tryptophan, however the sensitivity can be complemented by exogenously applied IAA. This complementation indicates that these proteins may act in the IAA biosynthesis pathway. BLASTp analysis indicates WEI8 is related to an alliinase-related aminotransferase or CS-lyase, however functional experiments for aminotransferase activity show that WEI8 can use tryptophan in an aminotransferase reaction. Additionally, reduced toxicity to 5-MT, a toxic tryptophan analog, is in line with the tolerant response seen in lines overexpressing known auxin biosynthesis genes. These results taken together suggest that WEI8 is responsible for performing an aminotransferase

function in the IAA biosynthesis pathway whereby tryptophan is converted to IPA. Furthermore, IAA is required for wild-type ethylene response in Arabidopsis.

Cloning and characterization of *WEI8*

The mutant *wei8-1* was originally identified by A. Stepanova from a population of activation tagged mutagenized Col-0 lines, and *wei8-2* by Larissa Benevente from a pool of EMS mutagenized Col-0 lines. Each mutant was selected for root-specific ethylene insensitivity after three days of growth in the dark. Complementation of ethylene insensitivity in the single mutants showed that sensitivity was restored in the presence of IAA, but not in the presence of tryptophan suggesting WEI8 acts downstream step in the IAA biosynthesis pathway. These findings are similar to those of *wei2* and *wei7* in that all are root-specific ethylene insensitive mutants which appear to reside in IAA biosynthesis-related pathways.

Few enzymes involved in IAA biosynthesis are known, hence cloning and sequence analysis of *WEI8* was performed to gain insight into the potential function of this protein and hypothesize a more precise location for WEI8 in the IAA biosynthetic pathway. Map-based positional cloning and subsequent BLASTn analysis revealed *WEI8* to have homology to an alliinase-related gene that encodes a protein with putative functions of aminotransferase or CS-lyase activity. Unfortunately, researchers have struggled to differentiate aminotransferase vs CS-lyase functions. In fact, the IAA biosynthesis-related proteins SUR1 and CORI3 were initially annotated as aminotransferases, but were later instead found to possess CS-lyase activity (Mikkelsen et al., 2004; Jones et al., 2003). Within the IAA biosynthesis pathway, there are only two known steps that are catalyzed by an

aminotransferase or CS-lyase. Work by Mikkelsen et al., (2004) with *SUR1* determined that this gene was non-redundant and codes for a CS-lyase, suggesting that WEI8 may be involved in the conversion of tryptophan to indole-3-pyruvic acid.

Auxin biosynthesis mutants have been difficult to identify in part because there are three independent pathways which are capable of generating IAA from tryptophan, and also in part due to the possibility of the presence of homologous proteins which can catalyze a single conversion step. We used BLASTp analysis to determine if WEI8 homologs existed in the Arabidopsis genome. Four homologs with varying levels of similarity were identified (Fig. 7). The expression profiles of the homologs were investigated and it was determined that *WEL2* expression mimics the pattern and level of *WEI8* expression in roots, flowers, and apex tissue. However the function of WEI8 and WEL2 can be identified only by performing functional assays.

Auxin defects are seen in *wei8 wel2*

Complementation assays using tryptophan and IAA suggest *WEI8* functions in the auxin biosynthesis pathway. One would expect mutants of the auxin biosynthetic pathway to display a series of auxin deficient phenotypes. Surprisingly, this is not the case in *wei8* single mutants, or in mutants of the *WEI8* homolog *wel2,* which have no obvious auxin defects and show normal gravitropism and apical dominance. This may be due to the high level of redundancy in the auxin biosynthesis pathway. Redundancy is evident not only by the presence of multiple pathways by which IAA can be generated (Woodward and Bartel, 2005), but also by the presence of multiple genes in each one of the steps of these pathways. Auxin defects are, however, seen in the *wei8 wel2* double mutant. Seedlings grown on 10uM

ACC show root-specific ethylene insensitivity and reduced response to gravity. Adult *wei8-1 wel2-2* double mutants have a loss of apical dominance, furthermore this defect is more severe in *wei8-1 wel2-1* homozygous lines suggesting that *wel2-1* is a stronger allele than *wel2-2*. Loss of apical dominance phenotypes are similar to those seen in known auxin-related mutants such as *axr1* (Lincoln et al., 1990). In addition, each of the double mutants displays morphological floral defects and reduced fertility. This auxin defect has also been seen auxin mutant *pin1* (Galweiler et al., 1998). Moreover, the *wei8 wel2* double mutant mimics very closely the phenotypes of triple and quadruple yucca mutants (Cheng et al., 2006). These phenotypic observations strongly suggest that WEI8 and WEL2 are involved in auxin biosynthesis and knocking out the function of both genes simultaneously reduces IAA levels to such a degree that morphological defects can be easily seen.

Complementation phenotypes observed in double mutant populations grown in the presence of low auxin levels provide further evidence for placing these proteins in the IAA biosynthesis pathway. Analysis of the progeny of *wei8/wei8 WEL2/wel2* plants shows that even the most ethylene insensitive seedlings (approximately 25%, likely representing the *wei8/wei8 wel2/wel2 individuals*) become sensitive to this hormone in the presence of exogenous IAA. However, in the same subset of the population, ACC sensitivity is not restored in the presence of tryptophan. Similar results were seen in single mutant analysis. This is an unseen profile compared with other mutants identified using identical screening criteria (Stepanova et al., 2005). Restoration of ethylene sensitivity by application of exogenous IAA and lack of complementation by tryptophan are consistent with the proposed role of WEI8 in the IAA biosynthetic pathway.

WEI8 functions as an aminotransferase

BLASTp analysis reveals WEI8 is highly conserved throughout the plant kingdom (Expect value =8e-04) (Expect value = number of hits you can expect by chance), however no significant similarity is seen throughout the protist or animal kingdoms based upon BLASTp analysis (Expect value = 0.003). This high level of conservation is at times associated with enzymes which are important in maintaining functions crucial to the survival and propagation of the plant, such as root growth and fertility.

WEI8 putatively encodes an alliinase-related aminotransferase. However to truly understand the role of WEI8 in auxin biosynthesis, it is essential to identify whether WEI8 has CS-lyase or aminotransferase function. There is evidence for CS-lyase and aminotransferase function at two different conversion steps in the auxin biosynthesis pathway. CS-lyase activity has been reported in the glucosinolate biosynthesis pathway and is coded for by *SUPERROOT1* (*SUR1*) (Mikkelsen et al., 2004). Aminotransferase activity is predicted to convert tryptophan to indole-3-pyruvic acid, however prior studies have not identified which protein was catalyzing this reaction. Unfortunately, the CS-lyase and aminotransferase function have been difficult to differentiate from one another (Mikkelsen et al., 2004) as they have similar reaction characteristics. Both require the cofactor, pyridoxial-5-phosphate, and convert amino acids to alpha-keto acids. In the case of CS-lyases, the reaction occurs as free ammonia is donated and pyruvate is released. Alternatively, aminotransferases transfer the amino group to the alpha-keto acid (Mikkelsen et al., 2004).

WEI8 sequence similarity to SUR1 or aromatic aminotransferases may have provided a clue to WEI8 function. However, surprisingly, based upon BLASTp analysis of WEI8 compared with SUR1 and three known aminotransferases, WEI8 does not appear to be more

similar to either the CS-lyase or the aminotransferases, indicating that whole protein sequence analysis is not a good predictor of function in this case. Ultimately, functional assays supply the most reliable evidence of function. The CS-lyase function present in the glucosinolate biosynthesis pathway, an offshoot within the IAA biosynthesis pathway which uses indole-3-acetaldoxime (IAOx) as a substrate, was found to be performed by SUR1 (Mikkelsen et al., 2004). Additionally, due to the complete abolishment of glucosinolates in the *sur1* mutant (Mikkelsen et al., 2004), SUR1 is believed to be non-redundant, hence aminotransferase function for WEI8 was investigated.

Aminotransferase function can be determined in cell-free protein extract assays (Baca et al., 1994) and in gel-based protein assays. Pedraza et al., (2004) were able to successfully show aminotransferase activity from nitrogen-fixing bacteria cell-free crude protein extracts which were immobilized using PAGE. Similarly, in our study, protein extracts from *E. coli* containing a *WEI8* overexpression vector were able to show aminotransferase activity. More specifically, WEI8 can utilize tryptophan as a substrate, a requirement if WEI8 is responsible for the generation of the auxin precursor indole-3-pyruvic acid *in planta*.

Another experiment which is often used to evaluate the in vivo function of a tryptophan-requiring enzyme in IAA biosynthesis is resistance to the toxic tryptophan analog, 5-MT. Overexpression mutants of the IAA biosynthesis pathway show reduced toxicity to 5-MT (Zhao et al., 2001, Zhao et al., 2002). Toxicity results from the integration of 5-MT into proteins. This likely causes the protein structure to be altered, which renders the protein to be less functional than in its wild-type confirmation. Overexpressing IAA biosynthetic genes results in a pool of 5-MT being converted into IAA instead of into protein, hence 5-MT is not toxic to the overexpression mutants. In our experiment, we show that

exogenously applied 5-MT results in severely dwarfed growth in wild-type, Col-0, seedlings, however *WEI8* overexpressed in the Col-0 background results in reduced sensitivity to 5-MT similar to *YUCCA* and *CYP79B2* overexpression mutants. *yucca* is a gain of function mutant of the flavin monooxygenase-like enzyme catalyzing the conversion of tryptamine to N-hydroxyl tryptamine and has been previously shown to be less sensitive to the toxic tryptophan analog, 5-MT (Zhao et al., 2001). Similarly, overexpression of CYP79B2, an enzyme that converts tryptophan to indole-3-acetaldoxime (IAOx), also confers resistance to 5-MT (Zhao et al., 2002). Hence, our findings of increased resistance to 5-MT in WEI8 overexpression lines combined with evidence of defects in auxin response (gravitropism, apical dominance) in *wei8-1 wel2-1* double mutants, is in line with findings of other auxin biosynthetic mutant studies.

If WEI8 is located within the IAA biosynthetic pathway as we believe it to be, then decreased levels of IAA due to mutation would likely reduce levels of IAA and therefore may reduce transcription of auxin-related genes. The auxin reporter *DR5-GUS* is a useful tool to visualize the level of auxin *in planta* (Ulmasov et al., 1997). Double mutant *wei8 wel2* seedlings show reduced DR5-GUS expression in the root compared with either wild-type or *wei8* single mutant. This suggests that WEI8 and WEL2 are necessary for maintaining wild-type levels of auxin in the root. This combined with recent findings by Ljung et al. (2005), which state that the root is a source of *de novo* IAA synthesis, supports our hypothesis that WEI8 is involved in IAA biosynthesis. Hence WEI8-mediated IAA biosynthesis is likely reduced in *wei8 wel2* double mutants, as indicated by decreased *DR5-GUS* expression. The strong ethylene insensitivity of the *wei8 wel2* double mutant further implicates auxin biosynthesis in the ethylene response.

Gravitropism defects are seen in *wei8 wel2* double mutants

It has been well established that IAA is involved in gravitational responses. Auxin-related mutants often show a clear defect in gravitropism. Rashotte et al. (2000) showed, using the auxin transport-related *eir1* mutant, that polar transport of IAA is required for root gravitropism in Arabidopsis. Additionally, the auxin response mutant, *axr1*, AUXIN RESISTANT1 (AXR1) has a delayed response to gravity compared to wild-type (Lincoln et al., 1990). In the case of *wei8*, the single mutant did not show any obvious gravity defects, however *wei8 wel2* double mutants had reduced responses to gravity when compared to Col-0 or *wei8-1*. Gravitational response is a complex process involving first the perception of gravity, and second, hormone-mediated differential growth. It is logical that reducing IAA levels through knockout mutation in WEI8 or WEL2 would cause gravity defects since this biological mechanism is reliant on hormone biosynthesis, transport, perception and signaling in the organism. The surprising fact that no gravity defects were seen in *wei8* or *wel2* may be explained by the high level of redundancy in the IAA biosynthesis pathways. Hence, small effects on the IAA level in the single mutant may be masked. However, evidence of reduced response to gravity in the double mutant seedlings may be due to a greater reduction of IAA in the seedling and supports the likelihood of WEI8 being an auxin-related protein.

Localization of WEI8

Many tools exist which predict the cellular localization of proteins based upon presence of specific motifs within the amino acid sequence. The predicted localization of aminotransferases are in the cytoplasm as well as multiple organelles, such as plastids,

mitochondria, and the peroxisomes of the cell (Liu and Huang, 1977; Schultz and Coruzzi, 1995; Wadsworth, 1997). However, amino acid sequence analysis using PROTCOMP predicts that WEI8 likely resides in the cytoplasm. This is supported by analysis using *in planta* confocal imaging which reveals GFP-tagged WEI8 localization in the cytoplasm (clearly showing lack of presence in the vacuole of the cell). This differs from the localization of alliinase in onion (*Allium cepa*) in the vacuole (Lancaster and Collin, 1981). Little is known on the subcellular localization of IAA and components of IAA biosynthesis in the cell. Enzymes of the Trp biosynthetic pathway reside in the plastid (Radwanski and Last, 1995). Additionally, YUCCA, the flavin monooxygenase enzyme of the IAA biosynthesis pathway is localized to the cytoplasm. Other IAA biosynthetic enzymes, such as CYP79B2 and CYP79B3, are believed to be targeted to the chloroplast based up on peptide sequence (Hull et al., 2000).

In summary, the identification of *wei8*, an auxin biosynthesis loss of function mutant which results in root-specific ethylene insensitivity, draws a link between ethylene response and production of IAA. Further studies to understand the effects of reduced IAA levels on various plant processes will be more easily executed using the *wei8 wel2* double mutant. The identification of loss of function mutants in the IPA-mediated IAA biosynthesis pathway provides an opportunity to study the role of specific branches of IAA biosynthesis in different developmental processes, as well as the ability of specific cells to produce IAA. However, the mechanism by which IAA regulates ethylene response remains nebulous and a topic for future research.

IAA has been shown to be required for a proper ethylene response. WEI8, as a likely biosynthetic protein, is responsible in part for maintaining IAA levels in the root based upon

DR5-GUS expression. One possible mechanistic explanation is that ethylene may induce the expression *WEI8* in the root in a manner similar to *WEI2* and *WEI7* (Stepanova et al, 2005), thereby increasing the level of IAA to a critical point which is necessary for ethylene response. However, the ethylene-mediated induction of IAA may generate a specific pool of IAA which is utilized during ethylene response. This model could be tested in part by determining if *WEI8p-GUS* transcription is induced in the presence of ethylene.

Another model for IAA-mediated ethylene response is that since IAA must be maintained at a critical level for ethylene biosynthesis, the IAA level may be reduced below the required threshold by knocking down one of the IAA biosynthesis pathways, hence resulting in ethylene insensitivity. These are only two possible scenarios which would explain the insensitivity to ethylene seen in the *wei8* single mutant and *wei8 wel2* double mutants. These models do not exclude the possibility that other mechanisms, such as auxin transport or post-transcriptional regulation, may be crucial to regulate ethylene response.

MATERIALS AND METHODS

Arabidopsis strains and growth conditions

All lines are in the Col-0 background. *DR5-GUS* reporter line was generously provided by Dr. T. Guilfoyle. *cyp79b2* and *yucca* overexpression lines were donated by Dr. Y. Zhao. *sur1* and *sur2* were obtained from the ABRC. *wei8-1* and *wei8-2* were identified in forward genetic screens using the Detlef Weigel activation tagged collection (ABRC) and an ethylmethanesulfonate (EMS) mutagenesis population, respectively. BC2 lines were generated from crossing mutants to Col-0 prior to phenotypic analysis. Seeds were surface sterilized in 50% bleach solution containing 0.005% Triton X-100 for 10 minutes, followed

by three rinses in autoclaved dH2O. Sterile seeds were planted on agar medium pH 6.0 (1X Murashige and Skoog (MS) medium, 1% (w/v) sucrose, and 0.8% (w/v) agar) and placed at 4 degrees for 3-5 days. Plates were then place in the light for two hours to promote germination, followed by 72 hours of growth in the dark at 22ºC. Seedlings transplanted to soil [1:1 mixture of MetroMix-200 (Scotts) and Fafard Germinating Mix (Conrad Fafard)] were grown under constant light for 16 hour light cycles or constant light at 22-25ºC.

Identification and Sequence Analysis

wei8-1 was identified by screening T-DNA insertion lines on agar medium supplemented with 10uM ACC grown under dark conditions for three days. A map-based technique was use to clone *WEI8*. The mutant, *wei8-1*, was crossed to Ler. A total of 487 F2 ethylene-insensitive individuals were selected and retested in the F3 generation. DNA was isolated from the selected lines according to Doyle and Doyle (1987) and subsequently genotyped using genome-wide classical and novel simple sequence-length polymorphic (SSLP) markers (Bell and Ecker, 1994) which were polymorphic between Col-0 and Ler. Markers F20P5-1, F5A18-1 and F23N20-1 were designed and have the following characteristics:

F20P5-1F (26.45Mb)	5'-GTTTCATCACCTACCCATGA-3'
F20P5-1R	5'-GATAGCAAAGCTTCGAGTGA-3'
F5A18-1F (26.735Mb)	5'-TGACCAAACTATTTATTTACACAAT-3'
F5A18-1R	5'-ATGTGTTCCTTTGAGAGTGA-3'
F23N20-F (25.84 Mb)	5'-AGAAGACATGGGACAAATGA-3'
F23N20-R	5'-AACAATGTTCTTGCCTTCC-3'

Fragments were amplified using polymerase chain reaction (PCR) for 40 cycles, where one cycle consisted of 15 seconds at 94ºC, 40 seconds at 52ºC, and 1 minute at 72ºC.

The following 15 Salk T-DNA insertion lines from eight candidate genes were examined for ethylene-insensitivity (*Salk_132567* from *At1g70530*; *Salk_095343*, *Salk_060907*, and *Salk_055543* from *At1g70550*; *Salk_127890*, *Salk_048897*, and *Salk_022743* from *At1g70560*; *Salk_042954*, and *Salk_011617* from *At1g70580*; *Salk_064783*, and *Salk_149453* from *At1g70590*; *Salk_024449* from *At1g70600*; *Salk_131260* and *Salk_059646* from *At1g70610*; and *Salk_108105* from *At1g70620*).

wei8 sequence analysis was conducted using the following gene-specific primers from *At1g70560* (Table 5):

F1 (5'-TGAAACTGGAGAACTCGAGGA-3')

F7 (5'-GCAATTATTGTATTTTCAC-3')

R1 (5'-GCTTTTAATGAGCTTCATGTTGG-3')

R3 (5'-CTTCATCATCTCACGACCAT-3')

R7 (5'-TTCAGAAACAACGCCCACTG-3')

R8 (5'-CATCAGAGAGACGGTGGTGAAC-3')

F9 (5'-CATGTGCAAAGACATCAGTAACA-3')

R5 (5'-ATAGATCTGCACTTGTCTAGC-3')

Fpromoter (5'-TTATGTGGTAATTCATTACCATC-3')

R-utr (5'-CTAAGAACTAGAACAGTTTCAC-3')

wei8 mutant alleles were sequenced using BigDye Terminator v3.1 kit (ABI Prism; PE-Applied Biosystems, Sunnyvale, CA).

Phylogenetic Analysis

Amino acid sequences for WEI8, WEL1, WEL2, WEL3, WEL4, SUR1, a known alliinase protein (GI|27573774) and three characterized aromatic aminotransferases, ABERRANT GROWTH AND DEATH2 (AGD2) (GI|3068977), an aspartate aminotransferase (GI|531555), and a tyrosine aminotransferase (GI|15076853), were analyzed using clustalW to determine sequence similarity. Phylogenetic trees were generated using TreeView (Page et al., 2001).

Expression Profile

Expression levels of *WEI8*, *WEL1*, *WEL2*, and *WEL3* were queried from AtGenExpress (Schmid et al., 2005) from the dataset "AtGenExpress: Expression Atlas of Arabidopsis Development" which included the developmental stages from Germination to senescence. *WEL4* was not present in the expression database, therefore was excluded. Expression was shown as mean expression across 237 microarray slides and was queried at http://jsp.weigelworld.org/expviz/expviz.jsp.

Gravitropism Analysis

Col-0, *wei8-1* and seeds from a segregating population of *wei8-/wei8-1 WEL2-1/wel2-1* were plated approximately one inch apart on square plates following surface sterilization as described previously. Following vernalization at 4ºC for 72 hours, the plates were placed vertically in a dark 22ºC incubator for five days, rotated 90 degrees, and then grown for two additional days. The plates were scanned on a standard flatbed scanner and

gravitropism response angles were measured using UTHSCSA Image Tool (Wilcox et. al., 2002). There were 37, 47, and 52 gravitational observations for Col-0, *wei8-1*, and *wei8-1 wel2-1*, respectively. Gravitational responses were graphed in a manually generated wheel diagram at 15 degree increments as frequency percentages.

Determining complementation by TRP and IAA

A complementation assay was performed using 10uM tryptophan and 5nM, 7.5nM or 10nM IAA. Seeds were sterilized and plated according to the protocol stated previously. Hypocotyl and root were prepared for length measurement by placing seedlings on a horizontal agar surface without selection, digitally capturing the seedlings, and with a digital pen trace the hypocotyl and root length of each seedling in Adobe Photoshop. Measurements were digitally obtained using Image Processing Tools (Reinder Graphics. www.reindeergraphics.com).

Double mutant generation and verification

wei8-1 was crossed to *wel2-1* and *wel2-2*. F1 plants were selfed and F2 etiolated seedlings were evaluated for ethylene insensitivitiy on 10uM ACC. Ethylene insensitive lines were transferred to soil and genotyped to verify presence of mutant *wei8-1* and *wel2* alleles. *WEI8* wild type genomic DNA sequence was detected by PCR using *WEI*8-F (5'-CATCAGAGAGACGGTGGTGAAC-3') and *WEI8*-R (5'-CTTCATCATCTCACGACCAT-3'). Presence of T-DNA insert in the *WEI8* gene sequence of the lines was assayed using *WEI8*-R and LB1 (5'-CATACTCATTGCTGATCCATGTAGATTTCC-3') (Weigel et al., 2000). Wild type genomic *WEL2* was determined using primers *WEL2*-F (5'-

GCACGCAAGTGAAGCTCCAAGC-3') and *WEL2*-R (5'-ATACTGTGGCCAATAGTAAGCC-3'). Presence of a *WEL2* located T-DNA insert in the lines was assayed using *WEL2*-F and LB1 (5'-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3').

Plant Transformation

Overexpression constructs (35S::*MYC9-WEI8*, 35S::*MYC9-WEL2*, 35S::*WEI8*, 35S::*GFP-WEI8*, and 35S::*WEI8-GFP*) were generated using the Gateway and Cre-Lox recombination systems (Kuhn, R., and Torres, R.M., 2002; Karimi et al., 2002). *Agrobacterium tumefaciens* was transformed with the vectors and Col-0 and *wei8-1* plants were transformed using the flower-dipping technique (Clough and Bent, 1998). T1 plants were screened on agar plates containing 100ug/ul kanamycin to identify transformants.

Confocal Imaging

Subcelluar localization of GFP in seedlings transformed with *35S::GFP-WEI8* was investigated using confocal microscopy. Multiple *35S::GFP-WEI8* seedlings with restored ethylene sensitivity in the root were take to The Cellular and Molecular Imaging Facility at North Carolina State University. Using a Leica MZ FLIII fluorescence stereo microscope, Dr. Eva Johannes performed image analysis on cell files from multiple seedlings.

Functional characterization – AAT assay

To determine if WEI8 functions as an aromatic amino acid transferase, WEI8 protein was extracted from a *E. coli* (strain DH5α) *T7-WEI8* transformed following induction with

IPTG (10uM final concentration). The cultures were grown for approximately four hours and pelleted at 14000 rpm. The pellets were resuspended in a Laemmli loading buffer as described in Pedraza et al. (2004). Each sample was sonicated five times, debris was pelleted, and the protein remaining in the supernatant was analyzed using PAGE. Protein was separated on polyacrylamide using 5% stacking gel with 12% separating gel. Activity was identified according to Pedraza et al., (2004). An aminotransferase activity stain was prepared in Trp-present and Trp-absent batches. The gels were stained overnight at 37ºC and images were digitally captured on a white light box. Coomassie blue staining was performed to determine the approximate size of the denatured WEI8 compared to known standards.

Toxicity study of 5-MT with overexpression lines and double mutants

Seeds from Col-0, CYP79B2 and YUCCA overexpression line, and *wei8 wel2* double mutant populations were surfaced sterilized and planted on plates containing 50uM 5-Methyltryptophan (5-MT). The lines were vernalized for 72 hours at 4ºC and grown under constant light for a total of eight days. Seedlings were analyzed for resistance to toxicity.

IAA Quantification

Segregating *wei8-1/wel2-1* F2 populations were grown on media in the absence of ethylene and double mutants (lacking a normal hook and gravitropism) were selected. Approximately 200 seedlings were bulked per timepoint, placed in liquid MS media containing ^{15}N-IAA and $^{2}H_5$-TRP, and harvested at 0, 2, 8, and 24 hours. IAA extraction was conducted according to an unpublished protocol from Jennifer Normanly (personal communication with potential proprietary value).

GUS Staining Assay

A cross of *wei8-1 DR5-GUS* to *wei8-1/wei8-1 WEL2-1/wel2-1* allowed us to generate homozygous *wei8-1* F2 lines all of which were segregating for *DR5-GUS* and *wel2-1*. Half of the lines were segregating at the *WEL2-1* locus. F2 plants were propagated and harvested. F2 lines which were homozygous *DR5-GUS* were identified using GUS expression as a marker. Lines which were homozygous for *DR5-GUS* were screened on ACC to identify those segregating for *wel2-1*. Segregating seedlings were selected for the ethylene insensitive root and abnormal hook (equivalent to *wei8-1 wel2-1* double mutant), ethylene insensitive root (equivalent to *wei8-1 wel2-1* heterozygotes), and ethylene insensitive root (equivalent to *wei8-1*).

GUS staining on seedlings was performed in the following manner. Seedlings were fixed in ice-cold 90% acetone, followed by two washes in wash buffer [50 mM $NaPO_4$ pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$]. Vacuum was applied twice at 25psi for one minute in staining buffer [50 mM $NaPO_4$ pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 1mg/ml cyclohexylammonium salt (X-gluc)]. The tissue was placed in a 15% EtOH solution to stop the staining process and photographs were taken of representative seedlings.

Table 1. Markers used for fine mapping of *WEI8*

Marker	Chromosome	Position (Mb)	Primer sequence	
			Forward	Reverse
F5A18-1	1	26.70	TGACCAAACTATTTATTTACACAAT	ATGTGTTCCTTTGAGAGTGA
F23N20	1	26.84	AGAAGACATGGGACAAATGA	AACAATGTTCTTGCCTTCC
F20P5-1	1	26.45	GTTTCATCACCTACCCATGA	GATAGCAAAGCTTCGAGTGA

Table 2. Salk T-DNA lines in the vicinity of *WEI8* which were screened for insensitivity on 10uM ACC

Gene	Position (Mb)	T-DNA Line	Ethylene Response
At1g70530	26.5939	Salk_132567	sensitive
At1g70550	26.6025	Salk_055543	sensitive
At1g70550	26.6027	Salk_095343	sensitive
At1g70550	26.6029	Salk_060907	sensitive
At1g70560	26.6091	Salk_127890	moderate insensitivity
At1g70560	26.6095	Salk_048897	moderate insensitivity
At1g70560	26.6100	Salk_022743	moderate insensitivity
At1g70580	26.6170	Salk_042954	sensitive
At1g70580	26.6195	Salk_011617	sensitive
At1g70590	26.6236	Salk_064783	sensitive
At1g70590	26.6237	Salk_149453	sensitive
At1g70600	26.6251	Salk_024449	sensitive
At1g70610	26.6291	Salk_131260	sensitive
At1g70620	26.6302	Salk_108105	sensitive
At1g70620	26.6329	Salk_059646	sensitive

Table 3. Expression levels of *WEI8*, *WEL1*, *WEL2*, and *WEL3* in multiple tissues at multiple stages in *A. thaliana*.

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEI8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
Root	Roots	Wt	7 days	ATGE_3	56.7571	3.9664	125.6195	176.4719
Root	Roots	Wt	17 days	ATGE_9	66.8143	3.8986	80.4333	174.3962
Root	root	Wt	15 days	ATGE_93	48.3804	4.3464	83.8117	534.6189
Root	root	Wt	8 days	ATGE_94	35.0788	3.9628	38.0844	284.5085
Root	root	Wt	8 days	ATGE_95	36.8004	4.1151	34.4147	528.4182
Root	root	Wt	21 days	ATGE_98	18.2911	4.0219	52.5136	333.2681
Root	root	Wt	21 days	ATGE_99	25.1760	4.0074	37.0498	562.6486
Stem	hypocotyl	Wt	7 days	ATGE_2	9.5368	4.0474	8.3280	62.0521
Stem	1st node	Wt	21+ days	ATGE_28	9.2442	3.9637	45.2238	211.8975
Stem	stem, 2nd internode	Wt	21+ days	ATGE_27	7.5500	4.2056	10.5713	1309.3902

Table 3. (continued)

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEI8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
Leaf	cotyledons	Wt	7 days	ATGE_1	46.7891	4.4431	53.0959	15.7017
Leaf	leaves 1 + 2	Wt	7 days	ATGE_5	30.0337	4.5406	125.3705	7.8117
Leaf	rosette leaf #4, 1 cm long	Wt	10 days	ATGE_10	31.1868	4.3574	132.4258	8.5781
Leaf	rosette leaf #4, 1 cm long	gl1-T	10 days	ATGE_11	25.8481	4.3605	51.5494	7.8348
Leaf	rosette leaf # 2	Wt	17 days	ATGE_12	12.7468	4.2703	37.4233	301.0393
Leaf	rosette leaf # 4	Wt	17 days	ATGE_13	11.5456	4.2608	22.0394	47.6985
Leaf	rosette leaf # 6	Wt	17 days	ATGE_14	6.7962	4.3903	24.4558	12.1913
Leaf	rosette leaf # 8	Wt	17 days	ATGE_15	7.6583	4.3199	29.0469	8.2385
Leaf	rosette leaf # 10	Wt	17 days	ATGE_16	14.2344	4.4107	29.9122	7.8001
Leaf	rosette leaf # 12	Wt	17 days	ATGE_17	24.2514	4.4895	73.0874	8.7390
Leaf	rosette leaf # 12	gl1-T	17 days	ATGE_18	20.8341	4.3024	83.1421	7.5674
Leaf	leaf 7, petiole	Wt	17 days	ATGE_19	9.3105	4.2753	14.8354	15.1791
Leaf	leaf 7, proximal half	Wt	17 days	ATGE_20	11.1137	4.4985	28.4014	9.9252
Leaf	leaf 7, distal half	Wt	17 days	ATGE_21	9.5622	4.6785	18.8241	7.8772
Leaf	leaf	Wt	15 days	ATGE_91	78.4744	4.4051	102.8816	15.2681
Leaf	senescing leaves	Wt	35 days	ATGE_25	19.9046	4.3316	5.9278	3539.6734
Leaf	cauline leaves	Wt	21+ days	ATGE_26	13.0515	4.2370	23.3080	1306.0639

Table 3. (continued)

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEI8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
whole plant	seedling, green parts	Wt	7 days	ATGE_7	78.5303	4.2684	153.5600	8.6827
whole plant	seedling, green parts	Wt	8 days	ATGE_96	64.4464	4.1580	38.5894	6.3110
whole plant	seedling, green parts	Wt	8 days	ATGE_97	76.6494	4.1537	43.0506	27.7040
whole plant	seedling, green parts	Wt	21 days	ATGE_100	15.7736	4.1548	29.0146	70.6521
whole plant	seedling, green parts	Wt	21 days	ATGE_101	16.6354	4.0416	34.4334	158.8953
whole plant	developmental drift, entire rosette after transition to flowering, but before bolting	Wt	21 days	ATGE_22	20.4098	4.3415	42.6271	14.3289
whole plant	as above	Wt	22 days	ATGE_23	23.2049	4.2954	56.1410	20.2329
whole plant	as above	Wt	23 days	ATGE_24	25.1055	4.3156	49.1489	34.3672
whole plant	vegetative rosette	Wt	7 days	ATGE_87	125.9806	4.3513	135.9237	8.2467
whole plant	vegetative rosette	Wt	14 days	ATGE_89	67.5690	4.2012	134.1031	8.8452
whole plant	vegetative rosette	Wt	21 days	ATGE_90	55.6436	4.2935	124.2199	6.5292

Table 3. (continued)

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEl8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
Apex	shoot apex, vegetative + young leaves	Wt	7 days	ATGE_4	53.5744	4.1973	134.2765	6.5347
Apex	shoot apex, vegetative	Wt	7 days	ATGE_6	88.0786	4.1624	130.2462	17.6638
Apex	shoot apex, transition (before bolting)	Wt	14 days	ATGE_8	201.0700	4.2138	345.3538	26.4287
Apex	shoot apex, inflorescence (after bolting)	Wt	21 days	ATGE_29	241.0030	4.1248	346.2138	40.2886
Apex	shoot apex, inflorescence (after bolting)	clv3-7	21+ days	ATGE_46	433.9559	4.2527	372.7800	65.0793
Apex	shoot apex, inflorescence (after bolting)	lfy-12	21+ days	ATGE_47	341.8335	4.1394	302.6740	46.5354
Apex	shoot apex, inflorescence (after bolting)	ap1-15	21+ days	ATGE_48	329.9149	4.3634	402.6964	116.8906
Apex	shoot apex, inflorescence (after bolting)	ap2-6	21+ days	ATGE_49	422.0846	4.1650	387.2280	33.5588
Apex	shoot apex, inflorescence (after bolting)	ufo-1	21+ days	ATGE_52	219.0263	4.2113	388.6252	25.9075
Apex	shoot apex, inflorescence (after bolting)	ap3-6	21+ days	ATGE_50	239.1655	4.7914	359.1343	32.7240
Apex	shoot apex, inflorescence (after bolting)	ag-12	21+ days	ATGE_51	194.9532	4.2444	376.3795	36.6413

Table 3. (continued)

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEI8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
Flowers	flowers stage 9	Wt	21+ days	ATGE_31	253.8179	3.8433	293.7009	22.6725
Flowers	flowers stage 10/11	Wt	21+ days	ATGE_32	201.5297	3.8772	281.7713	37.7624
Flowers	flowers stage 12	Wt	21+ days	ATGE_33	141.5384	3.9367	176.9918	30.7139
Flowers	flower stage 12; multi-carpel gynoeceum; enlarged meristem; increased organ number	clv3-7	21+ days	ATGE_53	267.5676	3.9684	188.8224	98.4037
Flowers	flower stage 12; shoot characteristics; most organs leaf-like	lfy-12	21+ days	ATGE_54	288.1352	4.0406	228.5687	102.9779
Flowers	flower stage 12; sepals replaced by leaf-like organs, petals mostly lacking, has secondary flowers	ap1-15	21+ days	ATGE_55	273.6079	4.2347	185.7030	51.1690
Flowers	flower stage 12; no sepals or petals	ap2-6	21+ days	ATGE_56	473.7983	4.1313	192.1842	22.2296
Flowers	flower stage 12; filamentous organs in whorls two and three	ufo-1	21+ days	ATGE_59	199.1132	3.9829	176.4388	28.3124
Flowers	flower stage 12; no petals or stamens	ap3-6	21+ days	ATGE_57	459.2823	3.9944	213.0883	158.8411
Flowers	flower stage 12; no stamens or carpels	ag-12	21+ days	ATGE_58	75.0861	4.0855	213.5561	78.4902
Flowers	flowers stage 15	Wt	21+ days	ATGE_39	72.3974	3.9179	41.0504	123.0903
Flowers	flower	Wt	28 days	ATGE_92	176.1144	3.9279	286.8074	79.0242

Table 3. (continued)

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEI8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
floral organs	flowers stage 15, pedicels	Wt	21+ days	ATGE_40	8.3743	4.3985	80.3629	26.4513
floral organs	flowers stage 12, sepals	Wt	21+ days	ATGE_34	11.6440	4.0977	29.3580	487.2636
floral organs	flowers stage 15, sepals	Wt	21+ days	ATGE_41	8.1686	4.1688	10.2703	938.9191
floral organs	flowers stage 12, petals	Wt	21+ days	ATGE_35	36.6966	4.1137	62.9669	20.0946
floral organs	flowers stage 15, petals	Wt	21+ days	ATGE_42	97.6047	4.1777	21.4240	276.7337
floral organs	flowers stage 12, stamens	Wt	21+ days	ATGE_36	24.5876	4.2467	84.7160	88.0152
floral organs	flowers stage 15, stamen	Wt	21+ days	ATGE_43	37.3517	4.5435	15.5075	204.2553
floral organs	mature pollen	Wt	6 wk	ATGE_73	21.7578	5.8369	25.6681	98.7347
floral organs	flowers stage 12, carpels	Wt	21+ days	ATGE_37	532.0767	4.0127	167.5738	18.1675
floral organs	flowers stage 15, carpels	Wt	21+ days	ATGE_45	233.5702	4.0638	68.0945	16.7672

Table 3. (continued)

Tissue cluster	Tissue	Genotype	Age	Sample Id	Expression value for *At1g70560* (*WEI8*)	Expression value for *At1g23320* (*WEL1*)	Expression value for *At4g24670* (*WEL2*)	Expression value for *At1g34040* (*WEL3*)
Seeds	siliques, w/ seeds stage 3; mid globular to early heart embryos	Wt	8 wk	ATGE_76	15.0857	4.0651	78.7357	36.4051
Seeds	siliques, w/ seeds stage 4; early to late heart embryos	Wt	8 wk	ATGE_77	70.5728	3.9652	42.1747	682.5975
Seeds	siliques, w/ seeds stage 5; late heart to mid torpedo embryos	Wt	8 wk	ATGE_78	94.7350	4.0220	31.1221	855.7404
Seeds	seeds, stage 6, w/o siliques; mid to late torpedo embryos	Wt	8 wk	ATGE_79	193.5289	4.2815	52.7719	754.1713
Seeds	seeds, stage 7, w/o siliques; late torpedo to early walking-stick embryos	Wt	8 wk	ATGE_81	214.2501	4.3772	47.5563	792.3121
Seeds	seeds, stage 8, w/o siliques; walking-stick to early curled cotyledons embryos	Wt	8 wk	ATGE_82	71.4606	4.8948	17.4086	122.4652
Seeds	seeds, stage 9, w/o siliques; curled cotyledons to early green cotyledons embryos	Wt	8 wk	ATGE_83	115.9248	5.2850	20.0575	86.9995
Seeds	seeds, stage 10, w/o siliques; green cotyledons embryos	Wt	8 wk	ATGE_84	131.1519	5.3432	12.4186	90.5128

Table 4. Global sequence comparision analysis of WEI8, Alliinase, SUR1 and three aminotransferases in Arabidopsis using the BLOSUM62 scoring matrix

		%	
Protein 1	Protein 2	Identical	Similar
WEI8	alliinase (from *Allium Sativum*) (GI|27573774)	28.72	44.84
WEI8	SUR1	13.82	25.00
WEI8	aspartate/tyrosine aminotransferase (GI|15076853)	11.76	25.57
WEI8	AGD2 (GI|3068977)	10.58	21.37
WEI8	aspartate aminotransferase (GI|531555)	9.83	21.97

Table 5. *WEI8* sequencing primers

Marker	Primer sequence
At1g70560-F1	TGAAACTGGAGAACTCGAGGA
At1g70560-F7	GCAATTATTGTATTTTCAC
At1g70560-R1	GCTTTTAATGAGCTTCATGTTGG
At1g70560-R3	CTTCATCATCTCACGACCAT
At1g70560-R7	TTCAGAAACAACGCCCACTG
At1g70560-R8	CATCAGAGAGACGGTGGTGAAC
At1g70560-F9	CATGTGCAAAGACATCAGTAACA
At1g70560-R5	ATAGATCTGCACTTGTCTAGC
At1g70560-Fpromter	TTATGTGGTAATTCATTACCATC
At1g70560-Rutr	CTAAGAACTAGAACAGTTTCAC

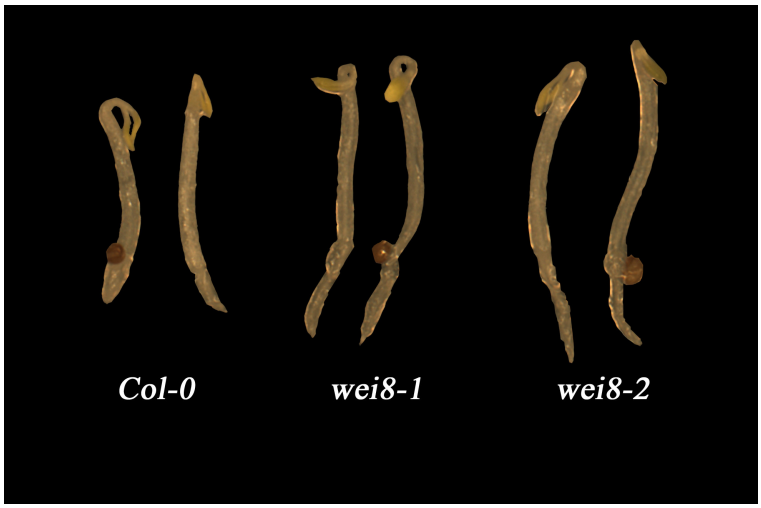


Fig. 1. Ethylene insensitive phenotypes of two *wei8* mutants. Seedlings were grown for three days in the dark in agar media supplemented with 10uM ACC. Col-0, *wei8-1* and *wei8-2* all show an exaggerated apical hook and short hypocotyls with radial swelling. However, *wei8-1* and *wei8-2* roots are less sensitive to ACC compared to Col-0.

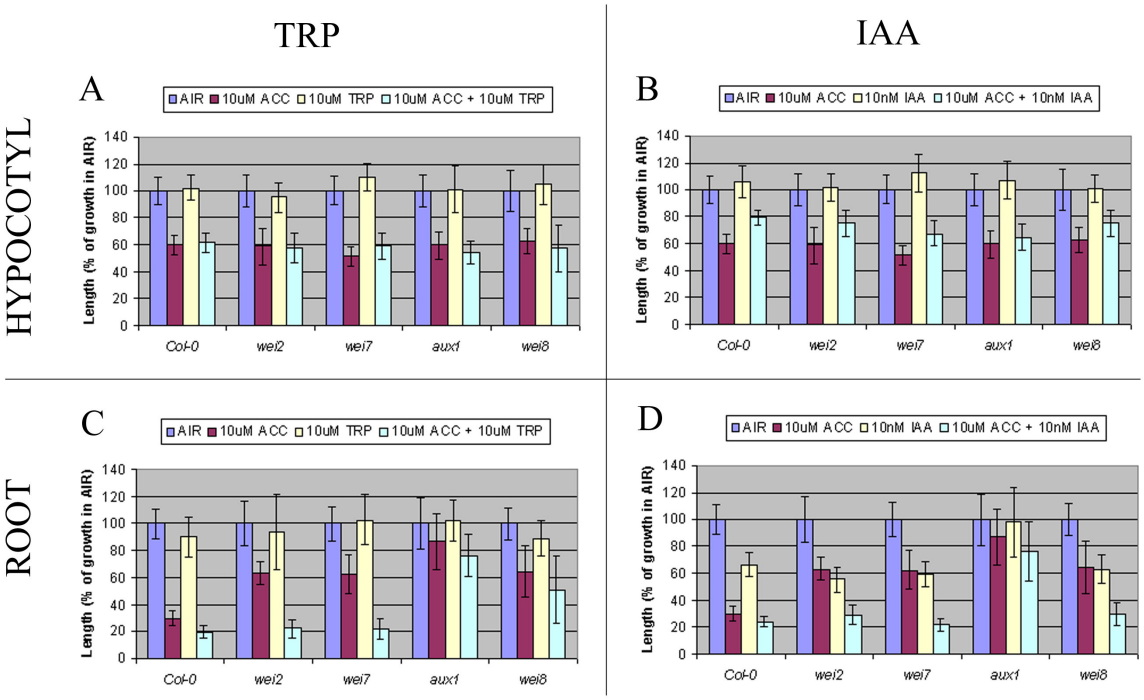


Fig. 2. Growth response of *wei8* in the presence of 10uM TRP, 10nM IAA, and 10uM ACC. Col-0, the tryptophan biosynthesis mutants *wei2* and *wei7*, auxin transporter mutant *aux1*, and *wei8* were grown for three days in the dark. Hypocotyl and root lengths were measured for an average of 20 seedlings per line (see table 1 for more details). *wei8* hypocotyls respond normally to ACC and neither TRP **(A)** or IAA **(B)** affect overall response compared to Col-0. However, *wei8* root response shows insensitivity to ACC. **(C)** Complementation of *wei8* ethylene-insensitivity is not seen upon application of 10mM TRP. **(D)** However 10nM IAA does restore root sensitivity to ACC.

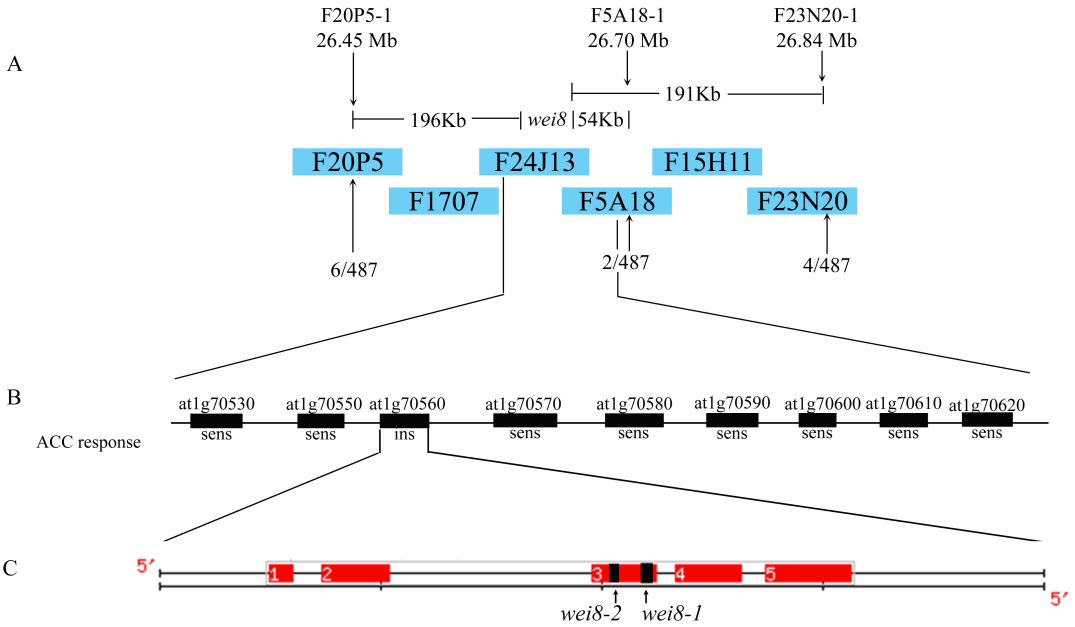


Fig. 3. *WEI8* gene structure. **(A)** Physical map of *WEI8* location on chromosome one. Three SSLP markers in F20P5, F5A18, and F23N20 are shown with six, two, and four recombinants, respectively. **(B)** SALK knockout lines were assayed for ethylene insensitivity in nine genes. Only *at1g70560* showed insensitivity. **(C)** Sequencing of *at1g70560* revealed a T-DNA insertion for *wei8-1* and a SNP for *wei8-2*.

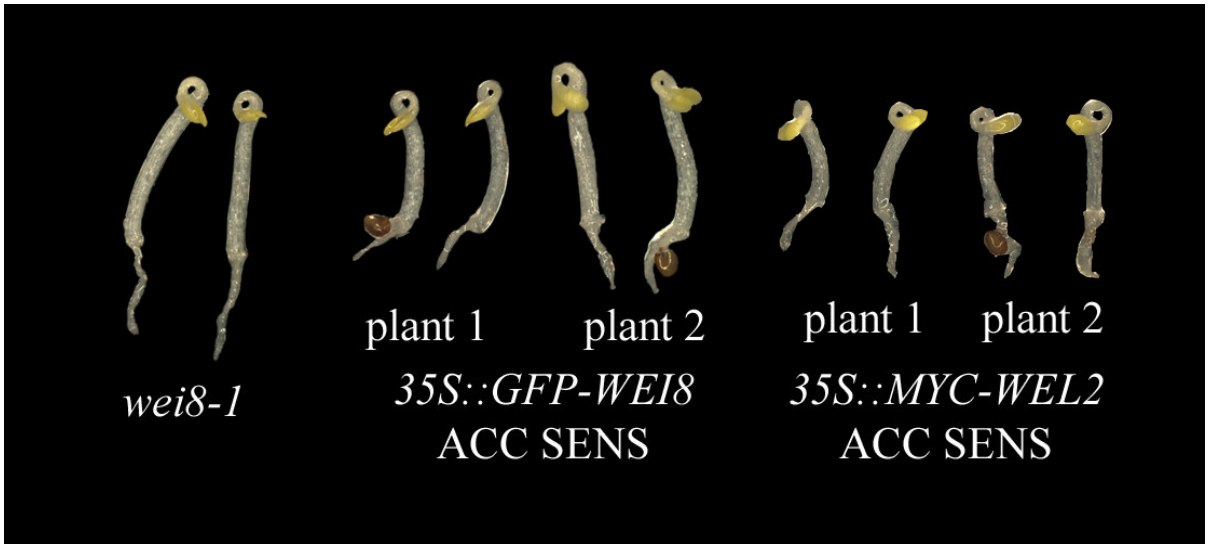


Fig. 4. Overexpression of *WEI8* or *WEL2* in *wei8-1* mutant background restores ethylene sensitivity. Four plants from two independently derived transformed lines grown in the presence of ethylene are shown. Complementation of ethylene insensitivity can be observed using both *35S::GFP-WEI8* and *35S::MYC-WEL2*. For comparision, a *wei8* seedling grown in the same conditions is shown.

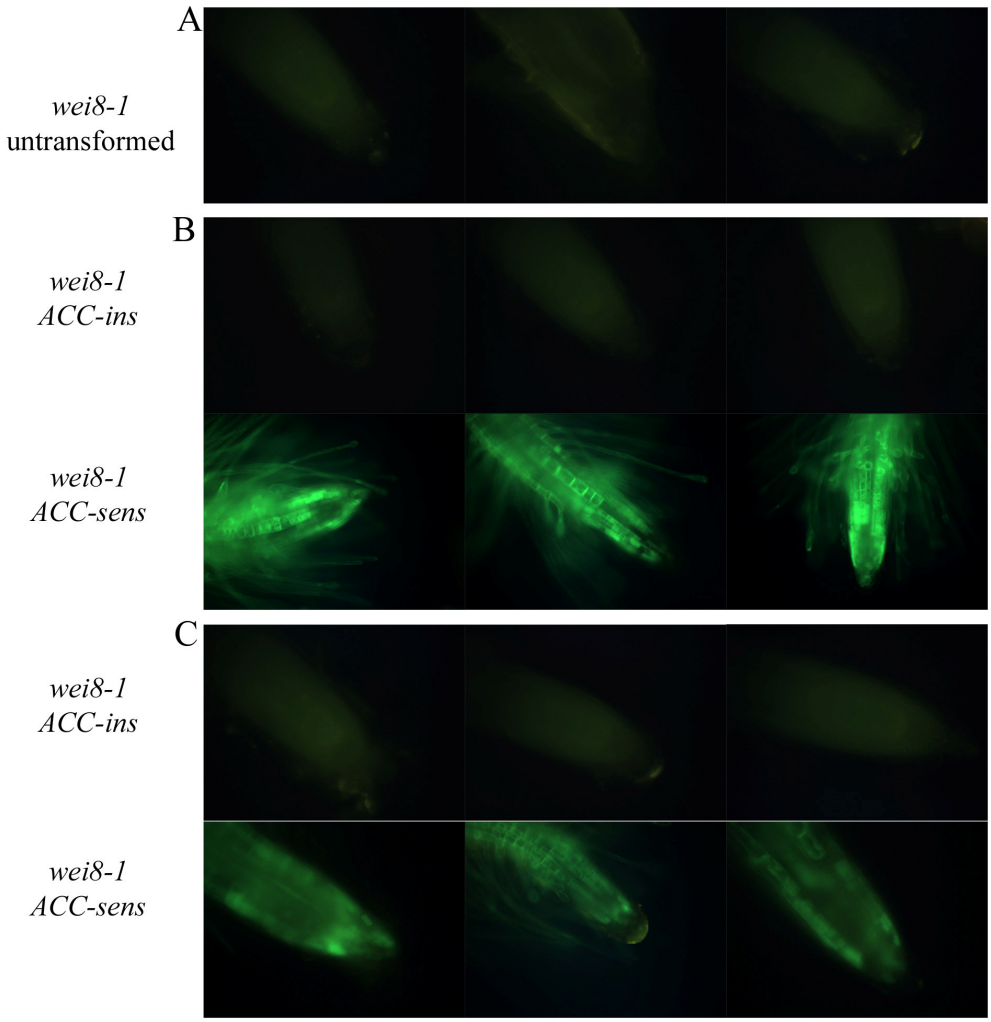


Fig. 5. GFP expression corresponds with ethylene sensitivity. **(A)** Untransformed *wei8-1* was used as an expression control. **(B)** and **(C)** Two independent segregating lines were shown to have GFP expression in ethylene-sensitive seedlings and GFP expression similar to control in ethylene-insensitive seedlings. All plants were in the *wei8-1* mutant background.

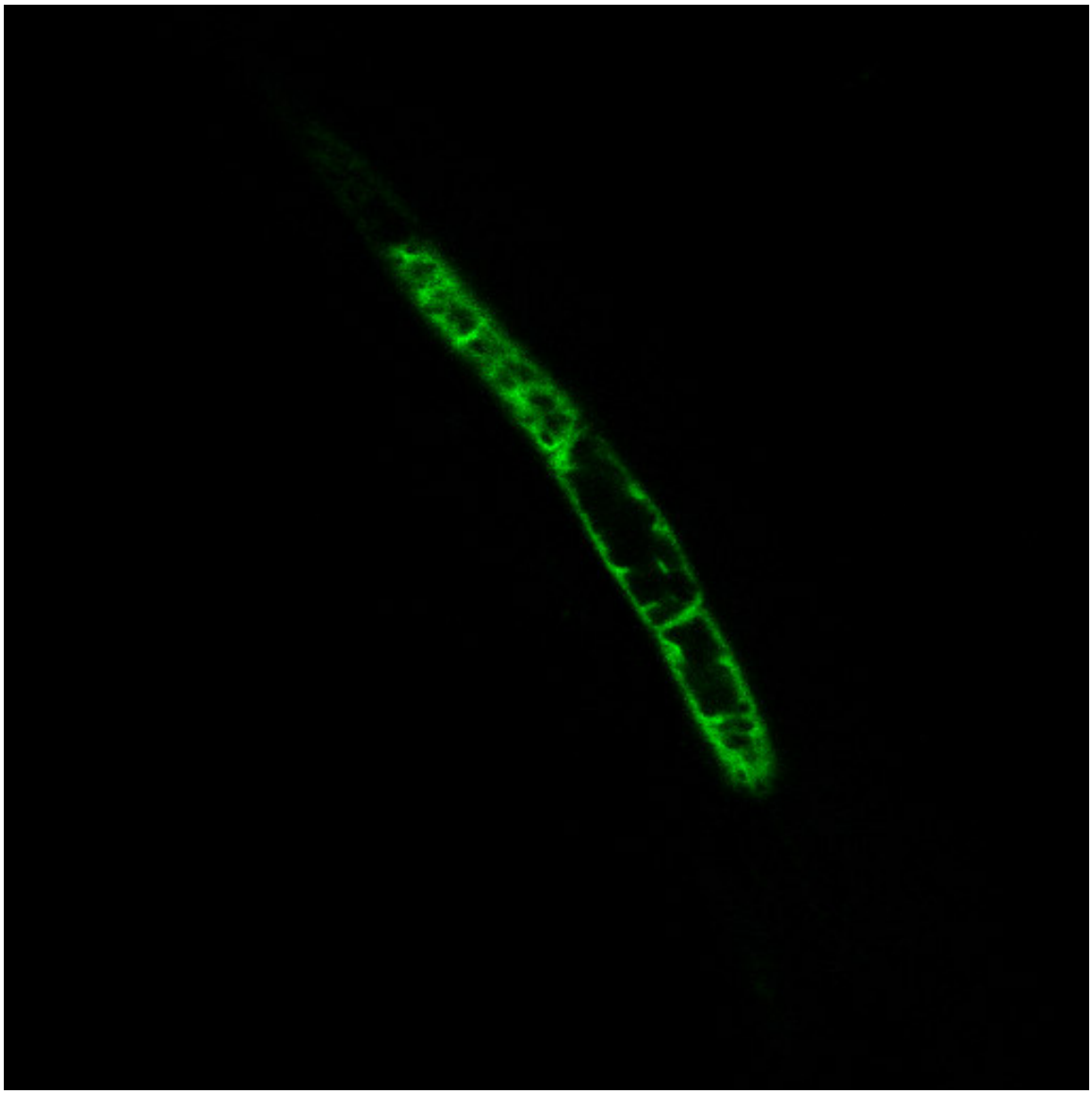


Fig. 6. Confocal image of GFP expression. *35S::GFP-WEI8* was expressed in *wei8* mutants and restore normal ethylene sensitivity. In these lines GFP was localized in the cytoplasm of the cell. In this figure three cells are shown, the top cell shows distributed cytoplasmic expression, whereas in the middle and lower cells, there is presence of a large vacuole with GFP expression evident in the periphery of the vacuole. Imaging was conducted by Dr. Eva Johannes at the Cellular and Molecular Imaging Facility at North Carolina State University.

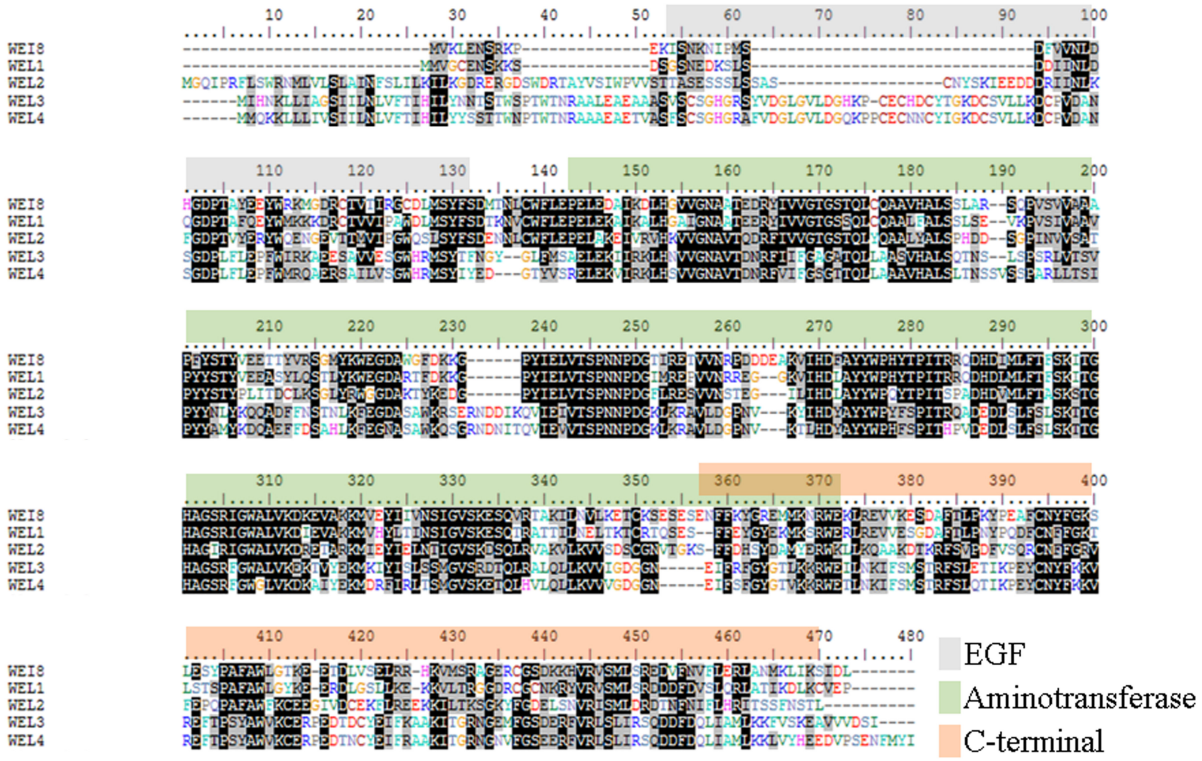


Fig. 7. Amino acid sequence analysis of WEI8 and homologs (WEL1 to 4). The Epidermal Growth Factor (EFG), aminotransferase, and C-terminal domain of the alliinase from *Allium sativum* (the alliinase homolgous to WEI8) is shown. Amino acids are shown in color to aid in visual differentiation. Residues that are at least 60% identical at a single position are shown with a black background. Grey background indicates at least 60% amino acid similarity.

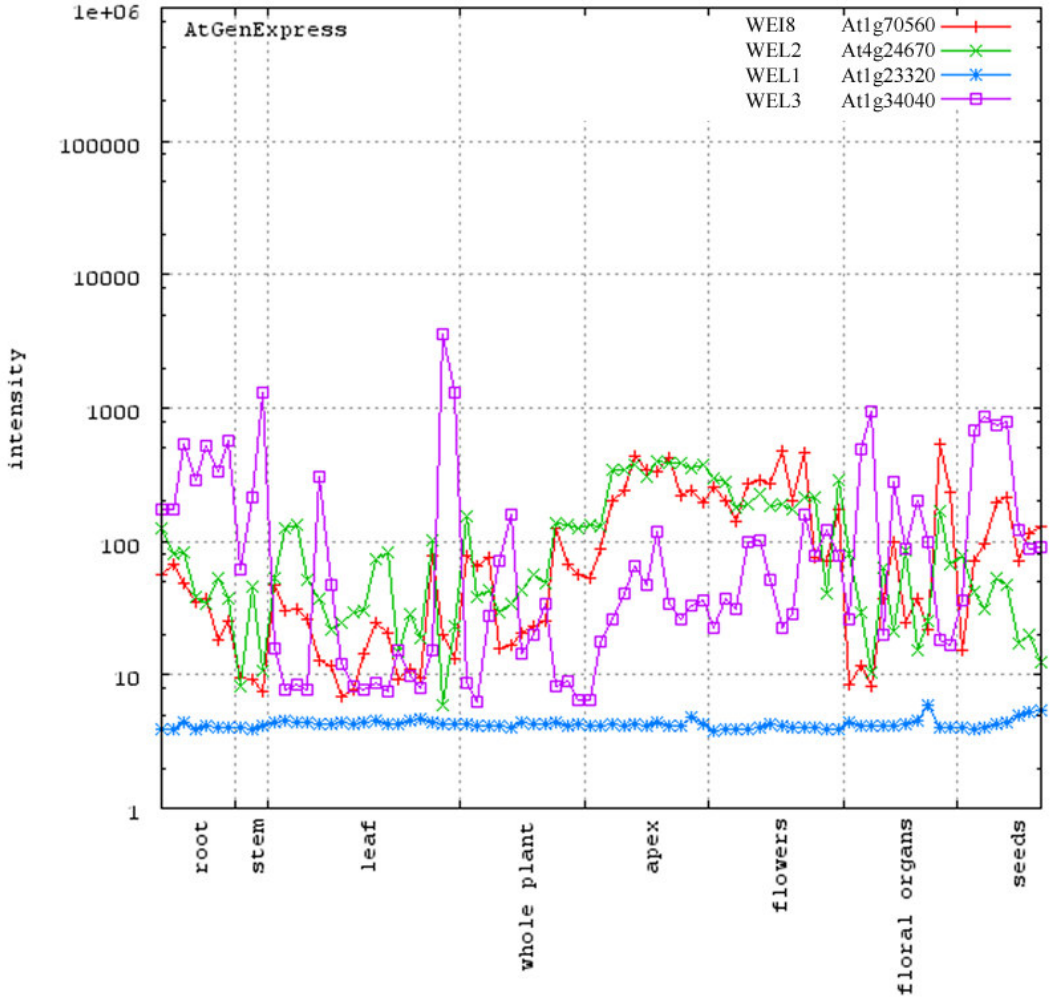


Fig. 8. Expression level of *WEI8* (*At1g70560*), *WEL1* (*At1g23320*), *WEL2* (*At4g24670*), and *WEL3* (*At1g34040*). Expression was measured in eight plant tissues (see table 2 for sampling characteristics for each tissue). Similar expression patterns are seen between *WEI8* and *WEL2* in the root, apex, and floral organs of the plant. Expression levels obtained from AtGenExpress. (Schmid et al., 2005)

Fig. 9. Ethylene insensitivity of *WEI8* homologs. Phenotype of 3-d-old seedlings grown in the dark on 10uM ACC. Root-specific ethylene insensitivity of *wei8-1* and the homologs *wel2-1* and *wel2-2* are shown.

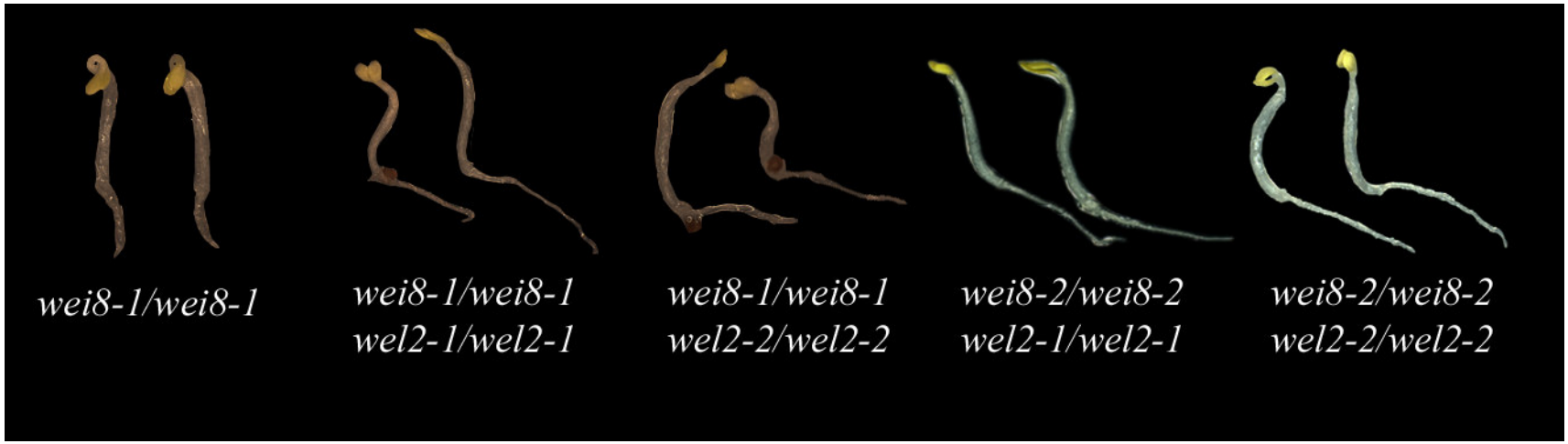


Fig. 10. Ethylene insensitive seedling phenotypes of *wei8 wel2* double mutants. Phenotype of 3-d-old etiolated seedlings grown on 10uM ACC. Root-specific ethylene insensitivity of all four *wei8-1 wel2* double mutant combinations are shown. *wei8-1* single mutant has a normal exaggeration of the apical hook and shortened hypocotyl with radial swelling, however root length is increased compared to the triple response. Homozygous *wei8 wel2* double mutants have defects in the apical hook In the presence of ethylene whereby no hook is formed. Similar to the triple response, the hypocotyls are shortened with radial swelling, however root insensitivity to ACC in the homozygous double mutants is markedly greater compared to the wei8 single mutant.

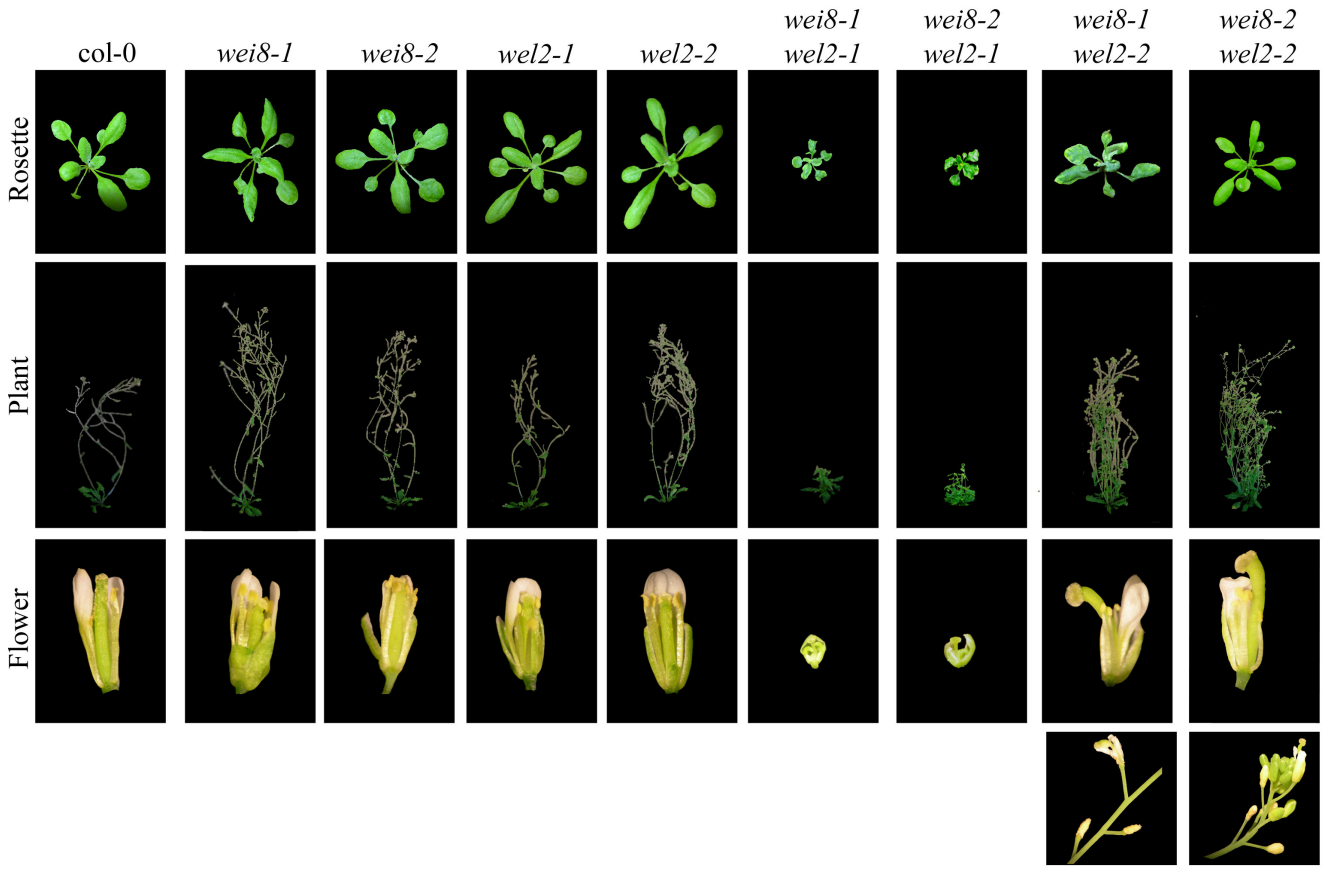


Fig. 11. Adult phenotypes of *wei8*, *wel2*, and double mutants *wei8 wel2*. Rosette, plant, and flower phenotypes approximate wild type for each of the single mutants. However *wei8 wel2-1* double mutants show extreme reduction in rosette size, plant growth and have very small flowers lacking proper stamen and petal structures. In general the double mutants containing the *wel2-2* allele had milder phenotypic alterations than those containing the *wel2-1* allele.

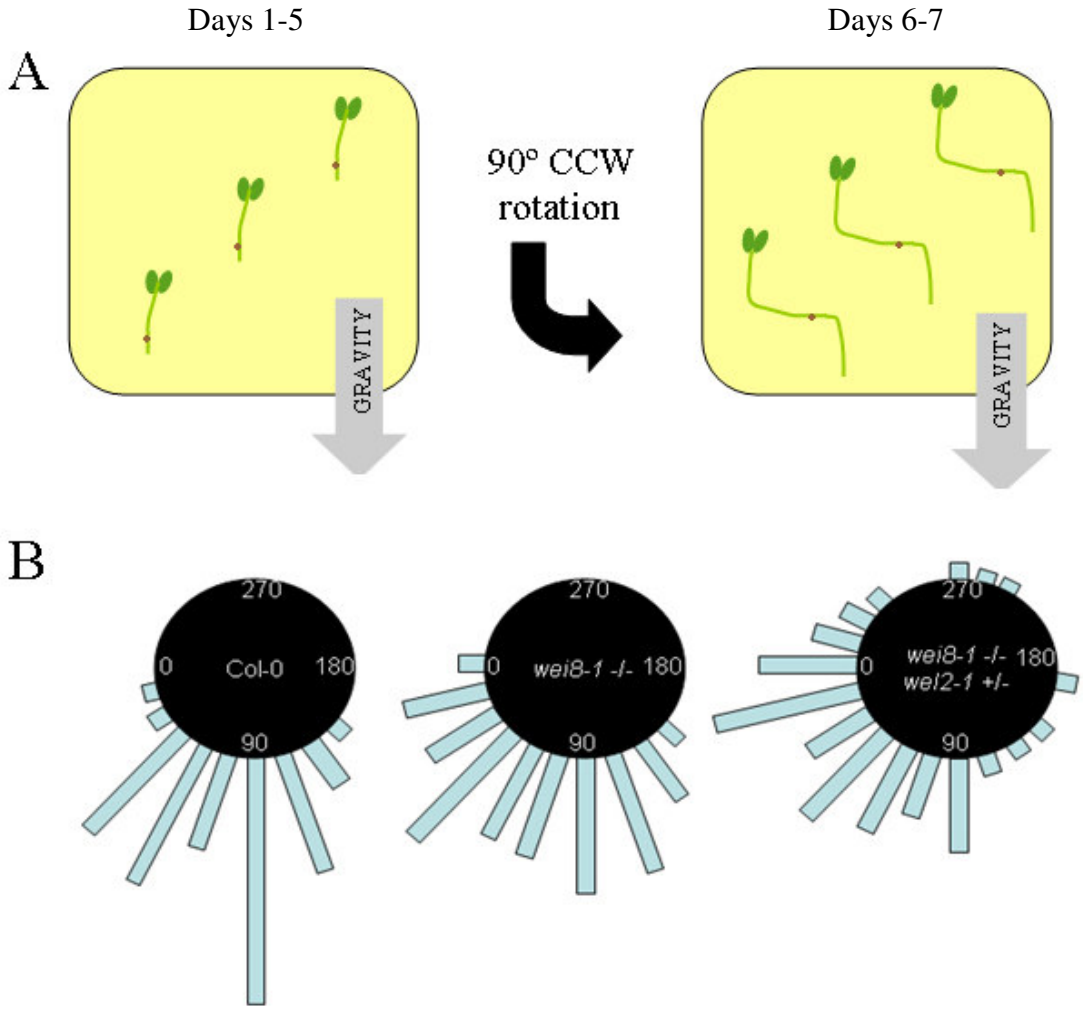


Fig. 12. *wei8* and *wel2* contribute to gravitropic defects in Arabidopsis. **(A)** Seedlings were grown on vertical plates for five days in the dark, then turned 90° and grown for an two additional days in the dark. Response to gravity was measured as the angle between the basal portion of the hypocotyl and the distal part of the root after the assay was concluded (90° turn is equal to normal response). **(B)** A total of 37, 47, and 52 seedlings were evaluated for Col-0, *wei8-1*, and the *wei8-1 wel2-1* population. *wei8-1* shows a mild level of gravity defects compared to wild type. Additionally, the population segregating for *wel2-1* has profound gravitropism defects.

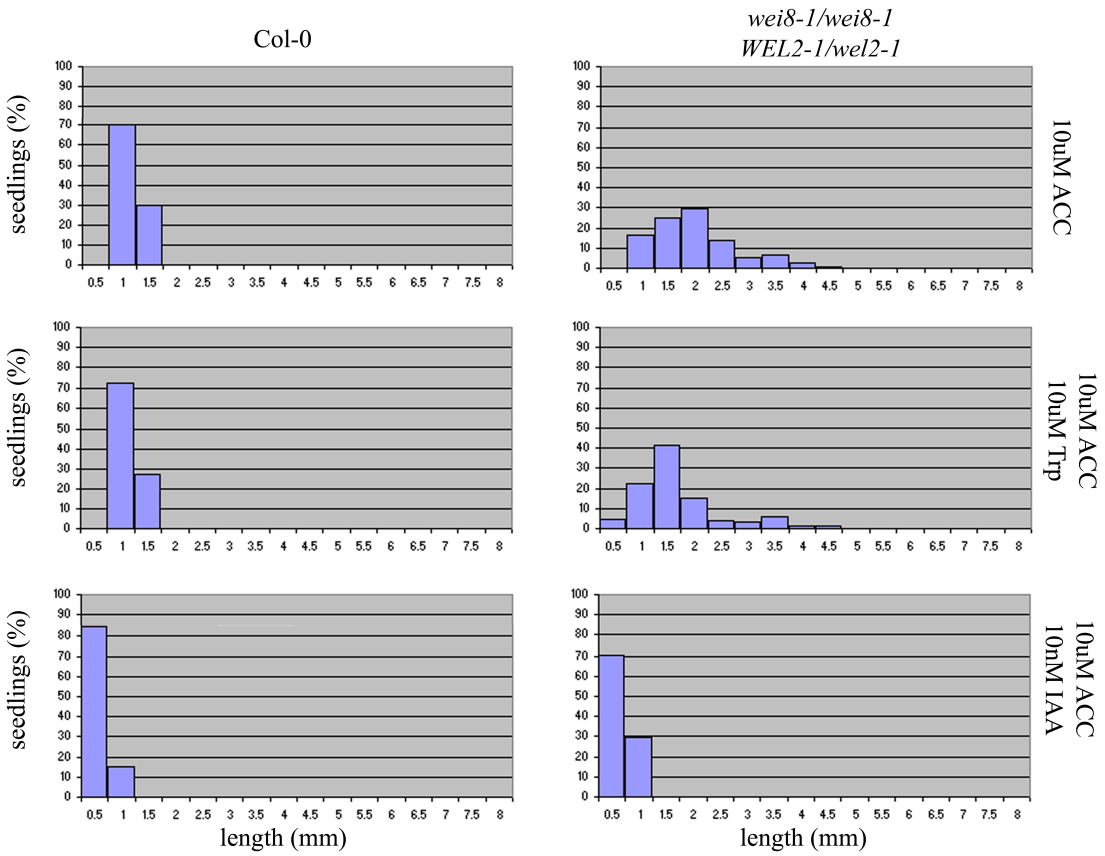


Fig. 13. Restoration of ethylene sensitivity in double mutant *wei8-1 wel2-1* population by IAA. Col-0 and the segregating progeny of *wei8-1*/*wei8-1 WEL2-1*/*wel2-1* plants were evaluated for ethylene sensitivity on 10uM ACC, 10uM ACC + 10uM Trp, and 10uM ACC + 10nM IAA. No complementation by tryptophan (Trp) was observed while the addition of exogenous IAA can restore normal ethylene response.

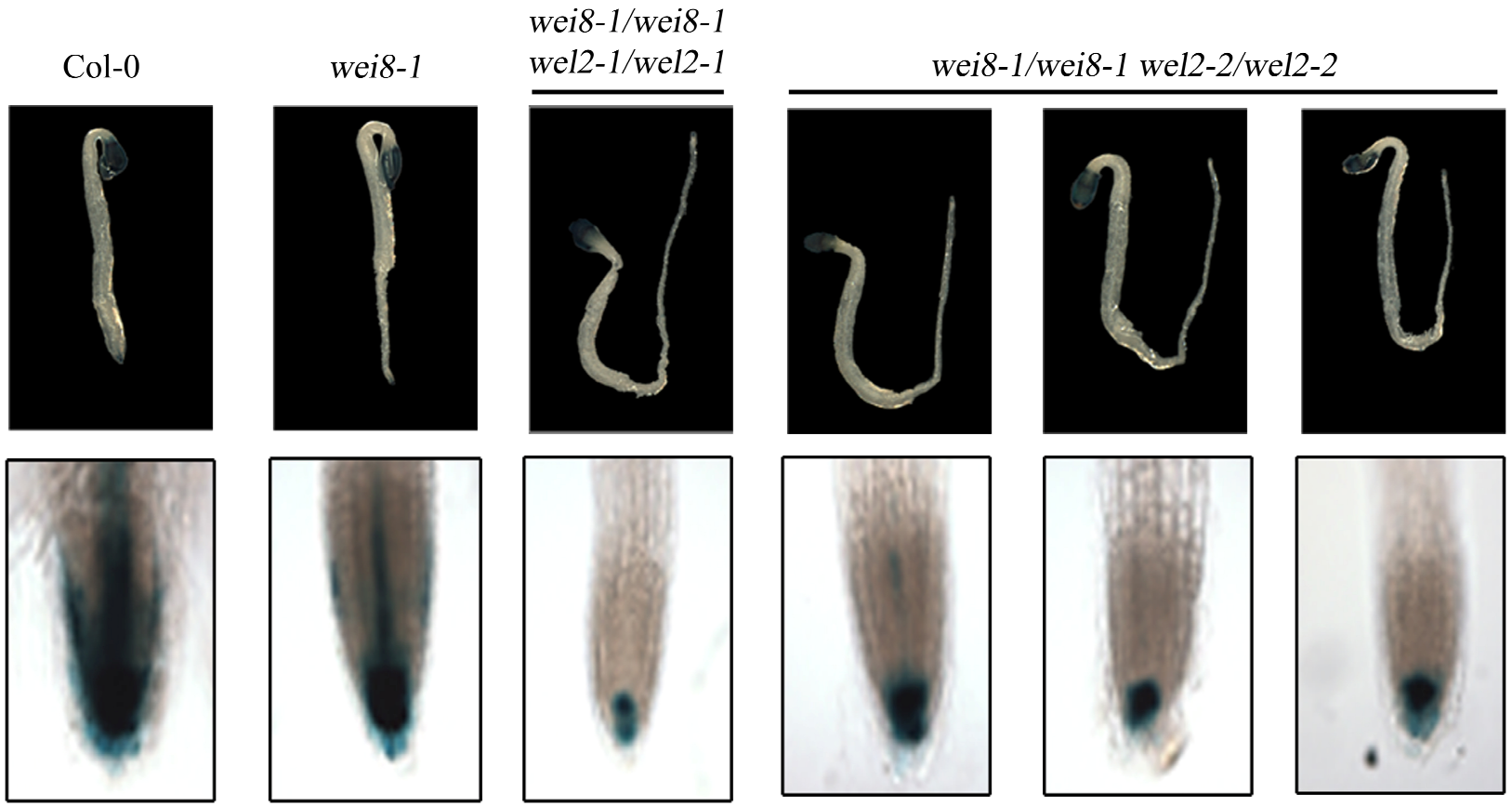
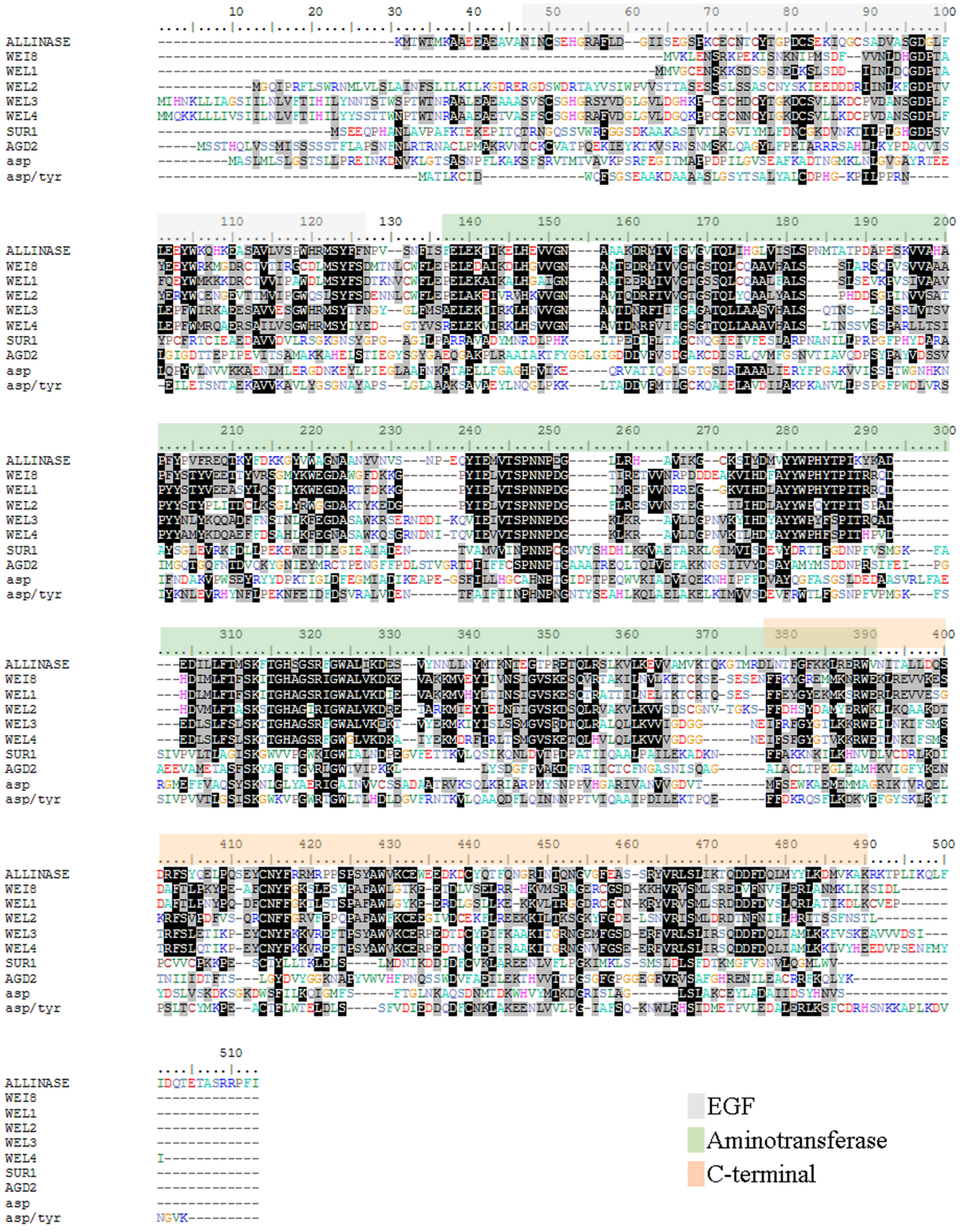


Fig. 14. DR5-GUS expression is reduced in single and double mutants compared to Col-0. Col-0, *wei8-1* and *wei8/wei8 Wel2/wel2* double mutant populations which were segregating for *wel2-1* or *wel2-2* were grown for three days in the dark on 10uM ACC. In the double mutant populations seedlings were selected based upon ethylene insensitive phenotype (agravitropic long root with open hook) which was shown to be correlated with homozygous mutation at both the *WEI8* and *WEI2* loci. DR5-GUS expression is slightly reduced in *wei8* compared to Col-0, while the double mutant *wei8 wel2* had a much more profound effect on the DR5-GUS expression. Reduced expression in the double mutants was stable and consistent across many independently derived lines.

Fig. 15. Multiple sequence alignment of WEI8, Alliinase, and SUR1, and known aminotransferases. Protein sequences from WEI8, WEI8 homologs, an *Allium Sativum* alliinase, three characterized aminotransferases (AGD2, an aspartate aminotransferase, and an aspartate/tyrosine aminotransferase), and one CS-lyase (SUR1) were analyzed using the ClustalW multiple alignment process. The Epidermal Growth Factor (EFG), an aminotransferase, and C-terminal domain of alliinase is shown. Analysis of WEI8 sequence shows greater similarity with alliinase in the aminotransferase domain compared with either SUR1 or the three aminotransferases.


ALLINASE
WEI8
WEL1
WEL2
WEL3
WEL4
SUR1
AGD2
asp
asp/tyr
EGF
Aminotransferase
C-terminal

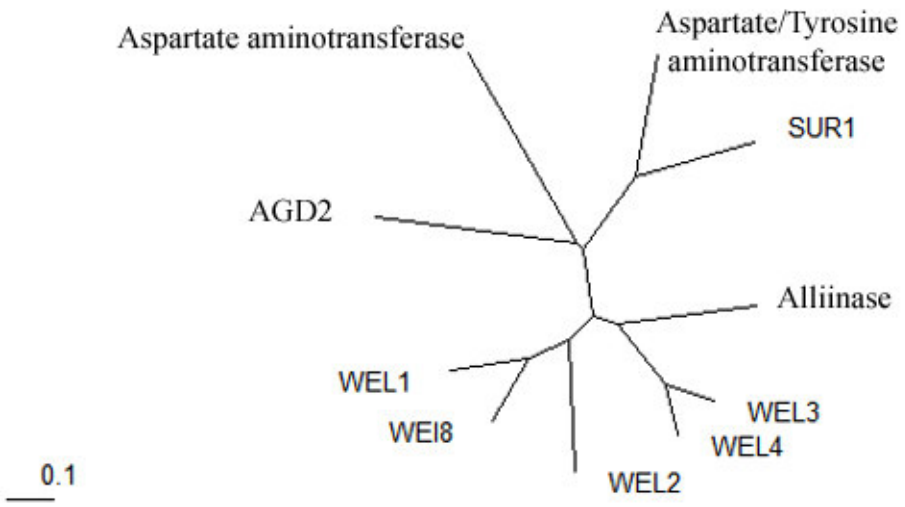


Fig. 16. Phylogenetic analysis of WEI8, Alliinase, and SUR, and known aminotransferases. Protein sequences from WEI8, WEI8 homologs, an *Allium Sativum* alliinase, three characterized aminotransferases (AGD2, an aspartate aminotransferase, and an aspartate/tyrosine aminotransferase), and one CS-lyase (SUR1) were analyzed using the ClustalW multiple alignment process. Treeview was used to generate a phylogenetic tree which shows WEI8 to cluster tightly with WEL1 and show no evidence of increased similarity for any of the aminotransferases or SUR1 when compared to one another.

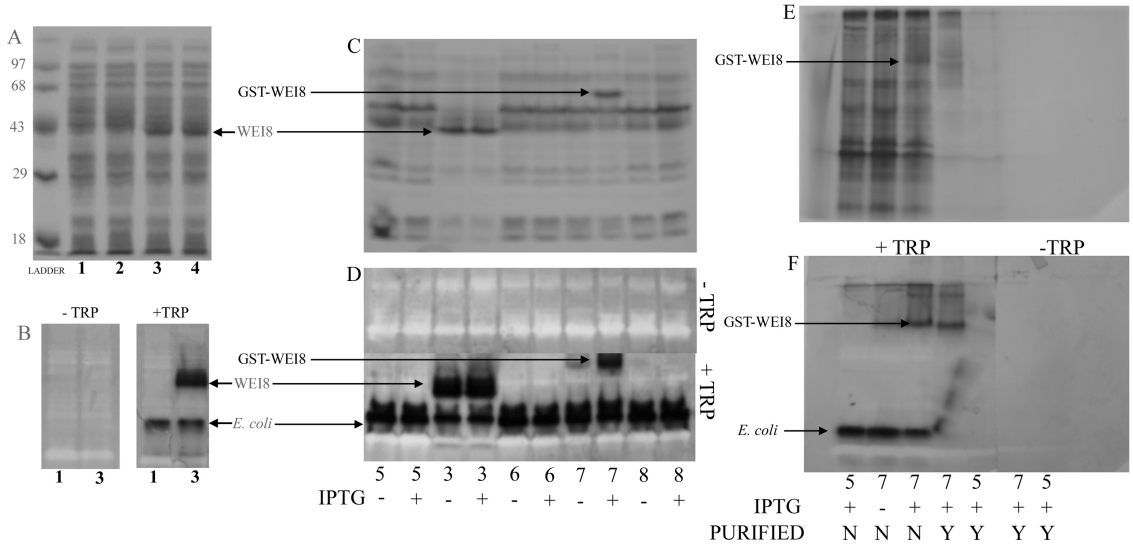


Fig. 17. Assay to detect aminotransferase activity in *WEI8*-transformed *E. coli*. *E. coli* strains are labeled as follows: (Lane) (1) *pHB1::MYC9*, (2) *pHB1::GST*, (3) *pHB1::MYC9-WEI8*, (4) *pHB1::GST-WEI8*, (5) *pT7::GST-EAD*, (6) *pT7::WEI8*, (7) *pT7::GST-WEI8*, (8) *pT7::WEI8-GST*. *pT7::GST-EAD* was used as a negative control to test the vector without *WEI8*. Strain DH5 (**A**, **B**) and BL21 (pLys) (**C**, **D**, **E**, **F**) of *E. coli* were used. IPTG was used for induction at a final concentration of 0.4 mM. All aminotransferase activity reactions were performed in tandem in the presence and absence of tryptophan (TRP). Denatured protein was analyzed using SDS-containing PAGE. Native protein was analyzed in aminotransferase activity reactions following non-SDS-containing PAGE. **(A)** Coomassie blue staining of total denatured protein. The molecular weight of WEI8 is approximately 40kDA, which is smaller than predicted (44.8 kDa). **(B)** Aminotransferase activity is shown in *MYC9-WEI8* transformed *E. coli*. **(C)** GST-WEI8 approximates predicted size in IPTG-induced BL21 (pLys) *E. coli*. The size of the GST-WEI8 protein approximates the expected weight of 63kDa. Additionally, IPTG-mediated expression is evident in the case of *pT7::GST-WEI8*. Lane designations are the same as in **(D)** **(D)** Aminotransferase activity of MYC9-WEI8 and GST-WEI8 is shown. (potential carry over into lane carrying un-induced GST-WEI8 sample during loading). **(E)** and **(F)** Purified GST-WEI8 is expressed and shows aminotransferase activity. Lane designations for **(E)** are the same as those indicated in **(F)**.(Residual staining from an overlapping gel during incubation is evident on **(F)**).

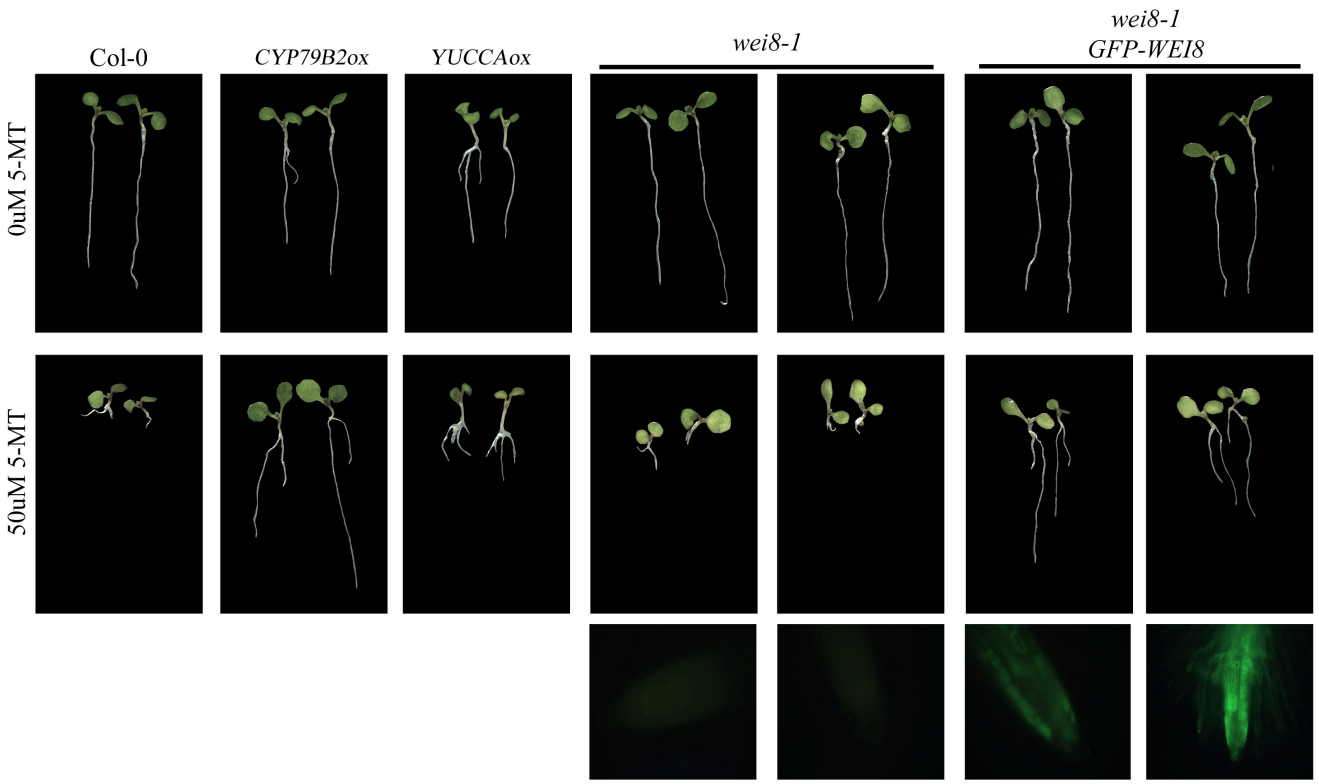


Fig. 18. Assay for tolerance to 5-MT in *GFP-WEI8* overexpression lines. Col-0, *CYP79B2ox*, *YUCCAox*, *wei8-1*, and *GFP-WEI8* transformed *wei8-1* were grown on 50uM 5-MT for eight days in the light. *wei8-1* shows no tolerance to 5-MT, similar to Col-0. However, overexpressing *WEI8* results in tolerance similar to the known overexpressors, *CYP79B2ox* and *YUCCAox,* in the IAA biosynthesis pathway. GFP expression is shown for transformed lines as a positive control of expression.

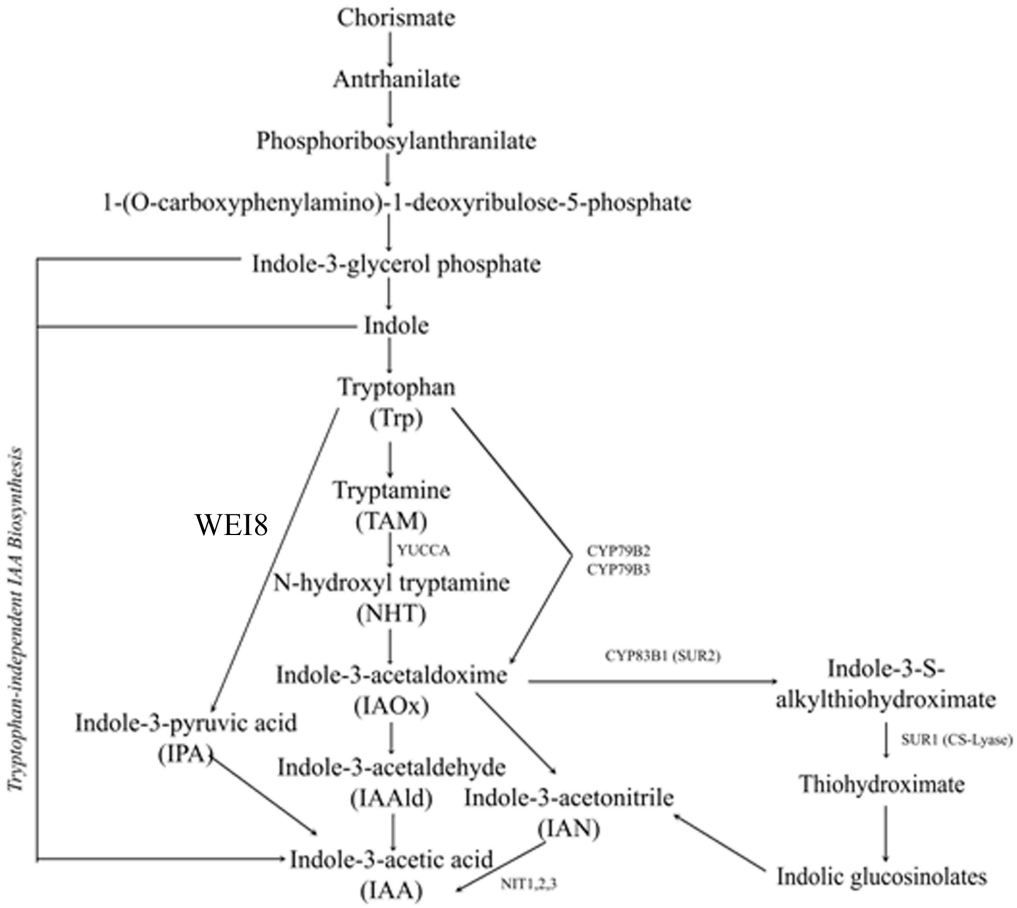


Fig. 19. WEI8 catalyzes the conversion of tryptophan to IPA in the IAA biosynthesis pathway. WEI8 has been found to use tryptophan as a substrate in an aminotransferase reaction. Additionally ethylene sensitivity is restored in *wei8* when treated with IAA, but not tryptophan. Hence, it is proposed that WEI8 catalyzes the conversion of tryptophan to IPA in the tryptophan-dependent IAA biosynthesis pathway.

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APPENDIX

Identification of an additional putative *aux1* mutant

The semi-dominant mutant, *t4d5-1*, in the Col-0 background, was identified in a screen of mutagenized lines by A. Stepanova as being ethylene and auxin insensitive. Additionally the mutant has agravitropic roots, and ethylene sensitivity can not be rescued by addition of exogenous tryptophan indicating that the auxin defect is not caused by reduction in tryptophan biosynthesis, but is likely due to reduced functionality of the auxin transport or response pathway.

Insensitive F2 lines from a cross of *t4d5-1* x Ler were selected for mapping. DNA was isolated from young leaves of 32 individuals. The genotype at marker nga168 (chromosome 2, 16.29MB) was determined by using marker specific primers to determine if there is evidence of linkage to *AUX1*, which encodes an auxin influx carrier, and whose mutant has an identical phenotype. Twenty-five of 32 lines show to be homozygous Col-0 at the nga168 locus. This observed frequency is significantly different from the expected frequency based on chi-square analysis (α=0.05). From this, it was determined that *t4d5-1* was likely a mutant allele of *AUX1* that maps in close proximity to nga168 and no further experiments were performed on this mutant.

The Search for Enhancers and Suppressors of the Ethylene-Insensitive *wei2* Phenotype

Many genes which encode proteins involved in ethylene insensitivity have been identified in recent years (Chen et al., 2005). Among these is *WEI2* (Stepanova et al., 2005), which was found to encode the alpha subunit of anthranilate synthase, an enzyme which converts chorismate to anthranilate in the first committed step of the tryptophan biosynthesis pathway. Ethylene-insensitive phenotypes resulting from loss of function mutations within the tryptophan biosynthesis pathway are of particular interest as tryptophan is a precursor of auxin biosynthesis.

WEI2 may be a point of interaction between ethylene and auxin-related response. This project focused on identifying mutants causing deviation in the ethylene-insensitive phenotype of *wei2*, and their subsequent characterization.

RESULTS

Fourteen of twenty-six EMS-mutagenized families were screened for enhanced or suppressed *wei2* phenotypes. As a result of the screening, 854 suppressors and 577 enhancers were initially identified. Only enhancers and suppressors from families one and two were retested due to time restriction. Eighteen of 206 suppressors and two of 61 enhancers were selected upon retest. This project was deprioritized and may be reinstated at a later date.

MATERIALS AND METHODS

Homozygous *wei2* seed in the Col-0 background were mutatgenized using EMS mutagenesis and selfed to the M2 generation (Alonso unpublished). M2 seed was grown on 10uM ACC and individual lines were selected which displayed either enhanced or reduced ethylene insensitivity compared to *wei2*. The selected M2 plants were retested in the M3 generation to verify the originally selected phenotype.

Crosses were made with *wei2*, Col-0 and Ler. Crossing to *wei2* removes additional mutations from the plant background and allows the evaluation of the mutation in a pure line. Crossing to Col-0 allows the enhancer or suppression mutation to be characterized in the absence of *wei2*. Finally, the cross to Ler is produced and selfed to an F2 mapping population which can then be used for subsequent cloning of the enhancer or suppressor.