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GENETIC ANALYSES OF AUXIN METABOLISM AND OF THE
TRANSITION TO FLOWERING IN THE MODEL PLANT
ARABIDOPSIS THALIANA

by

JAMIE LASWELL

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ABSTRACT

GENETIC ANALYSES OF AUXIN METABOLISM AND OF THE TRANSITION TO FLOWERING
IN THE MODEL PLANT ARABIDOPSIS THALIANA

by

Jamie Lasswell

The auxins are an important class of plant hormones involved in many aspects of plant development. The most common naturally occurring auxin is indole-3-acetic acid, or IAA. In Arabidopsis, up to 95% of the IAA pool is found conjugated to small molecules such as sugars and amino acids. However, the genes and enzymes involved in IAA conjugate metabolism are not yet well understood. A mutant, iar1, that is resistant to the inhibitory effects of multiple IAA-amino acid conjugates on root elongation was identified. The iar1 gene encodes a protein with numerous transmembrane domains and several histidine-rich regions. The IAR1 protein has homologs in other organisms, including Drosophila, C. elegans, and mammals, and is similar in molecular structure to the ZIP family of zinc transporters from Arabidopsis and yeast.

Plant reproduction requires precise control of the transition to flowering in response to environmental cues. We have isolated a late-flowering Arabidopsis mutant, fkfl, that is rescued by vernalization or gibberellin treatment. The mutant also exhibits a light-dependent hypocotyl elongation defect. We used a positional approach to clone FKFL, which encodes a novel protein with an N-terminal PAS domain similar to the flavin-binding region of certain photoreceptors, an F-box motif characteristic of proteins that target
ubiquitin-mediated degradation, and six kelch repeats predicted to fold into a β-propeller. 

*FKF1* mRNA levels oscillate with a circadian rhythm and the *fkf1* deletion mutation alters the rhythmic expression of other clock-regulated genes, implicating *FKF1* in regulation of the circadian clock.
ACKNOWLEDGMENTS

First and foremost, I would like to thank the members of my family who have seen me through all my many years of school: my parents, James and Kathleen Lasswell; my sister, Laurie Lippe, and her husband, Kary; and my grandmother Nelda Ruth Echols. I would also like to dedicate this thesis to the memory of my grandfather, E.K. Echols, a wonderful person who taught me to never give up and who truly understood the value of education.

I am also extremely grateful to my advisor, Dr. Bonnie Bartel. She has been unfailingly patient and encouraging and has taught me a great deal about science and about being a scientist. She is the best Ph.D. advisor anyone could ever hope for. I hope someday to be half as impressive as she is.

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TABLE OF CONTENTS

CHAPTER I: AUXIN
   I.A. Auxin effects 1
   I.B. Arabidopsis as a model system for genetic studies of auxin effects 2
   I.C. Genetic analysis of auxin response mutants of Arabidopsis 3
      I.C.1. Aux/IAA gene mutants 3
      I.C.2. Genes involved in ubiquitination 6
   I.D. Regulation of free IAA levels 9
      I.D.1. Synthesis 9
      I.D.2. Transport 12
      I.D.3. Conjugation 16
         I.D.3.a. Conjugates in plants 16
         I.D.3.b. Proposed functions of conjugates 19
            I.D.3.b.i. Storage 19
            I.D.3.b.ii. Transport 19
            I.D.3.b.iii. Hormonal homeostasis 20
         I.D.3.c. Molecular basis of conjugate metabolism 20
   I.D.4. Zinc 22
      I.D.4.a. Zinc transport 22
      I.D.4.b. Zinc and auxin levels 25
   I.E. Goals of this project 27

CHAPTER II: FLOWERING TIME AND LIGHT PERCEPTION
   II.A. Factors influencing flowering time 28
   II.B. Identified genes involved in the floral transition 29
   II.C. Pathways of floral promotion 30
      II.C.1. The autonomous pathway of floral promotion 32
      II.C.2. The photoperiodic pathway of floral promotion 32
      II.C.3. The gibberellin-responsive pathway of floral promotion 33
II.C.4. The vernalization-responsive pathway of floral promotion

II.D. Photoreceptors are involved in control of Arabidopsis flowering time
   II.D.1 Phytochromes in Arabidopsis floral promotion
   II.D.2. Cryptochromes in Arabidopsis floral promotion
   II.D.3. Phototropin mediates phototropism but not flowering time in Arabidopsis

II.E. The circadian clock is implicated in the control of Arabidopsis flowering time
   II.E.1. Arabidopsis photoreceptors mediate light input to the circadian clock
   II.E.2. Many Arabidopsis genes are associated with both flowering time control and the circadian clock

II.F. Goals of this project

CHAPTER III: MATERIALS AND METHODS

III.A. Plant materials and growth conditions
III.B. Isolation of mutants
III.C. Degenerate PCR
III.D. Identification of the splice site mutation of ILL5 in Col-0
III.E. Genetic mapping of the iar1 and fkfl mutations
III.F. Construction of a complementation library
III.G. cDNA isolation and manipulations
III.H. Promoter-reporter gene fusion
III.I. pBIN-FKF1g, pBINIAR1g, IAR1-GFP and IAR1-myc transgenic lines
III.J. Transgenic lines expressing the mouse KE4 cDNA
III.K. RNA blot analysis
III.L. Atomic absorption
III.M. Yeast
III.N. Yeast microsome isolation and analysis
III.O. Analysis of flowering time
III.P. Protein blot analysis
   III.P.1. Protein blot analysis of pBIN19IAR1-myc transgenic plants
III.P.2. Protein blot analysis of PhyA stability in fkl1  
III.Q. Enzyme assays with IAR3

CHAPTER IV: ILL5 IS A MEMBER OF THE ARABIDOPSIS AMIDOHYDROLASE GENE FAMILY
IV.A. Degenerate PCR to identify ILL1 homologs  
IV.B. ILL5 is most closely related to IAR3  
IV.C. Col-0 is an ill5 mutant  
IV.C. Discussion

CHAPTER V. MUTANT CHARACTERIZATION AND CLONING OF THE IAR1 GENE
V.A. Phenotype of the iar1 mutant  
V.A.1. iar1 is resistant to IAA-amino acid conjugates, but not to free IAA  
V.A.2. iar1 mutants elongate hypocotyls normally at high temperature and are resistant to hypocotyl elongation inhibition by IAA-Ala  
V.A.3. iar1 mutants may be early germinating  
V.A.4. The iar1 mutation partially suppresses the alf1 mutant phenotype  
V.A.5. The iar1 mutant responds normally to IBA, JA, MeJA, and JA conjugates  
V.A.6. The iar1 mutant phenotype is suppressed by manganese  
V.A.7. Identification of iar1 enhancers
V.B. Positional cloning of IAR1  
V.B.1. The iar1 mutation maps to the bottom of chromosome 1  
V.B.2. The iar1 phenotype is rescued by C37  
V.B.3. IAR1 encodes a protein with putative transmembrane domains and histidine-rich clusters

CHAPTER VI: MOLECULAR CHARACTERIZATION OF IAR1
VI.A. IAR1 expression
VI.B. IAR1 expression is induced by jasmonic acid and wounding but not by zinc
VI.C. Functional studies of IAR1
VI.C.1. Plants transgenic for an IAR1 overexpression construct are not more sensitive than wild type to IAA-Ala 121

VI.C.2. IAR1 does not confer IAA-Ala transport activity to yeast microsomes 121

VI.C.3. The mouse homolog of IAR1 rescues the iar1 mutant phenotype 123

VI.C.4. IAR1 does not rescue the phenotypes of the yeast pmrl or zrt1zrt2 mutants 124

VI.C.5. Atomic absorption studies 127

VI.C.6. Effects of metal ions on the growth of Arabidopsis seedlings 130

VI.C.7. Copper and zinc inhibit the hydrolase activity of the IAR3 enzyme 133

VI.D. Discussion 137

VI.D.1. iar1 mutants are resistant to the inhibitory effects of IAA-amino acid conjugates 137

VI.D.2. iar1 mutants may be early germinating 137

VI.D.3. The iar1 mutation partially suppresses the alf1 mutant phenotype 138

VI.D.4. The iar1 mutant responds normally to IBA, JA, MeJA, and JA conjugates 139

VI.D.5. IAR1 may be involved in wound responses 139

VI.D.6. IAR1 encodes a protein with predicted transmembrane domains and histidine-rich regions that shares characteristics with the ZIP family of metal transporters 140

VI.D.7. Models for IAR1 function in IAA conjugate metabolism 142

VI.D.7.a. Model 1: IAR1 transports IAA conjugates into the ER 142

VI.D.7.b. Model 2: IAR1 transports a conjugate hydrolase cofactor into the ER 143

VI.D.7.c. Model 3: IAR1 transports a conjugate hydrolase inhibitor out of the ER 144
VI.D.7.d. Model 4: IAR1 inhibits an IAA conjugate hydroxylase

CHAPTER VII: MUTANT CHARACTERIZATION AND CLONING OF THE FKF1 GENE

VII.A. Phenotype of the fkl mutant

VII.A.1. The fkl mutant is late flowering

VII.A.2. fkl flowering defects are rescued by vernalization or gibberellin

VII.A.3. The fkl mutant is defective in light-regulated hypocotyl elongation

VII.B. Cloning FKF1

VII.B.1. FKF1 is a member of a gene family in Arabidopsis

VII.B.2. FKF1 encodes a putative flavin-binding protein with an F-box and Kelch repeats

CHAPTER VIII: MOLECULAR CHARACTERIZATION OF FKF1

VIII.A. FKF1 is expressed in a variety of tissues

VIII.B. Constitutive expression of the FKF1 homolog ZTL1 leads to long hypocotyls and delayed flowering

VIII.C. The fkl mutation does not alter PhyA degradation rates

VIII.D. FKF1 transcripts oscillate with a circadian rhythm

VIII.E. Deleting FKF1 alters circadian transcript oscillation in continuous light

VIII.F. Discussion

VIII.F.1. The fkl mutant affects processes regulated by the circadian clock

VIII.F.2. FKF1 is expressed in a variety of tissues

VIII.F.3. The FKF1 transcript oscillates with a circadian rhythm

VIII.F.4. The flavin-binding domain suggests a role for FKF1 in light perception

VIII.F.5. An F-Box suggests that FKF1 functions in ubiquitin-dependent degradation

VIII.F.6. Possible roles for FKF1 in the plant circadian clock
VIII.F.7. Mutations in an *FKF1* homolog, *ZTL1*, also disrupt clock function 178

CHAPTER IX. CONCLUSIONS AND PERSPECTIVES 180
IX.A. *IAR1* 181
IX.B. *FKF1* 182
IX.C. Possible links between auxin and light perception 184

REFERENCES 184
Figure IV-2: PCR with degenerate oligos designed from conserved regions of ILR1, ILL1, and ILL2

Figure IV-3: Protein alignment of the Arabidopsis family of amidohydrolases

Figure IV-4: The predicted ILL5 protein is most similar to IAR3

Figure IV-5: Map positions of IAR3, ILL5, and the iar1 mutation on Arabidopsis chromosome 1

Figure IV-6: Col-0 is an ill5 splicing mutant

Figure IV-7: The mutation in ILL5 does not render Col-0 less sensitive to inhibition of root elongation by IAA-Ala

CHAPTER V. MUTANT CHARACTERIZATION AND CLONING OF THE IAR1 GENE

Figure V-1: The iar1 mutant is resistant to IAA-Ala but not to free IAA

Table V-1: Alleles of the iar1 IAA-Ala resistant mutant

Figure V-2: iar1 mutants are resistant to inhibition of root elongation by IAA-Ala, but are sensitive to IAA

Figure V-3: iar1 mutants are resistant to IAA-Phe and IAA-Leu

Figure V-4: iar1 mutants are resistant to various IAA-amino acid conjugates

Figure V-5: iar1 mutants elongate hypocotyls normally in response to high temperature

Figure V-6: iar1 mutants exhibit normal hypocotyl elongation

Figure V-7: IAA inhibits Arabidopsis hypocotyl elongation

Figure V-8: iar1 mutants are resistant to inhibition of hypocotyl elongation by IAA-Ala

Figure V-9: iar1 mutants may be early germinating

Figure V-10: The iar1 mutation suppresses the alfl root elongation phenotype

Figure V-11: iar1 mutants are not resistant to IBA

Figure V-12: iar1 is not resistant to JA, MeJA, or JA conjugates

Figure V-13: Manganese suppresses the iar1 IAA-Ala resistant phenotype

Figure V-14: iar1 enhancer J56 is highly resistant to IAA-Ala and resistant to IAA-Leu and IAA-Phe

Figure V-15: iar1 maps to chromosome 1 and is contained on BAC T7E4
Table V-2: Markers used to map the iar1 mutation

Figure V-16: C37 rescues the iar1 IAA-Ala resistant phenotype and the iar1-rescuing construct C37 contains three predicted open reading frames

Table V-3: Detection of iar1 mutant alleles

Figure V-17: Schematic representation of the IAR1 coding region

Figure V-18: IAR1 homologs are present in other organisms

Figure V-19: IAR1 and its homologs encode potential transmembrane proteins

Figure V-20: IAR1 is similar to the ZIP family of metal transporters

Figure V-21: IAR1 contains the ZIP family signature sequence

CHAPTER VI: MOLECULAR CHARACTERIZATION OF IAR1

Figure VI-1: IAR1 message is highest in roots and stems

Figure VI-2: IAR1-GUS staining

Figure VI-3: IAR1-GFP construct rescues the mutant phenotype

Figure VI-4: IAR1-myc lines express detectable levels of the fusion protein

Figure VI-5: IAR1 is induced by wounding and jasmonic acid

Figure VI-6: IAR1 expression may be down-regulated by high zinc

Figure VI-7: Plants transgenic for an IAR1 overexpression construct are not more sensitive than wild type to IAA-Ala

Figure VI-8: The mouse KE4 gene can functionally substitute for IAR1

Figure VI-9: The mouse gene KE4 rescues the iar1 IAA-Ala resistant phenotype

Figure VI-10: IAR1 does not rescue the yeast zrt1 zrt2 mutant

Figure VI-11: Results of atomic absorption analysis on Col-0 and iar1

Figure VI-12: Effects of metal ions on growth of Arabidopsis seedlings

Figure VI-13: iar1 mutants are neither resistant nor supersensitive to zinc

Figure VI-14: iar1 mutants may be hypersensitive to silver

Figure VI-15: Copper and zinc inhibit the IAA-Ala hydrolyzing activity of the IAR3 enzyme

CHAPTER VII: MUTANT CHARACTERIZATION AND CLONING OF THE FKF1 GENE

Figure VII-1: The fkl mutant is late flowering in long days

Figure VII-2: fkl mutants are late flowering in long days
Figure VII-3: The late-flowering phenotype of *fkfl* is rescued by vernalization or gibberellin treatment

Figure VII-4: *fkfl* has a short hypocotyl in the light

Figure VII-5: Positional cloning of *FKFL*

Figure VII-6: *FKFL* encodes a putative flavin-binding, kelch-domain, F-box protein

**CHAPTER VIII: MOLECULAR CHARACTERIZATION OF FKFL**

Figure VIII-1: Specificity of *FKFL* and *ZTL* expression

Figure VIII-2: Effects of constitutive expression of *ZTL* on hypocotyl elongation

Figure VIII-3: PhyA degradation rates are not altered in the *fkfl* mutant

Figure VIII-4: *FKFL* transcripts oscillate with a circadian rhythm

Figure VIII-5: Certain clock-controlled transcript oscillations are altered in the *fkfl* mutant
ABBREVIATIONS

2,4-D 2,4-dichlorophenoxyacetic acid
AAO ascorbic acid oxidase
ARF auxin response factor
AuxRE auxin responsive element
BAC bacterial artificial chromosome
bp base pairs
CaMV Cauliflower Mosaic Virus
CAPS cleaved amplified polymorphic sequence
CDF cation diffusion family
cM centimorgan
Col-0 Columbia
EMS ethyl methanesulfonate
FMN flavin mononucleotide
GA gibberellin/gibberellic acid
GC-MS gas chromatography-mass spectroscopy
GFP green fluorescent protein
GTPC1 GTP cyclohydrolase 1
GUS β-glucuronidase
His histidine
HFCA 2-chloro-p-hydroxyflourene-9-carboxylic acid
IAA indole-3-acetic acid
IBA indole-3-butyric acid
IAN indole-3-acetonitrile
IAA-Ala IAA-alanine
IAA-Asp IAA-aspartic acid
IAA-Gly IAA-glycine
IAA-Ile IAA-isoleucine
IAA-Leu IAA-leucine
IAA-Phe IAA-phenylalanine
IAA-Val IAA-valine
IAIns IAA-mpo-inositol
IAInsgal IAA-mpo-inositol-galactose
JA jasmonic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>JA-Ala</td>
<td>JA-alanine</td>
</tr>
<tr>
<td>JA-Phe</td>
<td>JA-phenylalanine</td>
</tr>
<tr>
<td>JA-Leu</td>
<td>JA-leucine</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LD</td>
<td>long day</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg erecta</td>
</tr>
<tr>
<td>LOV</td>
<td>light, oxidation, voltage</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>MeJA</td>
<td>methyl jasmonate</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NAA</td>
<td>2-naphthylacetic acid</td>
</tr>
<tr>
<td>NPA</td>
<td>1-N-naphthylphthalmic acid</td>
</tr>
<tr>
<td>PNS</td>
<td>plant nutrient sucrose</td>
</tr>
<tr>
<td>PP2A-A</td>
<td>protein phosphatase 2A regulatory subunit A</td>
</tr>
<tr>
<td>SAM</td>
<td>shoot apical meristem</td>
</tr>
<tr>
<td>SD</td>
<td>short day</td>
</tr>
<tr>
<td>SSLP</td>
<td>simple sequence length polymorphism</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TIBA</td>
<td>2,3,5-triiodo-benzoic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>UBQ</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>Ura</td>
<td>uracil</td>
</tr>
<tr>
<td>UV-A</td>
<td>ultraviolet A</td>
</tr>
<tr>
<td>WS</td>
<td>Wassilewskija</td>
</tr>
<tr>
<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
</tbody>
</table>
Chapter I: Auxin

In 1880, Charles Darwin and his son Francis observed that the tip of a growing shoot controlled the growth rate of a region some distance away. They hypothesized that some "influence" was moving from the tip to the growing region (Bandurski and Nonhebel, 1984). This influence was later identified as the plant hormone auxin. There are several naturally occurring auxins, including indole-3-acetic acid (IAA - see figure I-1), indole-3-butyric acid (IBA), 4-chloro-IAA, and others (Bandurski et al., 1995). IAA is the predominant form of auxin in most plants (Davies, 1995).

![Auxin molecule]

Figure I-1: Structure of indole-3-acetic acid

I.A. Auxin effects

Auxin is involved in many developmental processes in plants, including cell enlargement and division, vascular tissue differentiation, root initiation, apical dominance, and tropic responses such as gravitropism and phototropism (Davies, 1995; Estelle and Klee, 1994). The hormone can have either stimulatory or inhibitory effects on growth (Davies, 1995). Furthermore, the effects of auxin can vary in different plant species and even within the same species at different points in development (Bandurski and Nonhebel, 1984). It is likely that plants cannot survive without auxin, as nearly all plant cell cultures require it for growth and no auxin-deficient plant mutants have been identified (Gray and Estelle, 1998).
The mechanisms through which auxin exerts its effects are poorly understood. At the cellular level, the hormone is implicated in cell elongation (Cleland, 1995) and cell division (Aloni, 1995; Krikorian, 1995). Some of the earliest molecular responses to auxin include changes in plasma membrane H^+-ATPase activity, ion channel function, plasma membrane potential, and gene expression (Hobbie, 1998). Genes rapidly upregulated by the hormone include the SAURs, certain members of the Aux/IAA gene family, and others (Hobbie, 1998). Among genes upregulated more slowly by auxin are those encoding cell-cycle regulatory proteins and enzymes involved in cell wall metabolism (Hobbie, 1998). Genetic approaches currently being used to investigate this complex system will undoubtedly shed light on how these and other auxin effects combine to mediate a multitude of developmental processes in plants.

I.B. Arabidopsis as a model system for genetic studies of auxin effects

*Arabidopsis thaliana* is a widely used model system for genetic studies of plant processes. It is a small, diploid, rapidly flowering plant that can be grown inexpensively in the laboratory. It has a small nuclear DNA content with low amounts of repetitive DNA (Meyerowitz, 1992), which allows isolation of genes identified by mutation using positional cloning (Gibson and Somerville, 1992). The analysis of genes identified by function or homology is made easier because most of the nuclear genes are present in single copy or in small gene families (Meyerowitz, 1992). Furthermore, the sequence of the Arabidopsis genome is rapidly nearing completion. The entire sequence of two of the five Arabidopsis chromosomes was recently reported (Lin et al., 1999; Mayer et al., 1999), and the remainder of the genome will be sequenced soon, which will further facilitate gene cloning and analysis. For these reasons, Arabidopsis provides an excellent model system for the genetic analysis of IAA metabolism.
I.C. Genetic analysis of auxin response mutants of Arabidopsis

The identification of mutants with altered responses to IAA or IAA metabolites is an approach that has been successful in revealing information about this complex process. Many mutants resistant to the effects of exogenously applied auxins have been identified (Table I-1). Mutants thought to be involved in signal transduction will be discussed in this section; those involved in other processes, such as auxin biosynthesis and transport, will be discussed in the section on regulation of free IAA levels, Section I.D.

I.C.1. Aux/IAA gene mutants

Aux/IAA genes were first identified in peas on the basis of their rapid upregulation in response to application of exogenous auxin (Theologis et al., 1985). In Arabidopsis, approximately 20 Aux/IAA genes have been identified (Abel et al., 1995; Kim et al., 1997). Aux/IAA genes are expressed in various organs and have different profiles of auxin induction, ranging from rapid and strong to slow and weak upregulation (Abel et al., 1995). Expression of some Aux/IAA genes is also induced by treatment of plants with cycloheximide, an inhibitor of protein synthesis, which suggests the presence of short-lived repressors of Aux/IAA transcription (Theologis et al., 1985). It is theorized that the Aux/IAA proteins, in concert with other proteins such as the ARFs (auxin response factors), may be involved in auxin-responsive gene regulation. ARFs are transcriptional regulatory proteins that bind as dimers to auxin responsive elements (AuxREs) found in the promoters of many early and middle auxin response genes (Guilfoyle et al., 1998). Aux/IAA proteins can dimerize either with other Aux/IAA proteins or with ARFs (Guilfoyle et al., 1998). These interactions may mediate early and middle auxin responses. While the exact mechanisms of Aux/IAA action currently remain a mystery, their involvement in auxin-mediated processes is supported by evidence gained from the phenotypes of plants mutated in Aux/IAA genes.
Table I-1: Genes and mutants in auxin metabolic processes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cloned?</th>
<th>Mutant?</th>
<th>Gene Function:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conjugate Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILR1</td>
<td>YES</td>
<td>YES</td>
<td>hydrolase</td>
</tr>
<tr>
<td>ILL1</td>
<td>YES</td>
<td>NO</td>
<td>hydrolase</td>
</tr>
<tr>
<td>ILL2</td>
<td>YES</td>
<td>NO</td>
<td>hydrolase</td>
</tr>
<tr>
<td>IAR3</td>
<td>YES</td>
<td>YES</td>
<td>hydrolase</td>
</tr>
<tr>
<td>IAR4</td>
<td>NO</td>
<td>YES</td>
<td>unknown</td>
</tr>
<tr>
<td>FASS</td>
<td>NO</td>
<td>YES</td>
<td>unknown -- conjugation?</td>
</tr>
<tr>
<td>ILR2</td>
<td>NO</td>
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</tr>
<tr>
<td>ICR1</td>
<td>NO</td>
<td>YES</td>
<td>unknown</td>
</tr>
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<td>YES</td>
<td>PP2A phosphatase</td>
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The dominant auxin response mutant *axr3* (auxin resistant) is defective in the Aux/IAA gene *IAA17* (Rouse et al., 1998). *axr3* mutants are resistant to auxin and ethylene, have agravitropic roots, develop extra adventitious roots, display increased apical dominance, and ectopically express an auxin-induced reporter gene (Leyser et al., 1996). These phenotypes are consistent with an overresponse to auxin and provided the first direct evidence that Aux/IAA genes play a central, important role in auxin responses. The first *axr3* mutants isolated had dominant, gain-of-function mutations in a conserved domain of the encoded protein (Leyser et al., 1996). Because at least some Aux/IAA proteins are degraded rapidly (Abel et al., 1994), these dominant mutations may lead to increased AXR3 protein stability and therefore overactive AXR3 protein. Loss-of-function *axr3* mutants have much more subtle phenotypes than the gain-of-function mutants (Rouse et al., 1998), which may result from functional redundancy in the gene family.

Mutations in Aux/IAA genes in addition to *AXR3/IAA17* have also been identified. These mutants have varied and pleiotropic phenotypes, indicating that Aux/IAA proteins are involved in multiple auxin responses. All Aux/IAA gene mutants identified in primary screens have been gain-of-function mutants with alterations in the same conserved domain of the encoded protein (Nagpal et al., 2000; Rogg et al., 2000; Rouse et al., 1998; Tatematsu et al., 1999; Tian and Reed, 1999). Therefore, the phenotypes of these mutants are attributed to the overactivity of the encoded protein. For example, the shy2 mutant, originally isolated as a suppressor of the hypocotyl elongation phenotype of *phyB* (Kim et al., 1996; Reed et al., 1998), is defective in *IAA3*. shy2 plants exhibit many phenotypes that can be attributed to a disturbance in auxin signaling, including slight resistance to multiple phytohormones, reduced apical dominance, hyponastic leaves, and slightly agravitropic roots (Tian and Reed, 1999). Furthermore, they have fewer lateral roots than wild type, although they are able to induce lateral root formation in response to exogenous auxin (Rogg et al., 2000). *axr2* mutants also exhibit auxin-related defects such as lack of root hairs, extremely
agravitropic roots and shoots, and dwarfism (Timplte et al., 1994; Wilson et al., 1990). This mutant is defective in the Aux/IAA gene IAA7 (Nagpal et al., 2000). A mutant with gravity defects in its hypocotyls was found to have lesions in IAA19. This is msg2, which also has auxin resistant hypocotyls and weak phototropic responses (Tatematsu et al., 1999).

A particularly interesting Aux/IAA gene mutant has recently been characterized. iaa28 mutants are resistant to various phytohormones, including auxin, cytokinin, and an ethylene precursor. iaa28 plants have no overt gravity response defects, which makes them unique among the Aux/IAA mutants identified to date. Another intriguing aspect of the mutant phenotype is the reduced ability of iaa28 plants to make lateral roots, even in the presence of exogenous auxin. Adult plants are semi-dwarf and dark green, and make multiple secondary inflorescences which are much shorter than those of wild-type plants (Rogg et al., 2000). Preliminary evidence suggests that IAA28 expression may be downregulated in response to auxin (L. Rogg and B. Bartel, unpublished), rather than upregulated like the other Aux/IAA genes for which induction kinetics have been examined (Abel et al., 1995). This suggests that IAA28 normally functions as a repressor of the auxin response.

I.C.2. Genes involved in ubiquitination

A number of auxin response genes have been identified that are involved in ubiquitin-related processes. Ubiquitin (UBQ) is a highly conserved 76-amino acid protein found in all eukaryotic cells. Attachment of UBQ to a protein targets it for degradation by the 26S proteasome (Hochstrasser, 1995). The first step in this process is activation of UBQ by an E1 ubiquitin-activating enzyme. UBQ is then transferred to an E2 ubiquitin-conjugating enzyme, and is then attached to the target protein either directly or via an E3 ligase complex (Hochstrasser, 1995). The exact role of ubiquitination in auxin responses is unclear, but the ubiquitination machinery may target negative regulators of these responses for degradation. The specific targets for degradation are unknown, but proposed targets
include the Aux/IAA proteins (Gray et al., 1999), which may repress their own transcription and/or the transcription of other auxin response genes.

The phenotypes of the axr1 mutant (semi-dwarf, reduced apical dominance, reduced root gravitropism) are consistent with a decrease in auxin sensitivity and indicate that the AXR1 gene plays a fundamental role in the auxin response (Lincoln et al., 1990). AXR1 is similar to the N-terminal region of an E1 ubiquitin-activating enzyme (Leyser et al., 1993) and acts with ECR1, an Arabidopsis protein similar to the C-terminal portion of E1, to activate RUB1, a ubiquitin-related protein (delPozo et al., 1998). In yeast, Rublp may modulate the activity of SCF<sup>Cdc4</sup>, the E3 involved in ubiquitination of Sic1p. Sic1p is an inhibitor of CDK at the G<sub>1</sub> to S transition (Bai et al., 1996). Therefore, the AXR1/ECR1-mediated activation of RUB1 in Arabidopsis may serve in a similar regulatory role in auxin responses (figure I-2).

Mutants in the TIR1 gene also have reduced auxin responses (Ruegger et al., 1998). This gene encodes a protein which is similar to the yeast protein Grrlp and the human SKP2, and contains leucine rich repeats (LRRs) and an F-box (Ruegger et al., 1998). F-box proteins function in the SCF (Skp<sub>1</sub> Cdc53 F-box) ubiquitin ligase complex and give the complex its specificity toward particular targets (Patton et al., 1998). The F-box domain is thought to interact with Skp1 (Bai et al., 1996). Indeed, the F-box domain of TIR1 interacts with ASK1 and ASK2, Arabidopsis proteins related to Skp1. TIR1, along with the ASK proteins and a Cdc53-related protein, CUL1, forms an SCF-like complex (SCF<sup>TIR1</sup>) in plants (Gray et al., 1999). Additionally, mutations in the ASK1 gene result in an auxin resistant phenotype, further strengthening the evidence for SCF<sup>TIR1</sup> involvement in auxin responses (Gray et al., 1999). SCF<sup>TIR1</sup> may be the target of RUB1 modification mediated by AXR1/ECR1, and may be responsible for targeting negative regulators of auxin action for degradation by the ubiquitin pathway (figure I-2).
Figure I-2: Model for the function of AXR1 and TIR1 in auxin responses. See text for details. Modified from Gray et al., 1998.
Mutations in *sar1* suppress the *axr1* mutant phenotype (Cernac et al., 1997). This suggests that *SAR1* acts downstream of *AXR1* in the auxin response pathway. The gene mutated in *sar1* has yet to be identified, but cloning the *SAR1* gene will likely illuminate further the role of the ubiquitin pathway in auxin responses.

I.D. Regulation of free IAA levels

The central role of auxin in plant growth and development demands that plants have mechanisms to tightly regulate auxin levels. Levels of free, active IAA can be controlled by synthesis and degradation, by transport to and from specific sites, and by the formation or hydrolysis of inactive, conjugated forms of the hormone (Bandurski et al., 1995). Furthermore, zinc may play a role in determining levels of free IAA in plants (Cakmak et al., 1989; Hossain et al., 1997; Skoog, 1940; Tsui, 1948).

I.D.1. Synthesis

The exact pathways through which the *de novo* synthesis of IAA is carried out remain unclear, although it is likely that multiple pathways exist for auxin biosynthesis in plants. The different pathways may function at various times in development (Michalczuk et al., 1992), in different tissues (Koshiba et al., 1995; Müller et al., 1998) or in response to certain environmental conditions. For many years, the prevailing belief was that tryptophan (Trp) served as the major precursor for IAA and that IAA biosynthesis occurred via one of three Trp-dependent pathways: the indole-3-pyruvate pathway, the tryptamine pathway, and the indole-3-acetaldoxime pathway. Many experiments demonstrate the conversion of Trp to IAA (reviewed in Nonhebel et al., 1993). For example, cells in culture or excised portions of plants convert exogenously supplied, labeled Trp into IAA (Koshiba et al., 1995; Michalczuk et al., 1992; Ribnicky et al., 1996). However, there are several problems with Trp as the precursor of IAA. For example, Trp can be converted nonenzymatically to IAA
during routine handling. Also, in early experiments, the amounts of IAA produced from Trp were not carefully examined, making it difficult to determine if they were physiologically significant (reviewed in Normanly et al., 1995). New methods for quantifying IAA levels, such as stable isotope dilution GC-MS, have helped shed new light on the problem. Recent evidence suggests that there are indeed multiple routes for IAA biosynthesis (reviewed in Normanly et al., 1995), including Trp-dependent and -independent pathways and that a Trp precursor, rather than Trp itself, serves as the major IAA precursor in plants (reviewed in Bandurski et al., 1995; Normanly et al., 1995). A great deal of this evidence has been gained through the study of tryptophan auxotrophic mutants (reviewed in Bartel, 1997).

Several auxin overproducing mutants have been identified, among them trp2 and trp3. These mutants are defective in the last two steps of tryptophan biosynthesis (figure I-3) and accumulate high levels of IAA conjugates and the possible IAA precursor indole-3-acetonitrile (IAN)(Normanly et al., 1993). The fact that mutants defective in Trp synthesis have higher, rather than lower, amounts of total IAA points to a Trp-independent route for IAA biosynthesis. The accumulation of IAN in these plants indicates that this is a possible intermediate in IAA formation. Interestingly, the trp2 and trp3 mutants have normal levels of free IAA. Furthermore, the trp1 mutant, which is defective in an early step in the conversion of chorismate to Trp (figure I-3), accumulates anthranilate (Last and Fink, 1988) but has normal levels of IAA and IAA conjugates (Normanly et al., 1993). This suggests that the primary IAA precursor in plants is not Trp, but rather an intermediate between anthranilate and Trp. The trp5/amt-1 mutant is defective in feedback inhibition by Trp of anthranilate synthase, which converts chorismate to anthranilate. These mutants accumulate high levels of Trp (Li and Last, 1996) and total IAA (Ludwig-Müller et al., 1993), although levels of free IAA remain relatively normal (Ludwig-Müller et al., 1993).

Another clue to the auxin biosynthesis puzzle is provided by the Arabidopsis mutant rty, which has also been isolated as sur1, ivr, alfl, and hls1, and overproduces both free and
Figure I-3: Proposed pathways for IAA biosynthesis. Arabidopsis or maize mutants at various steps are shown in italics. Single arrows do not necessarily indicate single enzyme processes; hypothetical conversions are indicated by grey arrows. Modified from Bartel, 1997.
conjugated IAA (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995; Lehman et al., 1996). rty plants display phenotypes that can be copied by growing wild type plants on high auxin: increased numbers of adventitious and secondary roots, epinastic cotyledons and leaves, and sterility (Boerjan et al., 1995; King et al., 1995; Lehman et al., 1996). The gene defective in the rty mutant encodes a tyrosine aminotransferase (Gopalraj et al., 1996). This aminotransferase, directly or indirectly, inhibits accumulation of free IAA, although the mechanism by which this control occurs is still unknown.

I.D.2. Transport

Auxin transport can occur via either an active, energy-dependent, polar mechanism or a passive, nonpolar mechanism. Nonpolar auxin transport occurs in the vasculature of the plant, whereas polar auxin transport is extravascular (Normanly, 1997). It is polar auxin transport that is implicated in the control of various auxin-mediated processes, such as gravitropism and phototropism, elongation growth, and lateral root development (reviewed in Lomax et al., 1995).

Polar auxin transport was initially identified in excised plant sections by applying auxin to one end of the section and examining the amount of auxin transported through the tissue. Auxin is transported in a basipetal fashion (reviewed in Goldsmith, 1977) at a rate of approximately 5 to 10 millimeters per hour (Bennett et al., 1998). Transport may be driven by the proton motive force across the plasma membrane. In the intracellular space, where the pH is about 5.5, IAA is more protonated than inside the cell, where the pH is about 7.0. Protonated IAA can move into the cell, where it dissociates and accumulates as the anionic form, which is less able to cross the cell membrane. This is known as the chemiosmotic hypothesis of auxin transport (reviewed in Lomax et al., 1995). Auxin is also imported through a saturable 2H⁺/IAA⁻ symporter distributed more or less evenly about the cell membrane (reviewed in Lomax et al., 1995). The polar nature of auxin transport is
attributed to a basally-localized efflux carrier. The efflux carrier has been proposed to consist of at least three components: a transmembrane carrier protein, a phytotropin binding protein, and a third, labile component (reviewed in Bennett et al., 1998).

Polar auxin transport inhibitors are useful tools in the study of the auxin transport mechanism. These molecules act to block auxin efflux rather than influx (Lomax et al., 1995). The transport inhibitors NPA (1-N-naphthylphthalamic acid), a phytotropin, and quercetin, a flavinoid, are thought to block efflux via their affinity for the phytotropin binding component of the efflux carrier complex (Bennett et al., 1998). These compounds have been widely used in the biochemical analysis of auxin transport. More recently, they have been employed in genetic screens aimed at identifying components of the auxin transport machinery.

*tir3* was isolated as a transport inhibitor resistant mutant. These plants exhibit decreased apical dominance, short roots and stems, and reduced lateral root formation, which are all auxin-related phenotypes. Polar auxin transport and NPA binding are significantly reduced in this mutant (Ruegger et al., 1997), suggesting that *TIR3* encodes the phytotropin-binding component of the auxin efflux carrier. Another Arabidopsis mutant with altered responses to auxin transport inhibitors is *rcn1*. *rcn1* mutants, unlike wild type, exhibit root curling in the presence of NPA, are hypersensitive to NPA in hypocotyl elongation, and display increased sensitivity to NPA in auxin efflux. The gene defective in *rcn1* was cloned by transposon tagging and encodes a protein phosphatase 2A regulatory subunit A (PP2A-A) (Garbers et al., 1996). This PP2A-A may control the phosphorylation level and thereby the activity of a component of the auxin efflux system, although the target of RCN1 activity is currently unknown. The *pis1* mutant is thirty-six times more resistant to NPA than wild type (Fujito and Syono, 1997). Additionally, *pis1* is more sensitive to the effects of the synthetic auxin 2,4-D, a substrate of the influx carrier, on root elongation. This suggests that PIS1 might negatively regulate both the influx and efflux carriers, or that the accumulation of auxin in a cell affects the activity of PIS1 (figure I-4).
Figure I-4: Proposed mechanisms of polar auxin transport in Arabidopsis. Various auxins enter the cell through the influx carrier, AUX1 (Bennett et al., 1996), and exit the cell via a basally-localized efflux carrier. In roots, the transmembrane portion of the efflux carrier complex for IAA and 1-NAA is EIR1/PIN2/AGR1 (reviewed in Dolan, 1998); in shoots it is proposed to be PIN2 (Gälweiler et al., 1998, Steinmann et al., 1999). Other forms of auxin, such as IBA and 2,4-D, may exit the cell via a separate carrier. TIR3 (Ruegger et al., 1997) may be a component of the efflux carrier. PIS1 may negatively regulate both influx and efflux carriers (Fujito and Syono, 1997), and RCN1 may activate the efflux carrier (Garbers et al., 1996).
Further insight into the molecular mechanisms of polar auxin transport has been provided by mutants identified in various other types of screens. The aux/l mutant was initially identified due to its resistance to high concentrations of exogenous auxin (Maher and Martindale, 1980). Additional aux/l alleles were isolated based on resistance to NPA (Fujito and Syono, 1996) or ethylene (Pickett et al., 1990). The AUX/l gene encodes a transmembrane protein with similarity to amino acid permeases and is thought to encode the auxin influx carrier because auxin uptake in aux/l roots is significantly reduced (Bennett et al., 1996; Marchant et al., 1999). The substrates for this carrier include IAA and 2,4-D (Pickett et al., 1990), and preliminary evidence suggests that other forms of auxin, such as IBA and IAA conjugates, are also transported by AUX/l (LeClere, Zolman, and Bartel, unpublished data).

Mutants in the ERI/l/ PIN/l/ AG/l gene, which encodes a transmembrane protein with homology to bacterial transporters, are defective in root gravitropism, resistant to ethylene, and resistant to auxin transport inhibitors, but remain sensitive to auxin (reviewed in Dolan, 1998). Expression of ERI/l in yeast confers resistance to fluroindoles, which are toxic auxin-related compounds (Luschnig et al., 1998). Therefore, it is likely that ERI/l encodes a component of the auxin efflux carrier in roots. A homolog of ERI/l, PIN/l, is proposed to encode the transmembrane protein of the auxin efflux carrier complex in the shoot (Gälweiler et al., 1998; Steinmann et al., 1999).

Polar auxin transport may employ more than one efflux carrier. Roots of eir/l mutants, unlike those of wild-type plants, turn and enter vertically oriented agar containing IAA or NAA. This suggests that cells in contact with the agar take up IAA or NAA but do not efflux it, resulting in differential inhibition of growth leading to root bending (Utsuno et al., 1998). eir/l mutants do not display this response on media containing 2,4-D (Utsuno et al., 1998) or IBA (Zolman and Bartel, unpublished data), suggesting that these auxins are able to exit the cell via another mechanism. This mechanism may be passive or involve the activity of another, as yet undescribed, auxin efflux carrier (figure I-4). Interestingly, the
*EIR1/AGRI/PIN* gene family in Arabidopsis contains about ten members (Gälweiler et al., 1998).

**I.D.3. Conjugation**

The discovery of conjugated, or bound, auxins occurred in the 1930s. In 1934, Thimann proposed that some of the IAA in plants is bound (Thimann, 1934). In 1941, it was demonstrated that most IAA in plants is present in bound form (van Overbeek, 1941). In fact, about 95% of the IAA in a plant is present in conjugated form (Cohen and Bandurski, 1982). These conjugates fall into two general classes: esters, in which IAA is conjugated to another molecule through a carbon-oxygen-carbon bridge, and amides, where IAA is conjugated via a carbon-nitrogen-carbon bridge (Bandurski et al., 1995; figure I-5).

![Chemical structures](image)

Figure I-5: Structures of a) IAA-*myo*-inositol, an ester-linked conjugate of IAA, and b) IAA-alanine, an amide-linked conjugate of IAA

**I.D.3.a. Conjugates in plants**

Many experiments have identified conjugates formed when exogenous IAA is added to plants. The first conjugate to be chemically identified was indoleacetyl-aspartic acid (IAA-Asp), which is formed upon application of IAA to pea stems and roots (Andrae and Good, 1955). In 1961, Zenk found that IAA could be esterified to IAA-glucose when
applied to some plants (Bandurski and Nonhebel, 1984). Additionally, when the synthetic auxin naphthalene acetic acid (NAA) is incubated with tobacco protoplasts, it is converted to NAA-glucose and NAA-aspartic acid (Delbarre et al., 1994). In fact, plants ranging from mosses to hornworts to angiosperms metabolize exogenously applied auxin to form conjugates (Sztein et al., 1995). IAA-Asp may function as an intermediate in IAA destruction (Tsurumi and Wada, 1986; Tuominen et al., 1994), and this conjugate is formed in many plants following feeding of IAA (Barratt et al., 1999; Cohen and Bandurski, 1982; Östín et al., 1998). Arabidopsis has also been shown to form the conjugates IAA-glutamate, IAA-glucose, and IAA-glutamine upon feeding of IAA (Barratt et al., 1999; Östín et al., 1998).

Plants contain IAA conjugates even when they have not been exposed to exogenous IAA. The endogenous conjugates of the *Zea mays* (corn) kernel have been studied extensively. Labarca et al. (1965) identified four chromatographically distinct water-soluble esters from corn kernels, two of which contained IAA and myo-inositol whereas the other two contained IAA and arabinose (Labarca et al., 1965). One of these esters was later crystallized and more precisely identified as indole-3-acetyl-2-O-myoinositol (Nicholls, 1967). About half of the conjugates in corn kernels are water insoluble, high molecular weight esters; the remainder are water soluble esters such as IAA-myoinositol (IAInos) and IAA-myoinositol-galactose (IAInosgal) (Bandurski and Nonhebel, 1984). Only about 1% of the IAA in the kernel is free IAA (Cohen and Bandurski, 1982). In addition to being present in the corn kernel, IAInos is present in the shoots and other vegetative tissues of *Zea mays* (Chisnell, 1984; Chisnell and Bandurski, 1982), and in kernels of rice (Hall, 1980).

Endogenous amide conjugates that have been identified include IAA-Asp and IAA-glutamate, both of which are present in soybean seeds, cucumber, and tobacco (Cohen and Bandurski, 1982; Epstein et al., 1984; Sitbon et al., 1993; Sonnner and Purves, 1985). A number of other conjugates have been identified as IAA metabolites, including IAA-Ala, IAA-Leu, and IAA-Phe (Sztein et al., 1995). Additionally, Bialek and Cohen reported the
isolation of a conjugate from *Phaseolus vulgaris* which consists of a three kilodalton peptide with two IAA moieties in amide linkage (Bialek and Cohen, 1986).

Many IAA conjugates are biologically active. The IAA-2-O-*myo*-inositol ester from *Zea mays* kernels stimulates growth in flax callus culture and *Avena* coleoptile straight growth tests (Nicholls, 1967). In 1977, Feung et al. tested the biological activity of 20 L-α-amino acid conjugates on cell elongation of *Avena* coleoptile sections and in soybean cotyledon cultures. Most amino acid conjugates stimulate growth in both assays. The activity of IAA conjugates is dependent on the conjugating moiety. The most active conjugates include those with alanine, cysteine, lysine, serine, methionine, glycine, and aspartic acid whereas conjugates containing phenylalanine, histidine, and arginine were inactive (Feung et al., 1977). IAA conjugates are also active in callus induction of tomato and tobacco tissue cultures (Hangarter et al., 1980). IAA-L-Asp promotes growth of soybean hypocotyls at almost the same rate as IAA, while the D enantiomer is nearly inactive (Cohen and Baldi, 1983). Activity of amino acid conjugates also decreases with increasing size (Soskic et al., 1995).

Biologically active conjugates such as IAA-alanine (IAA-Ala) are metabolized in tissue culture to nearly the same products as free IAA, while inactive conjugates are not metabolized to these products. This led to the conclusion that IAA conjugates are hydrolyzed to yield free IAA and that their biological activity is related to the rate of hydrolysis (Hangarter and Good, 1981). When IAA amino acid conjugates are applied to excised bean sections, the rate of curvature of the section and therefore the biological activity of the conjugate is directly related to the amount of free IAA that is recovered from the section (Bialek et al., 1983). This further supports the idea that the activity of IAA conjugates is dependent on their hydrolysis to yield free IAA.
I.D.3.b. Proposed functions of conjugates

I.D.3.b.i. Storage

IAA conjugates have been proposed to function in several ways. Because IAA conjugates can be hydrolyzed to yield free, active IAA (Hangarter and Good, 1981), they may serve as hormone storage forms. In *Phaseolus vulgaris*, IAA-amide conjugates represent about 80% of the total IAA in the mature seed, which correlates with the amount of free and ester conjugated IAA lost during seed maturation (Bialek and Cohen, 1989). During germination in *Zea mays* and rice, there is a large loss of IAA conjugates from the seed (Cohen and Bandurski, 1982). The level of amide conjugates in seeds of *Phaseolus vulgaris* also drops substantially at the start of germination (Bialek and Cohen, 1992). Experiments with radiolabeled IAIInos in germinating *Zea mays* showed that esters of IAA are the immediate precursors of IAA in the shoot (Epstein et al., 1980), and that they are transported into the shoot at a rate sufficient to meet the need of the shoot for IAA (Nowacki and Bandurski, 1980). This evidence suggests that IAA conjugates do indeed function in IAA storage.

I.D.3.b.ii. Transport

Conjugates have also been implicated in the transport of IAA. When $^{14}$C-IAIInos was applied to the cut surface of the endosperm of a corn kernel, it was transported into the shoot of the germinating seedling 400 times faster than $^{14}$C-IAA. Furthermore, the IAA was most likely transported in conjugated form because 50% of the radioactivity in the shoot was still found as IAIInos (Nowacki and Bandurski, 1980). In soybean seedlings, significant amounts of radioactivity were found in the hypocotyl four hours after application of $^{14}$C-IAA-Asp to the cotyledons. Most of this radioactivity was no longer in IAA-Asp or IAA, however, but most likely in catabolic products (Cohen and Baldi, 1983). IAA-*myo*-inositol-galactose (IAInosgal) in *Zea mays* is hydrolyzed to IAIInos before being
transported into the shoot, indicating that different conjugates are transported differently (Komoszynski and Bandurski, 1986).

I.D.3.b.iii. Hormonal homeostasis

In addition to functioning in storage and transport, IAA conjugates are involved in protecting IAA from destruction and in maintaining hormonal homeostasis. Free IAA is very susceptible to oxidative attack. It is destroyed by horseradish peroxidase and peroxidases isolated from Zea mays and Pisum, but IAA conjugates endogenous to these plants are protected from degradation (Cohen and Bandurski, 1978). This is important in maintaining a readily available supply of the hormone, so that when IAA is needed it will not have to be synthesized de novo.

Conjugation may also be a mechanism by which plants permanently inactivate excess IAA. The conjugate IAA-Asp is an important intermediate in non-decarboxylative IAA catabolism in many plant species (reviewed in Normanly, 1997). Arabidopsis fed exogenous IAA convert the hormone to, among other products, IAA-Asp. IAA-Asp is oxidized on the indole ring, which renders it permanently inactive (Östlin et al., 1998). Oxidized IAA-Asp may be further metabolized to ring-hydroxylated forms (Östlin et al., 1998; Tuominen et al., 1994) as part of the catabolic process.

Hormonal homeostasis is defined as “maintenance of a steady state concentration of hormone in receptive tissue appropriate to any fixed environmental condition” (Cohen and Bandurski, 1982). IAA conjugates provide a mechanism for the plant to maintain this homeostasis by inactivating excess hormone and by providing a supply of IAA that can be quickly freed when needed.

I.D.3.c. Molecular basis of conjugate metabolism

While conjugation and deconjugation are likely mechanisms for the regulation of free IAA levels, the characterization of the enzymes and genes involved in these processes is
just beginning. Only one plant gene involved in conjugate formation has been identified. This is the *iaglu* gene from maize, which encodes an enzyme that esterifies IAA to glucose (Szerszen et al., 1994). Additionally, an Arabidopsis mutant that may be defective in the formation of IAA-amide conjugates has been identified. This mutant, *fass*, has two-fold less amide-linked IAA than wild type, has normal levels of ester-linked IAA, and accumulates higher than normal levels of free IAA (Fisher et al., 1996). The gene defective in the *fass* mutant has not yet been cloned. Another mutant with potential defects in conjugation is *sur2* (Delarue et al., 1998). This mutant produces adventitious roots, and some plants show increases in free IAA coupled with decreases in conjugated IAA (Delarue et al., 1998). The identity of the *SUR2* gene is also not yet reported.

IAA conjugate hydrolysis activity has been observed in a wide variety of plant species. Enzymes that hydrolyze IAA-glucose have been identified in maize, potato, oats, and beans (Jakubowska et al., 1993; Kowalczyk and Bandurski, 1990). Hydrolases specific for IAA-Ala have been partially purified from beans and carrots (Cohen et al., 1988; Kuleck and Cohen, 1993), and extracts of Chinese cabbage contain isozymes that will hydrolyze IAA-Ala, IAA-aspartic acid (IAA-Asp), and IAA-phenylalanine (IAA-Phe) (Ludwig-Mueller et al., 1996).

Mutants of Arabidopsis have been identified that respond abnormally to IAA conjugates. When grown on high concentrations of certain conjugates, Arabidopsis seedlings have abnormally short roots. Bartel and Fink (1995) screened a population of mutagenized seeds for plants able to grow long roots in the presence of IAA conjugates. They identified a mutant, *ilrl* (IAA-leucine-resistant), that had a long root even in the presence of high concentrations of IAA-leucine (IAA-Leu). Using map-based cloning, they identified the gene defective in the *ilrl* mutant and found it to encode an amidohydrolase specific for IAA-Leu and IAA-Phe, with lower affinity for IAA-Ala, IAA-Gly, and IAA-valine (IAA-Val) (Bartel and Fink, 1995). This was the first gene involved in conjugate hydrolysis to be identified. Three similar genes, *ILL1* (*ILR1*-like), *ILL2* (Bartel and Fink,
1995), and \textit{ILL3} (Davies et al., 1999) were identified based on sequence homology with the \textit{ILR1} gene. Additionally, the gene defective in the \textit{iar3} (IAA-Ala resistant) mutant was also found to encode an amidohydrolase. The IAR3 enzyme is specific for IAA-Ala (Davies et al., 1999). The mutants \textit{ilr2} (Magidin and Bartel, unpublished) and \textit{iar4} (LeClere and Bartel, unpublished) are resistant to IAA-Leu and IAA-Ala, respectively. Two other IAA-conjugate resistant mutants, \textit{icr1} and \textit{icr2}, have been isolated that are resistant to growth inhibition by IAA-Phe, IAA-Ala, and IAA-Gly (Campanella et al., 1996). The genes defective in these mutants remain unidentified.

\textbf{I.D.4. Zinc}

\textbf{I.D.4.a. Zinc transport}

Zinc is an essential micronutrient in all organisms and is involved in a wide variety of processes. It functions as a catalytic component of many enzymes, such as alcohol dehydrogenase, and as a structural component of others, including zinc-finger transcription factors. In yeast, for example, approximately three percent of all proteins contain domains with the potential to bind zinc (Eide, 1998). Because zinc ions are charged and hydrophilic, they must be transported across membranes rather than crossing them by passive diffusion (Guerinot and Eide, 1999).

Studies in yeast have aided in the general understanding of zinc transport. Kinetic studies indicate the presence of at least two uptake systems. The \textit{ZRT1} gene from \textit{Saccharomyces cerevisiae} encodes a transporter of the high-affinity uptake system. Overexpression of this gene causes increased high-affinity uptake, while mutations in the gene lead to elimination of high-affinity uptake. Consequently, the \textit{zrt1} mutant grows poorly on zinc-limited media (Zhao and Eide, 1996a). The related \textit{ZRT2} gene encodes a low-affinity uptake transporter. Mutations in this gene eliminate low-affinity transport, but have no affect on high-affinity transport, indicating that the two pathways are separate.
Finally, the \textit{zrt1/zrt2} mutant is viable, although it has growth defects on zinc-limited media. This indicates the presence of additional zinc uptake pathways in yeast (Zhao and Eide, 1996b). Transcription of both \textit{ZRT1} and \textit{ZRT2} is increased in response to low intracellular zinc concentrations (Zhao and Eide, 1996b; Zhao and Eide, 1997).

Proteins similar to the yeast \textit{ZRT1} and \textit{ZRT2} transporters are present in many different organisms. In fact, \textit{ZRT1} and \textit{ZRT2} were identified based on homology with the Arabidopsis \textit{IRT1} protein, which can transport iron, manganese, and zinc (Eide et al., 1996; Korshunova et al., 1999). Therefore, the family of \textit{ZRT-} and \textit{IRT1}-related proteins is referred to as the ZIP (\textit{ZRT}, \textit{IRT1} protein) family. Members of the ZIP family fall into two general classes based on amino acid sequence. In one class are the \textit{ZRT} proteins from yeast, as well as eleven proteins from plants and one from a protozoan. The second class is comprised of eight \textit{C. elegans} and two human proteins (Eng et al., 1998). Additionally, genes encoding more distantly related proteins have been found in the MHC region of the mammalian genome (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). ZIP family members have between five and eight predicted transmembrane domains and most contain a putative metal-binding motif of the type HXHXH (Eng et al., 1998). In \textit{ZRT1}, this metal binding motif (HDHTHDE) is located between membrane spanning domains III and IV and is predicted to be cytoplasmic (Eide, 1998). The highest homology among family members occurs in and around one of the predicted membrane spanning regions, designated spanner IV after its position in \textit{ZRT1}, \textit{ZRT2}, and \textit{IRT1}. A fifteen residue domain within this region, which contains fully conserved histadyl and glycyl residues, is diagnostic for ZIP family members (figure I-6) (Eng et al., 1998). Spanner IV and spanner V, which also contains a fully conserved histadyl residue, are thought to comprise part of a transmembrane channel through which the substrate metal can pass (Eng et al., 1998).

The Arabidopsis zinc transporters \textit{ZIP1}, \textit{ZIP2}, and \textit{ZIP3} were identified because they rescue the growth defect of the yeast \textit{zrt1 zrt2} mutant (Grotz et al., 1998). These were
Figure I-6: Fifteen amino acid signature sequence of the ZIP family of zinc transporters (Eng et al., 1998). A) Alignment of the ZIP family signature sequence region from Arabidopsis IRT1 (Korshunova et al., 1999), ZIP1, ZIP2, and ZIP3 (Grotz et al., 1998), and S. cerevisiae ZRT1 (Zhao and Eide, 1996a) and ZRT2 (Zhao and Eide, 1996b). B) Alternate residues possible in the fifteen amino acid ZIP motif.
the first zinc transporters to be identified in any plant species, and are members of the ZIP protein family. Zinc deficiency induces expression of the ZIP1 and ZIP2 genes in plant roots. An additional gene, ZIP4, has been identified by comparison to Arabidopsis genomic sequence. ZIP4 transcription is turned on in response to zinc deficiency in both the root and the shoot. ZIP1 and ZIP3 are thought to be responsible for zinc uptake from the soil; ZIP4 may transport zinc into chloroplasts as it contains a putative chloroplast targeting sequence. ZIP2 is predicted to be a plasma membrane protein, butZIP2 expression has not been detected (Grotz et al., 1998).

Genes involved in intracellular zinc transport have also been identified in yeast and plants. In yeast, overexpressing either the ZRC1 or COT1 gene confers resistance to high levels of zinc, while disruption of either gene leads to increased zinc sensitivity. COT1 appears to be localized to the mitochondria, but the localization of ZRC1 is not known (reviewed in Eide, 1998). Both proteins, which are 60% identical, are members of the cation diffusion family (CDF) of transporters. Members of this family, the ZnT proteins, transport zinc in mammalian cells (McMahon and Cousins, 1998), and two of these proteins are implicated in zinc transport into subcellular components (Palmiter et al., 1996a; Palmiter et al., 1996b). A CDF protein has recently been identified in Arabidopsis as well. Plants overexpressing the ZAT (zinc transporter of Arabidopsis thaliana) gene have increased zinc resistance and accumulate high levels of zinc in the roots (van der Zaal et al., 1999), suggesting that ZAT is involved in sequestration of zinc in a subcellular compartment. Intriguingly, CDF proteins contain a histidine-rich (HX)n repeat similar to that found in the ZIP family of zinc transporters (Eide, 1998).

I.D.4.b. Zinc and auxin levels

Zinc deficiency in plants retards growth, including reduced stem elongation and leaf expansion (Sekimoto et al., 1997). These morphological effects have been attributed to perturbed IAA metabolism. A connection between zinc nutrition and auxin levels was
established in the 1940s when Skoog and Tsui (Skoog, 1940; Tsui, 1948) observed that extracts from tomatoes grown under conditions of zinc deficiency had less auxin activity than those from zinc-sufficient plants. In fact, the decrease in auxin activity preceded the appearance of visible symptoms of zinc deficiency (Skoog, 1940). The relationship of zinc nutrition to auxin levels is supported by more recent reports indicating that application of zinc to French bean results in increased auxin content (Garg et al., 1986), and that free IAA levels in zinc-deficient Phaseolus vulgaris are only about 50% of those in control plants (Cakmak et al., 1989). Furthermore, application of zinc to Phaseolus returned IAA levels to normal within 96 hours (Cakmak et al., 1989). Different results have been reported for the effect of zinc on auxin content in radish shoots. Both free IAA (Domingo et al., 1992) and conjugated IAA (Hossain et al., 1998) in zinc-deprived radish shoots are present at fairly normal levels.

Several hypotheses regarding the role of zinc in auxin metabolism have been proposed, such as enhanced oxidation of the free hormone (Skoog, 1940) or disturbances in auxin biosynthesis (Takaki and Kushizaki, 1970; Tsui, 1948) under conditions of low zinc. One proposal is that reduced synthesis of tryptophan, an IAA precursor, is responsible for the reduction in auxin (Tsui, 1948). This is unlikely as many amino acids, including tryptophan, accumulate to high levels under conditions of zinc deficiency in maize (Takaki and Kushizaki, 1970) and bean (Cakmak et al., 1989). Takaki and Kushizaki (1970) suggest a role for zinc in the conversion of tryptophan to IAA. This conversion may be mediated by gibberellins, which are also known to be affected by zinc nutrition (Sekimoto et al., 1997). Another possibility is that auxin transport is somehow affected by zinc levels and that zinc-deficient plants are unable to transport the hormone to sites where it is needed (Cakmak et al., 1989). Other researchers argue that zinc is not required for auxin metabolism but, rather, for auxin activity. This is supported by the observation that radish shoots do not appear to have altered auxin content under conditions of zinc deficiency.
(Domingo et al., 1992; Hossain et al., 1998). However, this does not address the observations of decreased auxin content in other plant species under similar conditions.

It is also possible that other metals are involved in control of auxin homeostasis. For example, aluminum inhibits basipetal transport of IAA in maize roots (Hasenstein and Evans, 1988), which may play a role in aluminum toxicity in root elongation (Kollmeier et al., 2000). Also, preincubation of cultured radish roots in copper-containing medium decreases responses upon auxin application. The copper treatment increases ascorbic acid oxidase (AAO) protein levels in the roots, and the decreased auxin response may be due to oxidative decarboxylation of IAA by AAO (Kerk et al., 2000).

I.E. Goals of this project

Because IAA conjugates likely play an important role in regulating auxin levels within the plant, we are using a genetic approach to identify components involved in conjugate metabolism. Several mutants with altered responses to IAA conjugates have been isolated, and the identification of the genes defective in these mutants is beginning to reveal how IAA-amide conjugates function in maintaining auxin homeostasis. My goal was to clone and begin characterization of the gene defective in the iar1 mutant, a mutant that is resistant to the effects of exogenously supplied IAA conjugates.
CHAPTER II: FLOWERING TIME AND LIGHT PERCEPTION

It is essential that the transition of a plant from a vegetative state to a reproductive (flowering) state take place at the proper time in order to ensure that the next generation will encounter conditions favorable for survival. The floral transition takes place in the shoot apical meristem (SAM), a group of cells that serve as progenitors for the leaves, stems, and flowers of the plant. Environmental and endogenous cues determine when the SAM will undergo the transition from a vegetative to a floral meristem, a change that is mediated by the floral homeotic genes (reviewed in Weigel and Meyerowitz, 1994). The underlying molecular mechanisms that integrate information from environmental and endogenous signals to coordinate this transition are poorly understood. However, rapid advances have been made recently using genetic, biochemical, and physiological analyses of Arabidopsis mutants with altered flowering times. In this chapter, I will discuss advances in the field of flowering time that have been made using Arabidopsis as a model system.

II.A. Factors influencing flowering time

A variety of environmental and internal factors influence when a plant will undergo the transition to flowering. One of these factors is day length. Plants use day length, or photoperiod, as an indicator of season: days are longer in the summer and shorter in the winter. This allows plants to flower only during the time of year that is advantageous to the survival of the next generation. Different species of plants flower under different photoperiods; some are induced by short days (SD) while others are induced by long days (LD). Arabidopsis flowers earlier in long days than in short days, and is therefore considered a facultative long day plant (Rédei, 1962).

Other environmental influences on flowering time are the quality (wavelengths) and quantity (fluence) of light to which a plant is exposed. Blue and far-red light promote
flowering whereas red light inhibits flowering (Eskins, 1992). In Arabidopsis, the perception of light is mediated through various photoreceptors, which will be discussed in section II.D. of this chapter.

Temperature also plays a role in floral initiation. Exposing plants to a cold period (vernalization) simulates winter and promotes flowering (reviewed in Koornneef et al., 1998; Levy and Dean, 1998; Piñeiro and Coupland, 1998; Simpson et al., 1999). Vernalization causes decreased methylation of genomic DNA, and this methylation change may lead to altered transcription of genes involved in floral regulation. This hypothesis is supported by evidence that treatment of plants with demethylating agents can induce flowering and that late flowering mutants respond to such treatment in the same manner as they do to vernalization (reviewed in Koornneef et al., 1998).

The plant hormone gibberellin (GA) also plays a role in floral promotion as mutants disrupted in GA biosynthesis or response have correspondingly altered flowering times (Jacobsen et al., 1996; Jacobsen and Olszewski, 1993; Wilson et al., 1992). Furthermore, exogenous application of GA hastens flowering in Arabidopsis (Bagnall, 1992). Other mutants suggest a role for starch in flowering control: mutants defective in starch biosynthesis or mobilization are late flowering (Caspar et al., 1985; Caspar et al., 1991; Eimert et al., 1995; Lin et al., 1988).

II.B. Identified genes involved in the floral transition

The molecular mechanisms employed by plants to control the time of flowering in response to environmental and internal cues are not yet well understood. However, several components of this process have recently been identified through genetic analysis of late-flowering mutants and of natural variation in flowering time among Arabidopsis ecotypes (reviewed in Koornneef et al., 1998; Piñeiro and Coupland, 1998; Simpson et al., 1999), table II-1). There are currently about 80 genes and loci implicated in the control of flowering time (Simpson et al., 1999). The genes defective in a large number of flowering
time mutants remain to be identified, and the elucidation of the nature of these genes and their encoded products promises to offer great insight into the process of flowering time control.

However, the genes defective in a number of flowering time mutants have been identified and offer some initial insight into the possible mechanisms for floral promotion. For example, the genes defective in the late-flowering mutants co and ld (Rédei, 1962) encode transcription factors that promote flowering (Aukerman et al., 1999; Lee et al., 1994; Putterill et al., 1995). The FLC locus, originally identified through the ability of Landsberg alleles to suppress the late-flowering phenotypes of certain mutants (Koornneef et al., 1994; Lee et al., 1994), encodes a MADS box transcription factor that represses the floral transition (Michaels and Amasino, 1999; Sheldon et al., 1999). The gene defective in late-flowering fca mutants encodes a protein which contains two RNA binding domains and a WW protein interaction domain that may function in post-transcriptional control of components in the flowering time pathway (Macknight et al., 1997).

Loss-of-function mutations in the genes TFL and FT result in opposite flowering phenotypes (Kobayashi et al., 1999): tfl mutants are early flowering (Alvarez et al., 1992; Shannon and Meeks-Wagner, 1991) whereas ft mutants flower late (Koornneef et al., 1991). The protein products of these genes are 50% identical to one another and are putative phosphatidylethanolamine- and nucleotide-binding proteins related to a membrane-associated protein from mammals (Bradley et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999; Ohshima et al., 1997).

II.C. Pathways of floral promotion

Although a number of the genes involved in flowering time control have been identified, the interactions of these genes and other, as yet unidentified, components of the floral initiation machinery remain unclear. However, based on the responses of flowering
Table II-1: Cloned Arabidopsis genes involved in the transition to flowering.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence similarity / probable function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Promoters of flowering</strong></td>
<td></td>
</tr>
<tr>
<td>ADG1</td>
<td>ADP glucose phosphorylase, involved in starch metabolism</td>
</tr>
<tr>
<td>CO</td>
<td>Putative zinc finger transcription factor</td>
</tr>
<tr>
<td>DET2</td>
<td>Steroid 5-α reductase involved in brassinolide biosynthesis</td>
</tr>
<tr>
<td>FCA</td>
<td>RNA binding protein with protein-protein interaction domain</td>
</tr>
<tr>
<td>FPA¹</td>
<td>RNA binding protein with protein-protein interaction domain</td>
</tr>
<tr>
<td>FHA/CRY2</td>
<td>Cryptochrome 2, a blue-light photoreceptor</td>
</tr>
<tr>
<td>FPF1</td>
<td>Novel protein, may be involved in GA synthesis or response</td>
</tr>
<tr>
<td>FT</td>
<td>TFL1 homolog</td>
</tr>
<tr>
<td>GAI</td>
<td><em>ent</em>-kaurene synthetase A, an enzyme involved in GA biosynthesis</td>
</tr>
<tr>
<td>GAI</td>
<td>Member of novel family of transcription factors</td>
</tr>
<tr>
<td>GI</td>
<td>Novel protein with membrane spanning regions</td>
</tr>
<tr>
<td>LD</td>
<td>Glutamine-rich homeobox transcription factor</td>
</tr>
<tr>
<td>PGM</td>
<td>Phosphoglucomutase, involved in starch metabolism</td>
</tr>
<tr>
<td>PHYA</td>
<td>Light-labile red / far-red light photoreceptor</td>
</tr>
<tr>
<td><strong>Repressors of flowering</strong></td>
<td></td>
</tr>
<tr>
<td>CCA1</td>
<td>MYB-related transcription factor; LHY homolog</td>
</tr>
<tr>
<td>CLF</td>
<td>Homology to <em>Enhancer of Zeste</em>, a Drosophila polycomb-group gene</td>
</tr>
<tr>
<td>ELF3</td>
<td>Novel protein</td>
</tr>
<tr>
<td>ESD4</td>
<td>Novel protein</td>
</tr>
<tr>
<td>LHY</td>
<td>MYB-related transcription factor; CCA1 homolog</td>
</tr>
<tr>
<td>PHYB</td>
<td>Light-stable red / far-red light receptor</td>
</tr>
<tr>
<td>SPY</td>
<td>O-linked N-acetylglicosamine transferase, involved in modification of proteins</td>
</tr>
<tr>
<td>TFL</td>
<td>Similarity with phosphatidylethanolamine binding proteins</td>
</tr>
<tr>
<td>WLC</td>
<td>Novel protein</td>
</tr>
<tr>
<td>FLC²</td>
<td>MADS box transcription factor</td>
</tr>
</tbody>
</table>

Modified from Levy and Dean, 1998. ¹(Simpson et al., 1999); ²(Michaels and Amasino, 1999; Sheldon et al., 1999).
time mutants to various stimuli such as photoperiod, vernalization, and treatment with gibberellin, it is possible to group these components into different floral initiation pathways. The interactions between these pathways are not well understood, but it is likely that there is cross-communication among them.

II.C.1. The autonomous pathway of floral promotion

The autonomous pathway of floral promotion functions independently of daylength and is likely controlled by endogenous signals to allow eventual flowering regardless of day length. Mutants in this pathway, *fca, fve, fpa, fld* (Koornneef et al., 1991), and *ld* (Rédei, 1962), are late flowering in both inductive (long day) and noninductive (short day) photoperiods. The flowering defects in these mutants are generally rescued by vernalization or gibberellin treatment (reviewed in Koornneef et al., 1998; Piñeiro and Coupland, 1998; Simpson et al., 1999). Interestingly, *FLC* message levels are higher in the late-flowering mutants *fca, fpa, fve, and ld* than in wild-type plants (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* is a repressor of the floral transition (Michaels and Amasino, 1999; Sheldon et al., 1999), so it is possible that components of the autonomous pathway normally function to repress *FLC* transcription or play a role in post-transcriptional control of *FLC*. The latter is an attractive possibility because the products of the *FCA* and *FPA* genes are proteins that contain RNA binding motifs that may enable them to function in post-transcriptional control (Macknight et al., 1997; Simpson et al., 1999).

II.C.2. The photoperiodic pathway of floral promotion

The photoperiodic pathway of floral initiation promotes flowering in a daylength-dependent manner. Genes defective in mutants that flower late in long days but not in short days are considered part of this photoperiodic induction pathway. Mutants in this pathway, such as *fi, fe, fd, fha, fwa* (Koornneef et al., 1991), *gi* and *co* (Rédei, 1962) do not respond
to vernalization treatment (reviewed in Koornneef et al., 1998; Piñeiro and Coupland, 1998; Simpson et al., 1999). However, flowering time in these mutants, although not rescued, can be somewhat accelerated in long days by application of GA (Chandler and Dean, 1994).

The Arabidopsis phytochrome and cryptochrome photoreceptors play an integral role in the photoperiodic promotion of flowering, and will be discussed in section II.D.

II.C.3. The gibberellin-responsive pathway of floral promotion

Application of the hormone gibberellin (GA) hastens Arabidopsis flowering (Bagnall, 1992) and mutants disrupted in GA biosynthesis or response flower at correspondingly altered times. For example, the ga1 mutant, which is defective in GA biosynthesis, requires GA application for flowering to occur in short days but has relatively normal flowering in long days (Wilson et al., 1992). Mutations in the gene SPY, which encodes a tetratricopeptide repeat protein involved in transduction of GA signalling, cause constitutive GA response phenotypes and result in early flowering (Jacobsen et al., 1996; Jacobsen and Olszewski, 1993). Additionally, the gene FPF1 (flowering promoting factor) may be involved in mediating GA signalling during the floral transition. Transgenic plants overexpressing FPF1 flower earlier than wild-type plants in both short and long days (Kania et al., 1997). Epistasis experiments suggest that the GA pathway promotes flowering independently of the autonomous, photoperiod-dependent, and vernalization-response pathways (Simpson et al., 1999).

II.C.4. The vernalization-responsive pathway of floral promotion

Vernalization also promotes flowering in Arabidopsis (Simpson et al., 1999). As previously discussed, vernalization treatment can overcome the late-flowering phenotypes of mutants in the autonomous promotion pathway. Message levels of the FLC floral repressor are reduced in response to vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999), which may explain this phenomenon. Little else is known regarding the molecular
basis of floral promotion by vernalization, although decreases in DNA methylation levels may be involved (reviewed in Koornneef et al., 1998).

The mutants \textit{vrn1} and \textit{vrn2} were identified in an \textit{fca} autonomous pathway mutant background because they caused \textit{fca} plants to be less responsive to vernalization treatment (Chandler et al., 1996). These mutants can acclimate to cold temperatures as well as wild-type, indicating that they are defective in a vernalization-specific response rather than merely defective in cold perception (Chandler et al., 1996). The identification of the genes defective in these mutants will undoubtedly aid in the understanding of how the vernalization pathway functions and how it integrates with the other floral promotion pathways.

In summary, there are at least four separate pathways through which the floral transition can be promoted. These are the autonomous pathway, which is independent of environmental input; the photoperiodic promotion pathway, which promotes flowering in response to inductive photoperiods; the gibberellin-responsive pathway; and the vernalization-responsive pathway (figure II-1).

In addition to pathways of floral promotion, there are also mechanisms of floral repression in Arabidopsis. The gene \textit{FLC}, for example, is a key floral repressor, and plants with nonfunctional alleles of this gene flower early (Michaels and Amasino, 1999; Sheldon et al., 1999). The photoreceptor \textit{PhyB} also functions as a floral repressor (Goto et al., 1991; Reed et al., 1994). Many other genes and mutants implicated in floral repression have also been identified (table II-1; reviewed in Koornneef et al., 1998; Levy and Dean, 1998; Piñeiro and Coupland, 1998; Simpson et al., 1999), but their placement in pathways regulation floral development is less clear than that of many genes responsible for floral promotion.
Figure II-1: Proposed pathways controlling the transition to flowering in Arabidopsis. Arrows indicate positive influences; flat lines indicate inhibitory influences. Modified from Simpson et al., 1999.
II.D. Photoreceptors are involved in control of Arabidopsis flowering time

Arabidopsis encodes a number of photoreceptors that mediate light perception under various wavelengths and fluences. These include the red/far-red light-perceiving phytochromes and the blue/UV-perceiving cryptochromes and NPH1/phototropin. Cryptochromes and phytochromes are involved in transmitting daylength signals to plants, and mutations in these photoreceptors can lead to alterations in flowering time.

II.D.1 Phytochromes in Arabidopsis floral promotion

Phytochromes are red/far-red light receptors. The typical phytochrome is a dimer of identical ~124 kDa subunits, each of which contains a covalently bound tetrapyrrole chromophore in the N terminal region. Dimerization of the phytochromes is mediated through PAS domains. A PAS domain is an approximately 100 amino acid motif that was originally identified (Nambu et al., 1991) as two direct repeats in the Drosophila clock protein PER (Jackson et al., 1986), the mammalian transcription factor ARNT (Hoffman et al., 1991), and the Drosophila SIM protein (Crews et al., 1988). Phytochromes can exist in two different forms: the far-red light absorbing Pfr form and the red-light absorbing Pr form. Pfr is thought to be the biologically active form of the protein, whereas the Pr form is considered inactive. Conversion between the two forms is accompanied by conformational changes, which in turn lead to the activation of genes involved in light-mediated processes (Quail et al., 1995). The mechanisms by which phytochrome signaling takes place are mysterious, although a number of phytochrome-interacting partners have recently been identified and the phytochrome signaling pathway is beginning to be elucidated (Choi et al., 1999; Fankhauser et al., 1999; Nagy and Schäfer, 2000; Ni et al., 1998; Ni et al., 1999).

Arabidopsis has five characterized phytochromes, designated PhyA through PhyE (Clack et al., 1994; Sharrock and Quail, 1989). PhyA and PhyB are considered the primary Arabidopsis phytochromes. The PhyA protein accumulates to high levels in dark-grown
(etiolated) seedlings, but is rapidly degraded upon exposure to light in a process that is likely ubiquitin-dependent (Clough and Vierstra, 1997). The remaining phytochromes, PhyB through PhyE, remain stable in the light (Quail et al., 1995).

Mutations in Arabidopsis phytochromes lead to a variety of phenotypes, among them alterations in flowering time. phyB mutants flower early in both long and short days (Goto et al., 1991; Reed et al., 1994), and this early flowering phenotype is enhanced by phyD mutations (Aukerman et al., 1997). Night breaks, short periods of light that interrupt the usual dark period, normally promote flowering in Arabidopsis. Mutants defective in PhyA flower normally in long and short days, but respond less than wild type to night breaks (Reed et al., 1994), indicating that PhyA is involved in floral promotion in response to certain types of stimuli.

Another phenotype observed in phytochrome mutants is abnormal hypocotyl elongation. phyA mutants display longer than normal hypocotyls in far-red light but not in red light, whereas phyB mutants have longer hypocotyls in red but not far-red light. Double mutants have longer hypocotyls in red and far-red light than either of the single mutants (Reed et al., 1994). This evidence suggests that PhyA primarily controls perception of far-red light and PhyB controls perception of red light. Therefore, the opposite flowering time phenotypes of the phyA and phyB mutants can be explained by the quality of light perceived by each of these photoreceptors: far-red light, perceived by PhyA, promotes flowering whereas red light, perceived by PhyB, inhibits flowering (Eskins, 1992).

II.D.2. Cryptochromes in Arabidopsis floral promotion

The cryptochromes are blue/ultraviolet-A (UV-A) light receptors. In Arabidopsis there are two cryptochromes, encoded by the genes CRY1 (HY4) (Ahmad and Cashmore, 1993) and CRY2 (PHH1, FHA) (Hoffman et al., 1996; Lin et al., 1996). These proteins are similar in their N-termini to photolyases, flavoproteins that mediate DNA repair in a light-dependent manner (Ahmad and Cashmore, 1993; Hoffman et al., 1996). However, the
cryptochromes do not possess photolyase activity themselves (Malhorta et al., 1995). The CRY1 and CRY2 proteins have divergent C-termini: the C-terminus of CRY1 has similarity to tropomyosin (Ahmad and Cashmore, 1993) whereas CRY2 resembles neuromodulin in its C-terminal end (Lin et al., 1996). The CRY2 protein, like PhyA, is photolabile and is rapidly broken down in high-fluence blue light (Ahmad et al., 1998; Lin et al., 1998).

Mutations in the cryptochromes, like mutations in phytochromes, result in a variety of light-related phenotypes. The hy4 mutant is defective in the CRY1 gene and displays abnormally long hypocotyls in blue/UV-A light (Ahmad and Cashmore, 1993). Furthermore, hy4/cry1 mutants flower late in certain ecotypes (Bagnall et al., 1996). The cry2 mutant is allelic to the late-flowering mutant fha (Koornneef et al., 1991), and these mutants are late-flowering in white or red plus blue light, but not blue light alone (Guo et al., 1998). The fact that fha/cry2 mutants flower normally in blue light suggests that CRY2 may function to relieve PhyB-mediated floral repression (Guo et al., 1998).

II.D.3. Phototropin mediates phototropism but not flowering time in Arabidopsis

The nph1 mutant of Arabidopsis lacks blue-light mediated phototropic responses (Liscum and Briggs, 1995). The gene defective in nph1 encodes a light-dependent protein kinase that binds the chromophore flavin mononucleotide (FMN) (Christie et al., 1998; Christie et al., 1999; Huala et al., 1997). The N terminal FMN-binding region of the NPH1, or phototropin, protein is composed of two LOV (light, oxidation, voltage) domains, which are degenerate PAS domains. PAS domains can mediate protein dimerization (Huang et al., 1993) and have been identified in sensor domains of proteins from prokaryotes, eukaryotes, and archaeabacteria (Zhulin et al., 1997). For example, PAS domains, also known as S-boxes, mediate perception of oxygen and redox potential in the sensor modules of prokaryotic two-component regulatory systems (Zhulin et al., 1997). It is therefore likely that the LOV domains of NPH1/phototropin function in light sensing, and that NPH1/phototropin represents a new class of Arabidopsis photoreceptors involved in
mediating phototropic responses (Christie et al., 1998; Christie et al., 1999; Huala et al., 1997). However, phototropin does not appear to be involved in the floral transition as no flowering time defects have been reported in *nph1* mutants.

II.E. The circadian clock is implicated in the control of Arabidopsis flowering time

The circadian clock is an endogenous timekeeping mechanism that controls a variety of biological processes. Four characteristics define a circadian rhythm: a period of approximately 24 hours, entrainability to external stimuli, temperature compensation to preserve the period, and continued rhythmicity in the absence of exogenous periodic signals (Somers, 1999). The existence of the clock allows an organism to time the occurrence of certain metabolic and physiological processes so that they take place at an optimal time. Clock-controlled processes in Arabidopsis include stomatal opening and closing, periodic leaf movements (also called nyctinastic movements), and hypocotyl elongation (Dowson-Day and Millar, 1999; Somers, 1999). The expression of certain genes, including the *CAT2* and *CAT3* (Zhong and McClung, 1996) and *CAB (Lhcb)* genes (Millar and Kay, 1991), is also controlled by the clock, which allows experimental observation of clock cycling in Arabidopsis.

Circadian clocks have been well studied in other organisms, but the components of the Arabidopsis circadian clock are only beginning to be identified. In systems as varied as *Drosophila, Neurospora*, and mammals, the central oscillator is composed of a negative feedback loop that controls transcription and translation of clock components (reviewed in Dunlap, 1999). Arabidopsis equivalents of genes known to be involved in clock systems from other organisms have not been reported, but the Arabidopsis clock likely has the same general design as those in other organisms.
II.E.1. Arabidopsis photoreceptors mediate light input to the circadian clock

The generalized model of a circadian clock consists of input pathways, a central oscillator, and output pathways (figure II-2). One important input to the clock is light, and light input to the clock in Arabidopsis is mediated by the phytochromes and crytochromes. Mutations in either red- or blue-light photoreceptors perturb the Arabidopsis circadian clock (Millar et al., 1995; Somers et al., 1998a). *phyA* mutants have lengthened free-running circadian periods in low-fluence red light, whereas *phyB* mutants have longer free-running periods than wild-type plants in high-fluence red light (Somers et al., 1998a). Furthermore, *phyA* mutations result in period lengthening in low fluence blue light (Somers et al., 1998a). Loss-of-function *cry1* mutants have long periods in low- and high-fluence, but not intermediate-fluence, blue light. *cry2* mutations cause slight period shortening in low-fluence blue light, and period lengthening in high-fluence blue light (Somers et al., 1998a). Therefore, Arabidopsis photoreceptors influence not only flowering time, but the circadian clock as well.

II.E.2. Many Arabidopsis genes are associated with both flowering time control and the circadian clock

Several additional Arabidopsis genes that couple flowering time control to the circadian clock have been identified (reviewed in Somers, 1999). *toc1* mutants have a short circadian period in continuous light (Millar et al., 1995) and flower early in short days in certain ecotypes (Somers et al., 1998b). *FLC* encodes a MADS box transcription factor that acts in the autonomous pathway to repress the floral transition (Michaels and Amasino, 1999; Sheldon et al., 1999), and *flc* mutations also shorten the circadian period (Swarup et al., 1999). Transcript levels of the gene defective in the late-flowering mutant *gigantea* (*gi*) oscillate with a circadian rhythm, and *gi* mutants have altered patterns of clock-controlled gene expression (Fowler et al., 1999; Park et al., 1999), indicating that GI is necessary for proper clock cycling.
The Myb-related transcription factors LHY and CCA1 may link light input to the clock or may be part of the central oscillator itself (reviewed in McClung, 1998; Somers, 1999). Overexpressing either LHY or CCA1 disrupts the rhythmic accumulation of several clock-controlled transcripts, including LHY and CCA1 themselves (Schaffer et al., 1998; Wang and Tobin, 1998). CCA1 was originally isolated as a factor binding to a phytochrome-responsive region of the CABI (Lhcb1*3) promoter (Wang et al., 1997). CCA1 overexpressors exhibit both long hypocotyl and late-flowering phenotypes, the severity of which correlate with the amount of CCA1 protein present (Wang and Tobin, 1998). Constitutive LHY expression also causes a day length-insensitive flowering delay and an elongated hypocotyl (Schaffer et al., 1998).

The observation that several flowering time mutants also have hypocotyl elongation defects may reflect the control of both processes by the circadian clock. Hypocotyl elongation is clock-controlled and is maximal at dusk and arrests at dawn (Dowson-Day and Millar, 1999). This arrest is absent in the elf3 mutant (Dowson-Day and Millar, 1999), a photoperiod insensitive early-flowering mutant with a long hypocotyl (Zagotta et al., 1996).

Expression of a reporter gene from a clock-controlled promoter is arrhythmic when the elf3 mutant is shifted to constant light, but rhythmic when shifted to constant dark, suggesting that ELF3 is involved in light input to the oscillator (Hicks et al., 1996).

The identification of multiple genes involved in both the Arabidopsis circadian clock and the control of flowering time suggests that the circadian clock contributes to the control of flowering time. In fact, it is now generally accepted that the clock is responsible for time measurements that lead to photoperiodic induction of flowering (Samach and Coupland, 2000).
Figure II-2  Simplified model of the Arabidopsis circadian clock. Adapted from (Samach and Coupland, 2000).
II.F. Goals of this project

One allele of the iar1 mutant, in addition to being resistant to IAA-Ala, was late flowering. The late-flowering phenotype was not attributable to mutations in IAR1 as the six other alleles of this mutant flower normally. Also, the phenotype was not due to mutations in other flowering time control loci that map close to the position of IAR1 in the Arabidopsis genome. We therefore undertook to characterize the late flowering phenotype of this mutant in greater detail and to place the gene defective in the mutant in one of the pathways of floral promotion. We also wished to determine the molecular basis for the late-flowering phenotype of this mutant by cloning the gene responsible and characterizing its encoded product.
CHAPTER III: MATERIALS AND METHODS

III.A. Plant materials and growth conditions

*Arabidopsis thaliana* ecotypes Columbia (Col-0), Wassilewskija (WS) and Landsberg erecta tt4 (Ler) were used. Seeds were surface-sterilized (Last and Fink, 1988) and grown on PNS (plant nutrient media containing 0.5% sucrose; Haughn and Somerville, 1986) solidified with 0.6% agar. PNS either contained no supplement or was supplemented with 50 nM to 5 μM IAA (from a 10 mM stock in ethanol), 10 to 150 μM IAA-amino acid conjugates (from 100 mM stocks in ethanol), 10 μM IBA (from a 100 mM stock in ethanol), 5 μM JA (from an 1 mM stock in ethanol), 50 μM MeJA (from a 100 mM stock in ethanol) or JA-amino acid conjugates (from 100 mM stocks in ethanol), 15 μg/mL kanamycin (Kan; from a 25 mg/mL stock in water), 2.25 μg/mL BASTA (from a 15 mg/mL stock in 25% ethanol), or various concentrations of metals from 100 mM, 200 mM, 500 mM, or 1 M stocks in water. IAA and IAA conjugates were from Aldrich (Milwaukee, WI) or were synthesized by Sherry LeClere. JA and MeJA were from Aldrich; JA-amino acid conjugates were synthesized by Kristin Krukenberg and Seiichi Matsuda. Plates were wrapped in gas-permeable Leukopor surgical tape (Beierdorf Inc., Norwalk, CT) and grown in 24 hour illumination under unfiltered white light (120 μE m⁻² sec⁻¹) or under yellow long-pass filters (25 to 45 μE m⁻² sec⁻¹) to prevent breakdown of indolic compounds (Stasinopoulous and Hangarter, 1990). Plants grown on PNS were incubated at 22 °C unless otherwise specified. Plants transferred to soil (Metromix 200; Scotts, Marysville, OH) were grown at 22-25 °C under continuous illumination (~200 μE m⁻² sec⁻¹) by Sylvania Cool White fluorescent bulbs. For liquid growth experiments, seeds were sterilized and placed in 1/6 strength PN liquid with 0.1% sucrose.

For root elongation experiments, surface-sterilized seeds were plated on the indicated media and placed under the specified light condition at 22 °C. The longest root of
each seedling was measured to the nearest mm eight days after plating unless otherwise specified.

For hypocotyl experiments discussed in Chapters VII and VIII, surface sterilized seeds were plated on PNS, incubated at 4 °C for 18 hrs, under white light at 22 °C for 24 hrs, and then at 22 °C either in the dark, under unfiltered white light (120 μE m² sec⁻¹), or under white light filtered with yellow (plexiglass #2208) red (plexiglass # 2423) or blue (plexiglass # 2424) filters for five additional days. Hypocotyls were measured to the nearest mm. Hypocotyl experiments in Chapter V were performed in the same manner, except the incubation at 4 °C was omitted.

III.B. Isolation of mutants

The iar1-1 and iar1-2 mutant alleles were isolated from pools of WS seeds mutagenized with ethyl methanesulfonate (EMS) as described (Normanly et al., 1997). Surface-sterilized M₂ seeds were spread at a density of ~1000 seeds per plate on 150 X 25 mm Petri plates containing 100 mL PNS supplemented with 50 μM IAA-Ala. Approximately 24,000 seeds were screened. After two weeks, putative mutants with elongated roots were transferred to soil and allowed to set seed. The resultant M₃ seeds were screened separately for resistance to 50 μM IAA-Ala and sensitivity to 1 μM IAA. The iar1-3 and iar1-4 alleles were isolated in a similar manner from M₂ progeny of fast neutron-mutagenized Col-0 gl1-l seeds (purchased from Lehle Seeds, Round Rock, TX). The alleles iar1-5 and iar1-6 were identified from M₂ pools of EMS mutagenized ilrl-1 seeds on PNS plates supplemented with 100 μM IAA-Leu. Finally, iar1-7 was isolated from EMS mutagenized ilrl-1 M₂ pools screened on 70 μM IAA-Phe. iar1-1 through iar1-6 failed to complement one another, indicating that they represented alleles of the same gene; iar1-7 was identified as an iar1 allele by sequence analysis and was not tested for complementation of the other alleles. All seven alleles were shown to be independent by sequence analysis. iar1 mutants were back-crossed to the parental ecotype prior to
phenotypic analysis. In all experiments, the *iar1-1, iar1-2, iar1-3,* and *iar1-4* lines used had been back-crossed at least three times to their respective parental ecotypes. *iar1-5* and *iar1-6* were back-crossed twice to WS. In figure V-6, *iar1-2* had been out-crossed twelve times to Col-0 so that the mutation was in a Col-0 background.

The *fkl1* mutant was originally isolated as *iar1-4*. Both the IAA-alanine resistance (caused by deletion of the *IAR1* gene) and the late-flowering (caused by deletion of the *FKL1* gene) phenotypes behaved as recessive Mendelian traits. No normally flowering plants were detected in 50 IAA-alanine resistant F2 plants from a back cross to the wild type, indicating that the two lesions are tightly linked. Subsequent molecular analysis indicated that both genes, which are about 11 kb apart, were completely deleted in the mutant. Phenotypic characterization was performed on *fkl1* plants that had been back-crossed to the wild type (Col-0) three times to remove unlinked mutations.

**III.C. Degenerate PCR**

Oligonucleotides were from the Great American Gene Company (Ramona, CA). *ILR1*-like genes were identified by performing PCR with primers designed from regions similar among *ILR1, ILL1,* and *ILL2* (Figure IV-1). The forward primer degen. for (48-fold redundancy; table III-2) was designed to encode the amino acid sequence KIHENPE, and the reverse primer degen. rev (512-fold redundancy; table III-2) was the reverse complement that would encode the sequence FQP(G/A)EEG. These primers were used to amplify Col-0 genomic DNA by PCR using 30 cycles of 1 minute at 94° C, 1 minute at 55° C, and 1 minute at 72° C. This resulted in five discrete bands when separated on a 1.5% agarose gel ranging in length from 535 to 700 bp. These PCR products were gel purified using the Qiaex II gel extraction kit (Qiagen, Valenica, CA) and TA cloned into pT7Blue (Novagen, Madison, WI). Restriction analysis of the resulting clones identified the known amidohydrolase genes *ILR1, ILL1, ILL2* and *ILL3* (Table III-1). Subclones yielding restriction patterns other than those expected for *ILR1, ILL1,* and *ILL2* were sequenced
using T7-primed reactions on an Applied Biosystems (Foster City, CA) automated DNA sequencer by D. Needleman (University of Texas-Houston Medical School Molecular Genetics Core Facility) or C. Kolenda and J. Derr (Department of Veterinary Pathobiology, Texas A&M University, College Station).

Table III-1: Restriction digests used to differentiate clones in pT7Blue containing degenerate PCR products from known amidohydrolase genes.

<table>
<thead>
<tr>
<th>gene</th>
<th>restriction enzyme</th>
<th>HindIII</th>
<th>EcoRI</th>
<th>XhoI</th>
<th>BamHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIL1</td>
<td>vector + 426 bp or linearized</td>
<td>vector + 366 bp</td>
<td>uncut</td>
<td>vector + 133 bp</td>
<td>or vector + 567 bp</td>
</tr>
<tr>
<td>ILL1</td>
<td>linearized</td>
<td>vector + 273 bp or vector + 270 bp</td>
<td>uncut</td>
<td>linearized</td>
<td></td>
</tr>
<tr>
<td>ILL2</td>
<td>linearized</td>
<td>linearized</td>
<td>linearized</td>
<td>linearized</td>
<td></td>
</tr>
<tr>
<td>ILL3</td>
<td>linearized</td>
<td>linearized</td>
<td>uncut</td>
<td>vector + 126 bp or vector + 555 bp</td>
<td></td>
</tr>
</tbody>
</table>

*Fragments indicated as vector contain the 2.887 kb pT7Blue plasmid as well as some amount of DNA from the PCR product insert. Different size fragments were possible because the insert could be in either of two orientations in the vector.

III.D. Identification of the splice site mutation of ILL5 in Col-0

The region of ILL5 that contains the 3' splice acceptor site for intron two was PCR amplified from Col-0, WS, and Ler total genomic DNA using the forward primer ILL5-5 and the reverse primer ILL5-6 (table III-2). PCR conditions were 40 cycles of 30 seconds at 95 °C, 30 seconds at 56 °C, and three minutes at 72 °C. The resulting 1.2 kb PCR products were sequenced directly using the forward primer.
III.E. Genetic mapping of the iar1 and fkl1 mutations

To map the iar1 and fkl1 mutations, we used segregating populations from crosses between IAR1/IAR1 (ecotype Col-0) and iar1/iar1 (ecotype WS) plants. Genomic DNA was prepared (Celenza et al., 1995) from 684 IAA-Ala resistant F₂ plants and scored using published (Bell and Ecker, 1994; Konieczny and Ausubel, 1993) and new PCR-based polymorphic markers. The markers developed for mapping are listed in Table V-2; oligonucleotide sequences for these markers are in Table III-2.

III.F. Construction of a complementation library

50 µg T7E4 BAC (Arabidopsis Biological Resource Center) DNA was partially digested with the restriction enzyme Sau3AI (NEB) and run on a 0.8% agarose gel with 1 mM guanosine to prevent UV damage to the DNA (Gründemann and Schömig, 1996). Fragments running in the ~10 kb range were excised from the gel, purified using the Qiaex II gel extraction kit (Qiagen, Valenica, CA) then ligated into BamHI-digested pBIN19 plant transformation vector (Bevan, 1984) and transformed into E.coli strain DH5α. A contig of overlapping clones covering the region in which iar1 mapped was assembled using a combination of DNA blot hybridization and sequence analysis. Constructs from these clones were introduced into Agrobacterium tumefaciens GV3101 by electroporation (Ausubel et al., 1995). iar1-1, iar1-3, and iar1-4 fkl1 mutant plants were transformed with these constructs by floral dip (Clough and Bent, 1998), and transformants were selected by plating T₁ seeds on 15 µg/mL Kan. Kan resistant plants were transferred to soil, and the flowering time of iar1-4 fkl1 transformants was observed. T₂ plants were tested for rescue of the iar1 IAA-Ala resistant phenotype by plating on 40 µM IAA-Ala and examining root elongation after eight days.
Table III-2: Sequences of oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
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<td>T7E4-14</td>
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<td>T7E4-16</td>
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<td>degen_rev</td>
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<tr>
<td>GFP-Nxl</td>
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<tr>
<td>T7E4-38</td>
<td>CTTGCCAGATCGTGTCGACGCACCTTCATTTCCTGGTACGCAG</td>
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</table>

Sequences are 5' -> 3'. Oligos were synthesized by Great American Gene Company or One Trick Pony Oligos (Ransom Hill Biosciences, Ramona, CA). H indicates A, C, or T; N indicates A, C, G, or T; R indicates A or G; S indicates C or G; and Y indicates C or T. Underlined residues are those altered in oligo-directed mutageneses.
III.G. cDNA isolation and manipulations

A full-length *IARI* cDNA was isolated by hybridizing an Arabidopsis ecotype *Ler* cDNA library (Minet et al., 1992) with a 1.2 kb HindIII/PstI genomic fragment, subcloned from the C37 rescue construct, containing the last four predicted exons of the *IARI* gene as well as 43 bp 3' UTR. The NotI insert of the cDNA was subcloned into the NotI site of pBluescript KS (+) (Stratagene) to create KSIAR1c and sequenced using vector-derived and internal primers.

A full-length *FKFI* cDNA was isolated by hybridizing an Arabidopsis ecotype *Ler* cDNA library (Minet et al., 1992) with a 2.6 kb HindIII fragment, subcloned from T7E4, which contained all but the first 115 bp of the *FKFI* coding region. The NotI insert of the cDNA was subcloned into the NotI site of pBluescript KS(+) (Stratagene) and sequenced using vector-derived and internal primers. A full-length cDNA of *ZTL* was isolated by hybridizing a pSPORT-based (Gibco BRL) Arabidopsis ecotype Col-0 cDNA library (S. LeClere and B. Bartel, unpublished) with a probe made by PCR amplifying Col-0 genomic DNA with the oligos FKL2-1 and FKL2-2 (table III-2). This cDNA was sequenced using vector-derived and internal primers. DNA was sequenced by Lone Star Labs, Inc. (Houston, TX) using an automated sequencer.

*IARI*-overexpressing transgenic plants were made as follows. The 1.4 kb insert of the *IARI* cDNA was excised from pBluescript with NotI and ligated into the NotI site of 35SpBARN (LeClere and Bartel, unpublished), a binary vector modified from p1'Barbi (Mengiste et al., 1997) to contain a multiple cloning site flanked by the CaMV 35S promoter and the *nos* terminator. The overexpression construct, pBARNIAR1, was introduced into Col-0 plants (Clough and Bent, 1998). T1 seedlings were screened for resistance to BASTA by spraying with Finale herbicide (AgrEvo Environmental Health. Montvale, NJ) diluted in water to a concentration of 0.26 mg/ml glufosinate-ammonium at approximately one and two weeks after germination. Homozygous lines were identified by examining the pattern of BASTA resistance in the T3 generation.
The 2.3 kb insert of the ZTL cDNA was excised with SalI and NotI and ligated into XhoI/NotI-digested 35SpBARN (LeClere and Bartel, unpublished), and transformed into plants as described above.

III.H. Promoter-reporter gene fusion

To construct the IAR1-promoter GUS translational fusion, I introduced an XbaI site directly following the ATG of IAR1 by oligo-directed mutagenesis of the IAR1 promoter in pBluescript KS (-) with the oligo T7E4-42 (table III-2). A 2.5 kb XbaI/PstI fragment (the PstI site was from the polylinker of pBluescript) containing the IAR1 promoter was excised and cloned into the XbaI/PstI sites of pBI101.1 (Jefferson et al., 1987). The resultant construct, IAR1pGUS, encodes the initiator methionine from IAR1 fused to GUS.

To construct the FKF1-promoter GUS translational fusion, a BamHI site was introduced following the FKF1 initiation codon using oligo-directed mutagenesis with the primer T7E4-38 (table III-2). The 1.7 kb promoter fragment was excised using SmaI (from the polylinker) and BamHI and ligated into pBI101.1 (Jefferson et al., 1987) digested with HindIII, filled in with T4 DNA polymerase, and digested with BamHI. The resultant construct, FKF1pGUS, encodes the initiator methionine from FKF1 fused to GUS.

These constructs were transformed into Col-0 and transformed lines were selected on Kan as described above. β-glucuronidase activity was histochemically localized with 0.17 mg/mL (for FKF1pGUS) or 1 mg/mL (for IAR1pGUS) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) as previously described (Bartel and Fink, 1994). Each of the six independent FKF1pGUS lines examined showed a similar pattern of staining, although the intensity varied among the lines. Eight independent IAR1pGUS lines were obtained, of which only four showed staining in adult plants and one showed staining in seedlings.
III.I. pBIN-FKF1, pBINIAR1g, IAR1-GFP and IAR1-myc transgenic lines

To create the pBINIAR1g construct, which contains only the \textit{IAR1} coding region, 2 kb 5', and 1.7 kb 3' DNA, I subcloned a 6.45 kb EcoRI fragment from C37 into the EcoRI site of the plant transformation vector pBIN19 (Bevan, 1984).

To create transgenic Arabidopsis expressing IAR1-GFP and IAR1-myc fusion proteins, I made the following constructs: KSIAR1c+gGFP was made by mutagenizing the \textit{IAR1} cDNA in pBluescript KS (+) to create an NruI site just prior to the last codon. The oligo used for this mutagenesis was T7E4-44 (table III-2). A 500 bp BstEII/NotI fragment of the mutagenized cDNA was excised and cloned into BstEII/NotI digested \textit{IAR1} genomic DNA (replacing 825 bp \textit{IAR1} coding genomic DNA and the 3' genomic region) to create KSIAR1c+gNruI. KSIAR1c+gNruI contains approximately 2 kb genomic DNA from the 5' region of \textit{IAR1} and the first six \textit{IAR1} introns. However, it does not contain the last four introns and has the 3' UTR from the \textit{IAR1} cDNA. An NruI site was introduced 5' of the start codon of the \textit{GFP} coding region in psmGFP (Davis and Vierstra, 1996) by oligo-directed mutagenesis using the oligo GFP-NruI (table III-2) to create psmGFP-NruI. This construct was digested with SacI, the ends were filled using T4 DNA polymerase, then digested with NruI. The resulting fragment of approximately 700 bp was gel purified (Qiaex II gel extraction kit, Qiagen, Valencia, CA) and subcloned into the NruI site of KSIAR1c+gNruI to create KSIAR1c+g-GFP.

To make KSIAR1c+gmyc, the 200 bp insert of pT75Xmyc, which contains five copies of the myc epitope (Kolodziej and Young, 1991) was excised by digesting with DraI and SmaI, gel purified, and ligated into the NruI site of KSIAR1c+gNruI.

The inserts of KSIAR1c+gGFP and KSIAR1c+gmyc (about 5.5 kb and 5.2 kb, respectively) were excised with BsrBI (from the pBluescript polylinker) and ligated into the SmaI site of pBIN19 (Bevan, 1984) to create pBIN19IAR1c+gGFP and pBIN19IAR1c+gmyc.
Col-0 and iar1-3 plants were transformed with pBIN19IAR1g, pBIN19IAR1c+gGFP and pBIN19IAR1c+gmyc constructs as described above. Transformants were selected on 15 μg/mL Kan. Homozygous lines were identified by examining the pattern of Kan resistance in the T₃ generation.

A construct containing only the FKF1 gene and its promoter was made by digesting the rescuing subclone C575 (Figure VII-5A) with KpnI and SpeI and ligating the 4.3 kb fragment (FKF1 coding region, 1.7 kb of 5'-noncoding region and 0.7 kb of 3'-noncoding region) into pBIN19 (Bevan, 1984) cut with KpnI and XbaI. The resulting plasmid, pBIN-FKF1, was transformed into the fkl mutant using Agrobacterium-mediated transformation (Clough and Bent, 1998). Homozygous lines were identified by examining the pattern of kanamycin resistance in the T₃ generation, and two homozygous lines were assayed for flowering time and hypocotyl elongation.

III.J. Transgenic lines expressing the mouse KE4 cDNA

A full-length cDNA encoding the mouse IAR1 homolog KE4 in the vector pME18S-FL3 was purchased from Research Genetics (Huntsville, AL). The ~1.6 kb cDNA insert was excised with StuI and XmnI and ligated into the SmaI site of 35SpBARN (LeClere and Bartel, unpublished) to create 35SmKE4, with the mKE4 cDNA under the control of the 35S promoter. This construct was transformed into Col-0 and iar1-3 plants as described above.

III.K. RNA blot analysis

Total RNA was isolated from plants as described previously (Davies et al., 1999) or using Trizol reagent (Gibco BRL) according to the recommendations of the manufacturer. Plants for circadian experiments were grown atop sterilized filter paper on solid PNS media to facilitate harvest. Total RNA was electrophoresed on 1% agarose gels containing 0.37 M formaldehyde (Ausubel et al., 1995) and transferred to Bright-Star Plus nylon membrane
(Ambion; Austin, TX). 32P-labeled antisense RNA probes (Riboprobe *in vitro* transcription system, Promega) were hybridized in NorthernMax Prehyb/Hyb buffer (Ambion; Austin, TX) overnight at 65°C and washed at high stringency according to the recommendations of the manufacturer.

The *IAR1* riboprobe was made by linearizing the KSIAR1c plasmid with BamHI prior to probe synthesis using T7 RNA polymerase.

To avoid a cross-reacting message that hybridized to the 3’ end of the *FKF1* cDNA, we made a subclone in pBluescript KS (+) containing only the 5’ 1.3 kb of the cDNA for RNA probe synthesis. This plasmid was cut with EcoRV prior to RNA probe synthesis. A *ZTL* RNA probe was made by excising the *ZTL* cDNA from pSPORT with SmaI and XbaI, ligating it into pBluescript KS (+) cut with the same enzymes, and digesting the resultant plasmid with EcoRV. Other RNA probes were made after digesting the *CCA1* (EST 109K9T7) and *UBQ10* cDNAs (EST 193N23T7) with EcoRI and the *CAT2* cDNA (EST 47A1T7) with SmaI. The *FKF1* and *ZTL* probes were synthesized with T7 RNA polymerase, and *CCA1*, *CAT2*, and *UBQ10* with Sp6 RNA polymerase. A 28S rDNA probe labeled using random 12-mer oligonucleotides (Ausubel et al., 1995) was used to confirm equal loading of the lanes in figure VIII-1A; in figures VIII-1B, VIII-4, and VIII-5 *UBQ10* was the loading control.

**III.I. Atomic absorption**

Plants were grown in soil for four weeks or in 150 mL 1/6 strength liquid PN with 0.1% sucrose for two weeks prior to harvesting for atomic absorption analysis. Some liquid cultures were supplemented with 1 or 20 µM ZnSO₄ or contained 0 µM ZnSO₄ (1/6 strength PN normally contains 0.17 µM ZnSO₄). Harvested tissues were rinsed twice in milliQ water and dried in a 70 - 80 °C oven until completely dry (about three days). The dried tissue was ground to a fine powder with a mortar and pestle and sent to the
Department of Fruit and Vegetable Sciences at Cornell University for atomic absorption analysis of metal content using the dry ash method.

III.M. Yeast

Yeast strains used in this study are summarized in table III-X. Constructs for yeast transformations were made as follows: pFL61IAR1 was isolated from an Arabidopsis cDNA library ((Minet et al., 1992), see section III.G); pRS314GAL was constructed by Bethany Zolman by inserting the GALI promoter from pRS316GAL (Liu et al., 1992) into the XhoI site of pRS314 (Sikorski and Hieter, 1989); pRS314GALIAR1 was made by inserting the NotI fragment from pFLIAR1, which contains the IAR1 cDNA, into the NotI site of pRS314GAL; pRS316GALILR1 and pRS316GALIAR3 were made by Bonnie Bartel; pFL61 (Minet et al., 1992) and pFL61ZIP1 were kindly provided by Mary Lou Guerinot. pRS314GALIAR1-GFP was made by introducing an NruI site by oligo-directed mutagenesis (Ausubel et al., 1995) at the 3’ end of the IAR1 cDNA in the NotI site of pBluescript KS (+) immediately prior to the last codon. The oligo used for this mutagenesis was T7E4-44 (table III-2). The 700 bp SacI/NruI fragment of psmGFP-NruI was cloned into the NruI site of the mutagenized IAR1 cDNA to create KS(+)IAR1-GFP. The NotI insert of this construct, containing the IAR1 cDNA fused to GFP, was inserted into the NotI site of pRS314GAL to make pRS314GALIAR1.

Yeast transformations were carried out by growing a 5 mL overnight culture of the parent yeast strain in YPD (Ausubel et al., 1995). One mL aliquots of cells were spun at 10,000 rpm for 10 seconds in a microcentrifuge to pellet cells. The supernatant was aspirated off and the cells resuspended in 25 μL sterile water. 25 μL of salmon sperm carrier DNA (4 μg/μL) and 4 μL (200 ng/μL) of the transforming plasmid were added. The cells were mixed by gentle vortexing. 500 μL transformation buffer (40% PEG 3350, 0.1 M lithium acetate, 10 mM Tris pH 7.5, 1 mM EDTA, 0.1 M DTT) were added and the suspension mixed by gentle vortexing. The cells were incubated overnight (~12 to 15
hours) at room temperature, then pelleted at 10,000 rpm for 10 seconds. Cells were
resuspended in 100 μL sterile water, then plated on the appropriate selective media. For
transformations with yeast strain ZHY3, 1 mM ZnSO₄ was added to the transformation
reaction.

Yeast were grown on complete minimal (CM) medium (Ausubel et al., 1995). Yeast
strains containing pRS316- or pFL61-based constructs were selected by growth on -Ura
media, those containing pRS314-based constructs were selected by growth on -Trp media.
All strains derived from BZY2 were selected on -His media.

III.N. Yeast microsome isolation and analysis

Small initial cultures of Saccharomyces cerevisae strains JLY1, JLY2, JLY3, JLY7,
JLY8, JLY9, and JLY10 were grown overnight at 30 °C in CM -Trp, -Ura, -His, + 2%
glucose, diluted into 1:4 in fresh media, grown overnight, diluted 1:4 in fresh media and
grown overnight again. This culture was then diluted 1:4 in CM -Trp, -Ura, -His, + 2%
galactose to induce expression from the GALI promoter. Cultures were grown overnight,
and cells were pelleted by spinning at 6000g, 4 °C for 15 min. All procedures from this
point were carried out on ice. Each pellet was weighed, washed with sterile water, and
resuspended in sufficient microsome buffer (250 mM sucrose, 50 mM KOAc, 20 mM
HEPES, pH7.4, filter sterilized; 1 mM DTT and 1 mM PMSF were added immediately
prior to use) to make a 20% slurry of cells. Yeast cells were lysed using a French press and
spun at 6000g, 20 min. at 4 °C to remove cellular debris. The supernatants were transferred
to ultracentrifuge tubes and spun at 100,000g for one hour to pellet membranes. Each pellet
was resuspended in microsome buffer (~ 1 mL / 0.5-1g initial cell pellet weight) using a
Dounce homogenizer. Samples were divided into aliquots, flash frozen in liquid nitrogen,
and stored at -80 °C. Aliquots were also collected from the supernatant after
ultracentrifugation.
Table III-3: Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Parent strain</th>
<th>Made by</th>
<th>Genotype</th>
</tr>
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<tr>
<td>JBY575</td>
<td></td>
<td>Julie Brill and Gerald</td>
<td>ura3-52, trp1-Δ63, leu2-3,112, his3-Δ200, ade2</td>
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<td>BZY2</td>
<td>JBY575</td>
<td>Bethany Zolman</td>
<td>pha2::HIS3</td>
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<td>JLY1</td>
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<td>Jamie Lasswell</td>
<td>pRS14GALIARI1, pFLIAR3</td>
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<td>BZY2</td>
<td>Jamie Lasswell</td>
<td>pRS14GALIARI1, pFLILIR1</td>
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<td>BZY2</td>
<td>Jamie Lasswell</td>
<td>pRS14GAL, pFL61</td>
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<td>BZY2</td>
<td>Jamie Lasswell</td>
<td>pRS14GALIARI1, pRS16GALIIR1</td>
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<td>BZY2</td>
<td>Jamie Lasswell</td>
<td>pRS14GALIARI1, pRS16GAL</td>
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<td>BZY2</td>
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<td>pRS14GAL, pRS16GALIIR1</td>
</tr>
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<td>BZY2</td>
<td>Jamie Lasswell</td>
<td>pRS14GAL, pRS16GAL</td>
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<td>BZY2</td>
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<tr>
<td>DY1457</td>
<td></td>
<td>Mary Lou Geurinot and</td>
<td>ade6, can1, his3, leu2, trp1, ura3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elizabeth Rogers</td>
<td></td>
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<td>ZHY3</td>
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<td>zrt1Δ zrt2 Δ</td>
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<td>pFL61</td>
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<td>zrt1/zrt2, pFL61ZIP1</td>
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<td>L5708</td>
<td>KCX184-6C</td>
<td>Kyle Cunningham and</td>
<td>pmr1::HIS3/crp2::URA3</td>
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<td>Gerald R. Fink</td>
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<td>L5713</td>
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<td>crp2^hisG</td>
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<td>Jamie Lasswell</td>
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</table>

All strains have the same genotype as the parent strain with the modifications indicated in the genotype column. Strains DY1457 and ZHY3 were a gift from Dr. Mary Lou Geurinot (Dartmouth College); L5708 and L5713 were provided by Dr. Kendal Hirschi (Baylor College of Medicine).
To assay for possible IAA-amino acid or JA-amino acid conjugate uptake by the purified microsomes, I incubated 50 μL of microsomes from each yeast strain with 50 μL microsome buffer containing 2 mM of the conjugate to be tested, 2 mM MnCl₂ (as a cofactor for the hydrolases), 10 mM MgCl₂, and 10 mM ATP. Control reactions were set up in the same manner with addition of 0.1% TritonX-100 or using supernatant rather than microsomes in the reaction. Conjugates tested were: IAA-Ala, IAA-Leu, IAA-Phe, JA-Ala (more and less polar forms), JA-Leu, and JA-Phe. Reactions were incubated at room temperature for 12 - 48 hours. Two μL samples from each reaction and 1 μL unreacted control were spotted on TLC plates and resolved using a solvent of 49.5% hexane, 49.5% ethyl acetate, and 1% acetone. Plates were stained with Van Urk-Salkowski reagent (Ehmann, 1977) for IAA conjugates or with anisaldehyde for JA conjugates. Alternately, 180 μL of JLY8 and JLY10 microsomes were incubated with 400 μL microsome buffer containing 2 mM of the conjugate to be tested, 10 mM ATP, and 10 mM MgCl₂ for 12 hours at room temperature. Reactions were spun down in a tabletop ultracentrifuge at 100,000g to pellet microsomes. Each pellet was resuspended in 100 μL ethanol. Three μL of the microsomes were run along with one μL of the supernatant on TLC plates as described above.

III.O. Analysis of flowering time

For flowering time studies, seeds were plated on PNS and grown in 16 hr. white light:8 hr. dark (LD conditions) for 9 days, then transferred to soil and maintained in LD conditions. For short day experiments, seeds were sown directly in moist soil and grown in 8 hr. white light, 16 hr. dark (SD conditions). Plants were examined daily for the first sign of bud development in the shoot apical meristem. Days to flowering was recorded as the number of days from plating seeds on PNS until bud identification. Leaf number was counted for each plant on the day of bud identification, and included mature and immature leaves present in the rosette.
III.P. Protein blot analysis

III.P.1. Protein blot analysis of pBIN19IAR1-myc transgenic plants.

To determine the best expressing lines of pBIN19IAR1-myc transgenics, Col-0 plants homozygous for the pBIN19IAR1-myc construct were grown in soil. Buds and immature siliques were harvested into liquid nitrogen and stored at -80 °C. Protein was extracted from the samples as described (Purugganan, 1997), and sample concentrations were determined using the Pierce BCA Assay (Pierce, Rockford, IL). 30 μg of each sample were separated on a 6% SDS-PAGE (Ausubel et al., 1995). The gel was electroblotted for 45 min. at 24 volts in Tris-glycine buffer, pH 8.3 using a Genie electrophoretic blotter (Idea Scientific, Minneapolis, MN) onto Hybond ECL nitocellulose membrane (Amersham, Arlington Heights, IL). The blot was blocked in blocking buffer: 1X TBS (diluted from 10X TBS: 0.15 M Tris HCl, 0.04 M Tris base, 1.37 M NaCl, pH 7.6), 0.1% Tween-20, 5% milk for one hour at room temperature. The blot was then washed three times for five minutes with 1X TBS, 0.1% Tween-20, then probed with the anti-myc antibody diluted 1:1000 in blocking buffer (Santa Cruz Biotechnology, Santa Cruz, CA). The blot was washed (three times for five minutes in 1X TBS, 0.1% Tween-20), then probed with the secondary anti-mouse HRP conjugated antibody (NEB1) diluted 1:1000 in blocking buffer. Blots were again washed (three times for five minutes in 1X TBS, 0.1% Tween-20) and imaged by incubating in Lumiglo reagent (NEB) followed by autoradiography.

Membranes were isolated from tissue samples from expressing lines as follows: tissue (approximately 2 g, combined from four pBIN19IAR1-myc lines) was ground in liquid nitrogen with a mortar and pestle, resuspended in 10 mL cold extraction buffer (290 mM sucrose, 2 mM EDTA, 250 mM Tris-HCl, pH 8.5, 2 mM PMSF, and 76 mM β-mercaptoethanol), then spun at 6000g, 20 min. at 4 °C to remove intact organelles and cell walls. The resulting supernatant was centrifuged at 100,000g, 4 °C, for one hour to pellet
membranes. The membrane pellet was resuspended in 0.5 mL extraction buffer using a Dounce homogenizer, and stored at -80 °C. Two-phase partitioning was used to separate plasma membranes from organelle membranes as described (Larsson, 1985).

**III.P.2. Protein blot analysis of PhyA stability in ft/f1**

Col-0 and ft/f1 mutant seeds were sterilized and plated on sterile filter paper atop PNS. Plates were incubated at 22 °C in white light for 24 hours, then transferred to continuous darkness. After six days, plates were transferred to white light and samples harvested into liquid nitrogen at 0, 1, 2, 4, 6, and 8 hr. (the 0 time point was harvested prior to transfer to light). Protein was extracted from the samples as described (Purugganan, 1997), and sample concentrations were determined using the Pierce BCA Assay (Pierce, Rockford, IL). 20 μg of each sample were separated on a 6% SDS-PAGE (Ausubel et al., 1995). The gel was electroblotted for 45 min. at 24 volts in Tris-glycine buffer, pH 8.3 using a Genie electrophoretic blotter (Idea Scientific, Minneapolis, MN) onto Hybond ECL nitocellulose membrane (Amersham, Arlington Heights, IL). Probing was as described in section III.P.1, except the blot was probed with the O73D anti-PhyA antibody at a dilution of 1:1000 (Shanklin, 1988); kindly provided by Dr. Richard Vierstra, UW-Madison) rather than anti-myc as the primary antibody.

**III.Q. Enzyme assays with IAR3**

Purified recombinant IAR3 protein (Davies et al., 1999; provided by R. Tellez) was incubated at room temperature with 1 mM IAA-Ala and 1 mM of the metal cofactor to be tested or 1 mM MnCl₂ plus 0.2, 0.5, 1, 2, or 5 mM CuSO₄ or ZnSO₄ for 5 hours. Three μL of each reaction were run on a TLC plate using a 49.5% hexane/49.5% ethyl acetate/1% acetic acid solvent and stained for indoles with Van Urk-Salkowski reagent (Ehmann, 1977).
Table III-4: Plasmids constructed for this study

<table>
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<th>plasmid name</th>
<th>description</th>
<th>strain number</th>
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<tbody>
<tr>
<td>pBARNIAI1</td>
<td><em>IAI</em> cDNA in plant transformation vector</td>
<td>540</td>
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<tr>
<td>pBARNZTL1</td>
<td><em>ZTL1</em> cDNA in plant transformation vector</td>
<td>670</td>
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<td>IAR1pGUS</td>
<td><em>IAI</em> promoter fused to <em>GUS</em> gene in pBl101.1</td>
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<tr>
<td>FKFIpGUS</td>
<td><em>FKFI</em> promoter fused to <em>GUS</em> gene in pBl101.1</td>
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<td><em>IAI</em> cDNA in pBluescript</td>
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<td><em>IAI</em> cDNA mutagenized to contain NruI site prior to last codon</td>
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<tr>
<td>KSIAIR1c+gNruI</td>
<td>hybrid of <em>IAI</em> genomic and cDNA in pBluescript. First 8 exons are genomic, has <em>IAI</em> promoter. Last four exons are cDNA, has 3’ UTR. Mutagenized to contain NruI site prior to last codon.</td>
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<td>psmGFP-NruI</td>
<td>psmGFP (Davis and Vierstra, 1996) mutagenized to contain NruI site prior to start codon of GFP</td>
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<td>KSIAIR1c containing Sacl/NruI fragment of psmGFP-NruI. Encodes IAR1-GFP fusion</td>
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<td>KSIAIR1c+gmyc</td>
<td>KSIAIR1c+gNruI containing 5Xmyc tag at 3’ end of <em>IAI</em>. Encodes IAR1-myc fusion</td>
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<td><em>IAI</em> cDNA under control of <em>GAL1</em> promoter in yeast expression vector pRS314</td>
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<td>pRS314GALIAIR1 -GFP</td>
<td>IAR1-GFP fusion in yeast expression vector. Under control of <em>GAL1</em> promoter</td>
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<td>6.4 kb genomic EcoRI fragment containing <em>IAI</em> in plant transformation vector pBIN19</td>
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<td>pBINFKF1g</td>
<td>4.3 kb fragment (<em>FKFI</em> coding region, 1.7 kb of 5’-noncoding region and 0.7 kb of 3’-noncoding region) in pBIN19</td>
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<tr>
<td>pBIN19IAR1c+g</td>
<td>insert of KSIAIR1c+gGFP in plant transformation vector pBIN19</td>
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<tr>
<td>pBIN19IAR1c+gmyc</td>
<td>insert of KSIAIR1c+gmyc in plant transformation vector pBIN19</td>
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</table>
CHAPTER IV: ILL5 IS A MEMBER OF THE ARABIDOPSIS AMIDOHYDROLASE GENE FAMILY

Part of the work discussed in this chapter has been published (Davies et al., 1999); the degenerate oligonucleotides used to begin the project were designed by Bonnie Bartel.

IV.A. Degenerate PCR to identify ILR1 homologs

The IAA-conjugate resistant phenotype of iar1 plants suggested that the gene defective in the mutant might encode an amidohydrolase similar to ILR1. Several genes encoding amidohydrolases in the ILR1 family had been previously identified (Bartel and Fink, 1995). To determine whether IAR1 also encoded an amidohydrolase, we identified additional ILR1-like genes and compared their map positions to that of iar1. Degenerate PCR primers were designed from highly conserved regions of ILR1, ILL1, and ILL2 (figure IV-1). PCR with these primers on Arabidopsis total genomic DNA resulted in five discrete bands ranging in size from 535 to about 700 bp when separated by electrophoresis on an agarose gel (figure IV-2). I gel purified these PCR products, TA cloned them into the pT7Blue vector, and transformed them into E.coli. I then restriction mapped the products to determine the identity of the gene from which they were amplified. I was able to identify ILR1, ILL1, ILL2, ILL3, IAR3, and one new gene, ILL5, by this method. Also amplified by the degenerate primers was a 1.3 kb band (figure IV-2) that is probably from the amidohydrolase-like GRI gene identified as a tagged gene in a mutant susceptible to Peronospora infection (GenBank accession number AJ010735).

I attempted to isolate an ILL5 cDNA by hybridizing an Arabidopsis cDNA library (Minet et al., 1992) with radiolabeled degenerate PCR product from ILL5, but all clones hybridizing to the probe were determined to be IAR3 by restriction mapping. I therefore
Figure IV-1: Degenerate oligos designed from regions conserved among ILR1, ILL1, and ILL2 for PCR of *ILRI*-like genes from Arabidopsis. Black triangles indicate the positions of introns in the genes encoding these proteins. In the oligo sequences, H indicates A, C, or T; N indicates A, C, G, or T; R indicates A or G; S indicates C or G; and Y indicates C or T.
Figure IV-2: PCR with degenerate oligos designed from conserved regions of ILR1, ILL1, and ILL2. The genomic structure of the amidohydrolase genes is shown at left: exons are indicated by rectangles and introns by connecting lines. The arrows indicate the location of degenerate PCR primers used to amplify genomic DNA. The gel shown at right is a 2% agarose gel stained with ethidium bromide. Lane 1 contains the product of PCR amplification of Arabidopsis genomic DNA (ecotype WS); lane 2 contains molecular size markers, given in kb to the right of the gel.
turned to a genomic library (Olszewski et al., 1988). A cosmid hybridizing to an IAR3 probe was provided by David Goetz, a former undergraduate in our laboratory. This cosmid was found to contain two HindIII fragments that hybridized to the ILL5 probe. Because there were no HindIII sites in the probe, I reasoned that the two bands represented two separate genes. The restriction map of IAR3 indicated that the smaller band represented IAR3, and that the larger 5.5 kb band therefore contained ILL5. This band was gel purified and subcloned into pUC19 and used to obtain the sequence of ILL5 (Figure IV-3).

IV.B. ILL5 is most closely related to IAR3

ILL5 (GenBank accession #AF085806) is very similar in sequence to IAR3 (GenBank accession #AF081067), which encodes an amidohydrolase specific for IAA-Ala (Davies et al., 1999). In fact, the predicted ILL5 and IAR3 proteins are 83% identical, whereas ILL5 is only 43.3% to 55.8% identical to the other known amidohydrolase genes of Arabidopsis (figures IV-3, IV-4). This suggests ILL5 might also be an amidohydrolase with activity toward IAA-Ala.

However, ILL5 is not the gene defective in the iar1 mutant because it maps too close to IAR3 on chromosome 1. ILL5 and IAR3 were found on the same 20 kb cosmid, whereas IAR3 and the iar1 mutation map 30 cM apart on chromosome 1. Because, in Arabidopsis, a cM is approximately 200 kb (Koornneef, 1994), the distance between iar1 and IAR3 can be estimated as 6000 kb. Even when variability in recombination frequencies is taken into account, this is still a distance obviously much larger than 20 kb (figure IV-5).

IV.C. Col-0 is an ill5 mutant

Once I had the genomic sequence of ILL5, I again tried to clone an ILL5 cDNA. I was not able to identify a cDNA for this gene from Col-0 by RT-PCR. The reason became clear upon close examination of the ILL5 genomic sequence: the 3' splice acceptor site of intron 2 in the Col-0 sequence is mutated from the conserved AG:G (Brown et al., 1996)
Figure IV-3: Protein alignment of the Arabidopsis family of amidohydrolases. Sequences were aligned with the Megalign program (DNASTar, Madison, WI) using the Clustal method (Higgins and Sharp, 1989). Residues identical in at least four of the six sequences are shaded in black. The ILL5 sequence is from a conceptual splice of the genomic sequence because no ILL5 cDNA has been identified; other sequences are derived from cDNAs.
<table>
<thead>
<tr>
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<th>ILL2</th>
<th>ILL3</th>
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Figure IV-4: The predicted ILL5 protein is most similar to IAR3. Percent identity and percent divergence for the amidohydrolase family of proteins in Arabidopsis was determined in the Megalign (DNA Star) program.
Figure IV-5: Map positions of *IAR3*, *ILL5*, and the *iar1* mutation on Arabidopsis chromosome 1. Positions of the markers nga248, nga111, and nga280 (Bell and Ecker, 1994) are from the February 1998 version (http://genome-www.stanford.edu/Arabidopsis/ww/Feb98RImaps/index.html) of a Col-0/Ler recombinant inbred map (Lister and Dean, 1993). Approximate map positions in centimorgans of *IAR3*, *ILL5*, and *iar1* are shown in parenthesis.
to AT:G (figure IV-6A). To confirm that the mutation was not an artifact of the cloning process and to determine if other ecotypes harbored this mutation as well, I performed PCR on genomic Col-0, WS, and Landsberg erecta (Ler) DNA with primers flanking the intron 2/exon 3 junction. I then sequenced the PCR product directly. Col-0 indeed has the G → T mutation, while WS and Ler have the conserved AG:G sequence (figure IV-6B, WS data not shown). Because all Arabidopsis introns have a G at this position (Brown et al., 1996), Col-0 is likely an ill5 mutant.

IAR3 is an amidohydrolase with specificity toward IAA-Ala, and iar3 mutants are resistant to root elongation inhibition by IAA-Ala (Davies et al., 1999). Because of the similarity between IAR3 and the predicted ILL5 protein, I tested the response of Col-0 (a presumed ill5 mutant), WS, and Ler seedlings to 10, 20, 30, and 40 μM IAA-Ala to determine whether the ill5 mutation renders Col-0 less sensitive than WS or Ler to inhibition of root elongation by this conjugate. Col-0 plants are not less sensitive than the other ecotypes to IAA-Ala; in fact, they are more sensitive at some concentrations, although only slightly (figure IV-7).

IV.C. Discussion

Arabidopsis encodes a family of proteins similar to the ILR1 IAA-amino acid conjugate hydrolase ILR1 (Bartel and Fink, 1995; Davies et al., 1999). The predicted ILL5 protein is a member of this family, and is most closely related (83% identical) to IAR3, an IAA-amino acid conjugate hydrolase with specificity for IAA-Ala (Davies et al., 1999). It is therefore possible that ILL5 is an IAA-amino acid conjugate hydrolase, and its similarity to IAR3 suggests that the ILL5 enzyme might cleave IAA-Ala. However, ILL5 might also cleave conjugates other than IAA-Ala; the proteins ILL1 and ILL2 are 87% identical at the amino acid level but have different activity profiles of conjugate cleavage (Tellez and Bartel, unpublished). It is also possible that ILL5 does not have activity toward any IAA-amino
acid conjugate but cleaves the amide bond of molecules such as IAA-peptide conjugates, dipeptides, or amino acid conjugates of other hormones, such as jasmonic acid (JA).

Most members of the ILR1-like family of proteins terminate with the amino acid sequence (H or K)DEL or a derivative thereof. This sequence signals plant proteins to be retrieved to the lumen of the endoplasmic reticulum, or ER (Bednarek and Raikhel, 1992). The predicted ILL5 protein ends in KDEL, suggesting that it, like IAR3, ILR1, ILL1, and ILL3, might reside in the ER.

I was unable to identify an ILL5 cDNA from a Ler cDNA library (Minet et al., 1992) or by RT-PCR on Col-0 RNA. The lack of an ILL5 cDNA in Col-0 can be explained by the fact that the intron 2/exon 3 junction of ILL5 in this ecotype is mutated from the consensus AG:G (Brown et al., 1996) to AT:G. This change in the splice acceptor site is likely to cause mis-splicing of the ILL5 message. Therefore, Col-0 may represent an ill5 mutant and ILL5 protein may be present only in certain ecotypes of Arabidopsis.

The ILL5 splice site mutation was not present in the other ecotypes examined (WS and Ler), indicating that they might properly splice ILL5. The fact that no ILL5 cDNA was obtained from a Ler library may reflect the relative abundance of IAR3 and ILL5 message; clones from the library that hybridized to an ILL5 probe were in fact IAR3 cDNAs. Another possibility is that ILL5 is a non-transcribed pseudogene that is no longer expressed in Arabidopsis.

To determine whether the ill5 mutation of Col-0 caused this ecotype to be less sensitive to root elongation inhibition by exogenous IAA-Ala than other ecotypes, I tested the response of Col-0, WS and Ler seedlings to IAA-Ala. Col-0 plants were not more resistant to the effects of IAA-Ala than WS or Ler. In fact, they are slightly more sensitive than the other ecotypes (figure IV-7). There are several possible explanations for this observation. First, the ILL5 protein may not cleave IAA-Ala but, rather, another amino acid conjugate. Testing the response of the various ecotypes to additional conjugates would begin to address this possibility. Second, ILL5 may cleave IAA-Ala but not normally
Figure IV-6: Col-0 is an ill5 splicing mutant. A). The splicing mutation identified in ill5 from Col-0 DNA alters the canonical AG:G splice acceptor sequence of intron 2 to AT, which may lead to mis-splicing of the ill5 message. B). The presence of this mutation in Col-0 (B1) was confirmed by PCR amplifying and sequencing the region from genomic DNA. The region was also sequenced from Ler (B2) and WS (not shown) DNA, which do not have the mutation. The site of the G -> T change in Col-0 is shaded in gray.
Figure IV-7: The mutation in *ILL5* does not render Col-0 less sensitive to inhibition of root elongation by IAA-Ala. Seeds of Col-0, WS, and Ler ecotypes were plated on the indicated concentrations of IAA-Ala. After eight days under yellow-filtered light at 22 °C, the longest root of each seedling was measured. Data were standardized against growth on unsupplemented media. Asterisks indicate significant difference from Col-0; diamonds indicate significant difference from WS; and filled circles indicate significant difference from Ler by Student’s t test, *p* < 0.0001. Error bars are standard deviations of the means (*n* = 20).
function in the root. Third, *ILL5* might not be expressed in any of the ecotypes tested. In this case no difference in Col-0, WS, or Ler response to IAA-Ala could be attributed to *ILL5*.

The isolation of an *ILL5* cDNA is a key step in understanding the role of *ILL5* in auxin conjugate metabolism. If a cDNA can be identified, then the expression of the gene in at least some ecotypes of Arabidopsis would be confirmed. This would allow the expression and purification of recombinant *ILL5* protein, which would facilitate the determination of *ILL5* substrate specificity. An alternative approach would be to monitor various Arabidopsis ecotypes for *ILL5* expression by RT-PCR with *ILL5*-specific primers.
CHAPTER V. MUTANT CHARACTERIZATION AND CLONING OF THE IAR1 GENE

The iar1-1, iar1-2, iar1-3 and iar1-4 alleles were isolated by Bonnie Bartel. iar1-5 and iar1-6 were isolated and mapped by Mindy A. Cohen, and iar1-7 by Rosie Tellez and Melanie Stagger. Bonnie Bartel performed the iar1 mutagenesis, iar1 backcrosses, the initial iar1 mapping, and the iar1/alf1 crosses and phenotypic analysis. Luise E. Rogg and Catherine Rongey carried out most of the DNA preparation from F2 mapping plants and analysis of molecular markers in these plants, and assisted in the development of new molecular markers. Luise E. Rogg assisted with construction of clones for complementation of the mutant phenotype and in some phenotypic analysis. David C. Nelson constructed the Sau3AI library from BAC T7E4.

V.A. Phenotype of the iar1 mutant

V.A.1. iar1 is resistant to IAA-amino acid conjugates, but not to free IAA

Regulation of free IAA levels within a plant is essential for proper plant development. IAA conjugates are likely to play a significant role in this regulation, but the genes and enzymes involved in conjugate formation and hydrolysis are only beginning to be characterized (reviewed in Normanly and Bartel, 1999). To identify possible components of the IAA conjugate metabolic machinery, we screened for mutants resistant to the inhibition of root elongation by IAA-amino acid conjugates. The iar1 (IAA-Ala resistant) mutant was originally identified in this screen because it is able to elongate roots on normally inhibitory concentrations of IAA-Ala (figure V-1). Seven different alleles of the iar1 mutant have been identified in four separate screens (table V-1). iar1-1 and iar1-2 were isolated in the WS ecotype from progeny of EMS-mutagenized seeds; iar1-3 and iar1-4 are in the Col-0
Figure V-1: The *iar1* mutant is resistant to IAA-Ala but not to free IAA. WS (wild type) or *iar1-1* mutant seeds were sown on unsupplemented media or media containing 0.2 μM IAA or 40 μM IAA-Ala and grown for eight days under continuous yellow-filtered light. Seedlings were photographed after eight days.
background and were generated by fast-neutron bombardment, which is known to cause deletions in Arabidopsis (Bruggemann et al., 1996); iar1-5, iar1-6, and iar1-7 were isolated as EMS-induced enhancers of the IAA-Leu resistant mutant ilrl (Bartel and Fink, 1995) in the WS ecotype.

Table V-1: Alleles of the iar1 IAA-Ala resistant mutant

<table>
<thead>
<tr>
<th>iar1 allele</th>
<th>ecotype</th>
<th>mutagen</th>
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<th>isolated by:</th>
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<td>IAA-AlaR</td>
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<td>iar1-3</td>
<td>Col-0</td>
<td>fast neutrons</td>
<td>IAA-AlaR</td>
<td>B. Bartel</td>
</tr>
<tr>
<td>iar1-4</td>
<td>Col-0</td>
<td>fast neutrons</td>
<td>IAA-AlaR</td>
<td>B. Bartel</td>
</tr>
<tr>
<td>iar1-5</td>
<td>WS</td>
<td>EMS</td>
<td>ilrl enhancer</td>
<td>M. Anderson</td>
</tr>
<tr>
<td>iar1-6</td>
<td>WS</td>
<td>EMS</td>
<td>ilrl enhancer</td>
<td>M. Anderson</td>
</tr>
<tr>
<td>iar1-7</td>
<td>WS</td>
<td>EMS</td>
<td>ilrl enhancer</td>
<td>R. Tellez and M. Stagger</td>
</tr>
</tbody>
</table>

To quantitate the IAA-Ala resistance of six of these alleles (iar1-1 through iar1-6), I plated seeds of each mutant allele along with the corresponding wild type parent strains (Col-0 for iar1-3 and iar1-4; WS for iar1-2, iar1-2, iar1-5 and iar1-6) on media containing 20 or 40 μM IAA-Ala. The allele with greatest resistance to inhibition of root elongation by IAA-Ala is iar1-4, which was completely resistant to both 20 and 40 μM IAA-Ala (figure V-2A). The weakest allele is iar1-5, which is only slightly resistant to 40 μM IAA-Ala (figure V-2C).

To determine if the iar1 mutant is specifically resistant to IAA conjugates and not generally auxin resistant, I tested the effects of free IAA on root elongation in the mutant.
Figure V-2: *iar1* mutants are resistant to inhibition of root elongation by IAA-Ala, but are sensitive to IAA. Wild type (Col-0 or WS) seeds and *iar1* mutant seeds were sterilized and plated on media containing 20 or 40 μM IAA-Ala (A, C) or 50, 100, or 200 nM IAA (B,D). After eight days the longest root of each seedling was measured. Data were normalized against growth on unsupplemented media. Asterisks indicate measurement is significantly different from wild type by Student's t test, *p* < 0.0001. Error bars indicate standard deviations of the means (*n* = 12).
iarl plants are not significantly resistant to IAA (figure V-2B,D; figure V-1), indicating that
the mutation specifically alters conjugate sensitivity.

As previously mentioned, several of the iarl alleles were isolated in screens for
effectors of the ilrl amidohydrolase mutant. The ilrl mutation confers resistance to
inhibition of root elongation by IAA-Leu but not IAA-Phe (Bartel and Fink, 1995), although
the amidohydrolase encoded by ILRL hydrolyzes both of these conjugates (Bartel and Fink,
1995). The sensitivity of ilrl mutants to IAA-Phe may reflect redundancy in substrate
specificity among the Arabidopsis amidohydrolase family; several of the enzymes cleave
IAA-Phe efficiently (Tellez and Bartel, unpublished). The iar1-5 and iar1-6 mutants were
isolated in an ilrl background by screening for increased resistance to IAA-Leu. The iar1-
7 allele was identified as an enhancer of ilrl that is resistant to IAA-Phe. The isolation of
iarl alleles in the ilrl background that led to IAA-Phe resistance and increased resistance to
IAA-Leu suggested that the iarl mutation alone might also lead to resistance to these
conjugates. I tested the effects of these conjugates on root elongation in iar1-3 mutant
plants over a range of concentrations from 10 μM to 40 μM. The mutant is resistant to
IAA-Phe (figure V-3) at all concentrations tested, but is resistant to IAA-Leu only at low
concentrations (10 - 20 μM; figure V-3). Therefore, the iarl mutation affects plant
responses not only to IAA-Ala, but to other conjugates as well, albeit to varying degrees.

I then tested the response of the mutant to 20 μM concentrations of IAA-Gly, IAA-
Gln, and IAA-Glu, three additional IAA-amino acid conjugates that have IAA-like effects on
Arabidopsis root elongation (LeClere and Bartel, unpublished). The conjugated moieties of
these molecules encompass a range of characteristics, from small and uncharged (Gly) to
larger and negatively charged (Glu) or neutral (Gln). iarl roots are resistant to elongation
inhibition by all of these conjugates (figure V-4), providing additional evidence that IAR1 is
involved in IAA conjugate sensitivity in a general, rather than conjugate-specific, manner.
Figure V-3: *iar1* mutants are resistant to IAA-Phe and IAA-Leu. Col-0 wild type and *iar1-3* mutant seeds were plated on media containing the indicated concentration of IAA-Phe or IAA-Leu. After eight days, the longest root of each seedling was measured. Data were standardized against growth on unsupplemented media. Asterisks indicate significant difference from wild type by Student's t test, *p* < 0.0001. Error bars are the standard deviations of the means (*n* = 14).
Figure V-4: iar1 mutants are resistant to various IAA-amino acid conjugates. Wild type and iar1 mutant seeds were plated on the indicated conjugates at 20 μM. After eight days, the longest root of each seedling was measured. Data were normalized to growth on unsupplemented media. Asterisks indicate significant difference from wild type using the Student's t test, $p < 0.001$. Error bars are standard deviations of the means ($n = 18$).
V.A.2. *iarl* mutants elongate hypocotyls normally at high temperature and are resistant to hypocotyl elongation inhibition by IAA-Ala

IAA is involved in various growth responses, including hypocotyl elongation in light-grown plants. Arabidopsis seedlings display increased hypocotyl elongation at high temperatures (28 °C versus 22 °C), which is correlated with increased endogenous auxin concentrations (Gray et al., 1998). Additionally, auxin transport is required for hypocotyl elongation in light-grown seedlings, but not in dark-grown seedlings (Jensen et al., 1998). I therefore determined whether *iarl* mutants displayed defects in auxin-mediated hypocotyl elongation at high temperature or in various light conditions. I compared hypocotyl elongation in *iarl* and wild type plants grown at 22 and 28 °C in yellow light, in the dark, and under various light conditions to determine if the mutant has any hypocotyl elongation defects in these conditions. Most alleles of *iarl* mutant seedlings elongate their hypocotyls normally in all conditions tested, including high temperature (figure V-5), dark (figure V-6), and white, yellow-, red- or blue-filtered light (figure V-6). *iarl-4* seedlings have shorter hypocotyls than wild type when grown in the light (figure V-5), but this phenotype is due to a mutation in a gene other than *IAR1* (see Chapter VII) and the mutant still elongates its hypocotyls in response to high temperature and dark (figure V-5).

IAA and IAA conjugates, when included at high concentrations in the growth medium, can inhibit elongation of Arabidopsis hypocotyls (figures V-7, V-8). Because the *iarl* mutant is resistant to root elongation inhibition by IAA conjugates but not by free IAA, I tested the response of the mutant to these compounds in hypocotyl elongation. The *iarl* mutant remains sensitive to hypocotyl elongation inhibition by free IAA in all cases (figure V-7), but *iarl* mutants are resistant to the inhibitory effects of IAA-Ala on hypocotyl elongation (figure V-8). This data suggests that the IAR1 protein functions in the hypocotyl as well as in the root of Arabidopsis seedlings.
Figure V-5: *iar1* mutants elongate hypocotyls normally in response to high temperature. Wild type and *iar1* mutant seeds were plated on sterile media and grown for eight days in the indicated temperature. The hypocotyl length of each seedling was measured. Asterisks indicate measurements significantly different from wild type by the Student's t test, $p < 0.0001$. Error bars are standard deviations of the means ($n = 12$).
Figure V-6: iar1 mutants exhibit normal hypocotyl elongation. Col-0 wild type and iar1 mutant seeds (iar1-2 seeds used in this experiment had been outcrossed to Col-0 twelve times) were plated on unsupplemented media and grown in the indicated light condition for seven days at 22 °C. For the dark-grown seedlings, plates were placed in white light for 24 hours prior to transfer to darkness to aid in germination. The hypocotyl lengths of the seedlings were measured. No measurements were significantly different from wild type by Student’s t test, p < 0.0001. Error bars are standard deviations of the means (n = 18).
Figure V-7: IAA inhibits Arabidopsis hypocotyl elongation. Wild type and iar1 mutant seeds were plated on media containing the indicated concentrations of IAA. After eight days in yellow-filtered light at 22 °C, the hypocotyl of each seedling was measured to the nearest mm. Data were normalized against growth on unsupplemented media. No measurements showed significant difference from wild type using the Student's t test, $p < 0.0001$. Error bars are standard deviations of the means ($n = 20$, except for iar1-2 measurements, where $n = 10$).
Figure V-8: *iarl* mutants are resistant to inhibition of hypocotyl elongation by IAA-Ala. Wild type and *iarl* mutant seeds were plated on media containing either no IAA-Ala or 150 µM IAA-Ala. After eight days in yellow-filtered light at 22 °C, the hypocotyl length of each seedling was measured. Asterisks indicate significant difference from wild type using the Student's t test, $p < 0.0001$. Error bars are standard deviations of the means ($n = 20$, except for *iarl*-2 measurements, where $n = 10$).
V.A.3. *iarl* mutants may be early germinating

IAA conjugates may serve as a reservoir of IAA for germinating seedlings. In *Phaseolus vulgaris*, IAA-amide conjugates represent about 80% of the total IAA in the mature seed, which correlates with the amount of free and ester conjugated IAA lost during seed maturation (Bialek and Cohen, 1989). During germination in *Zea mays* and rice, there is a large loss of IAA conjugates from the seed (Cohen and Bandurski, 1982). The level of amide conjugates in seeds of *Phaseolus vulgaris* also drops substantially at the start of germination (Bialek and Cohen, 1992). In Arabidopsis, conjugate hydrolases are expressed early in seedling development, and may function to provide IAA to the germinating seedling (Tellez and Bartel, unpublished). If *iarl* mutants are defective in their ability to metabolize IAA conjugates, they might display germination time defects as a result. I therefore tested the germination time of the *iarl* mutant. To control for variables such as seed age, storage conditions, and growth conditions of the parent plant, I used a segregating F2 population from a cross of wild-type Col-0 to the *iarl*-3 mutant. I plated these seeds on unsupplemented media and examined them periodically for germination. To determine which plants were *iarl*-3 homozygotes, I transferred germinated seeds to IAA-Ala containing media and examined seedling root elongation several days later. Surprisingly, IAA-Ala resistant plants were earlier germinating than IAA-Ala sensitive plants in this experiment (figure V-9). This is contrary to the expected result, which is that seedlings unable to properly metabolize IAA conjugates would germinate later than wild type seedlings.

V.A.4. The *iarl* mutation partially suppresses the *alf1* mutant phenotype

The Arabidopsis mutant *alf1* (Celenza et al., 1995), also isolated as *surl*, *ivr*, *hls*3, and *rty* (Boerjan et al., 1995; King et al., 1995; Lehman et al., 1996) overproduces both free and conjugated IAA. *alf1* plants have shortened primary roots, display increased
Figure V-9: iar1 mutants may be early germinating. F₂ seeds from a cross of iar1-3 to Col-0 were sterilized and plated on unsupplemented growth media. Plates were placed in yellow light at 22 °C. Seeds were examined periodically under a dissecting microscope, and germination was defined as the time when the radicle emerged from the seed coat. Germinated seeds were transferred to plates containing 50 μM IAA-Ala, and root elongation was examined after five days. IAA-Ala resistant plants are presumably iar1-3 homozygotes. n = 184. Percent IAA-Ala sensitive plants was 83, percent IAA-Ala resistant plants was 17. This experiment has not yet been repeated.
numbers of adventitious and lateral roots, and are completely sterile (Celenza et al., 1995). These phenotypes can be copied by addition of exogenous auxin to wild-type plants (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995; Lehman et al., 1996). To investigate whether the IAR1 gene influences IAA levels in vivo, we crossed the iar1 mutant to an alf1/ALF1 heterozygote. Root elongation on unsupplemented media of resultant iar1/iar1 alf1/alf1 homozygotes was measured after eleven days and compared to that of wild type and the parent mutants. The iar1 mutation suppresses the root elongation phenotype of the alf1 mutant (figure V-10). iar1 also partially rescues the sterility defect of alf1 as double mutant plants yielded a few seeds compared to no seeds from the alf1 mutant alone. The fact that mutations in IAR1 are able to alleviate auxin-related defects in the alf1 auxin overproducing mutant indicates that the IAR1 gene product normally plays a role in increasing free IAA levels or IAA sensitivity in vivo.

V.A.5. The iar1 mutant responds normally to IBA, JA, MeJA, and JA conjugates

Indole-3-butyric acid (IBA) is a naturally occurring form of auxin that is present at levels lower than those of IAA in Arabidopsis tissues (reviewed in Epstein and Ludwig-Müller, 1993). IBA has IAA-like effects on plant growth, although it is controversial whether these effects are due to the conversion of IBA to IAA within the plants or if IBA mediates signalling independent of IAA. There is evidence to support the former as the peroxisome is presumed to be the site of IBA to IAA conversion by a mechanism similar to fatty acid β-oxidation (reviewed in Gerhardt, 1992), and several IBA-resistant, IAA-sensitive mutants have been identified that have peroxisomal defects (Zolman and Bartel, unpublished). However, this does not preclude the presence of both pathways in mediating IBA effects. To determine if iar1 mutants have altered responses to this naturally occurring auxin, I tested the effects of IBA on root elongation in mutant seedlings. iar1 is not significantly different from wild type in root elongation on IBA (figure V-11),
Figure V-10: The iar1 mutation suppresses the alf1 root elongation phenotype. Seeds of WS wild type, the iar1-1 and alf1 single mutants, and the iar1-1/alf1 double mutant were plated on unsupplemented media. After eleven days in yellow-filtered light at 22 °C, the root of each seedling was measured. Error bars are standard deviations of the means (n ≥ 4).
Figure V-11: *iar1* mutants are not resistant to IBA. *iar1* mutant and wild type seeds were plated on media containing 10 μM IBA. After eight days in yellow-filtered light at 22 °C, the longest root of each seedling was measured. Data were standardized against growth on unsupplemented media. No measurements were significantly different from wild type by Student’s t test, $p < 0.0001$. Error bars are standard deviations of the means ($n \geq 11$).
indication that the *IAR1* gene is likely not involved in IBA signaling or metabolism.

Jasmonic acid (JA) is a plant hormone involved in plant defense responses. Application of JA or its methyl ester, methyl jasmonate (MeJA), to plants can induce expression of a number of genes implicated in defense processes (Staswick, 1995). Interestingly, a cDNA named *JR3*, which comes from the *IAR3* gene, was identified as JA-induced by differential display (Titarenko et al., 1997). *IAR3/JR3* is also rapidly and transiently upregulated by wounding, and this wound- and JA-induced expression is blocked in the JA-insensitive mutant *coil* (Rojo et al., 1998; Titarenko et al., 1997), suggesting a role for IAA conjugates in wound responses. JA and JA conjugates can inhibit root elongation. Therefore, I tested the response of *iar1* to JA, MeJA, and various JA-conjugates in root elongation and found no significant differences between wild type and the mutant (figure V-12).

### V.A.6. The *iar1* mutant phenotype is suppressed by manganese

The Arabidopsis amidohydrolases characterized to date all require a metal cofactor for optimal hydrolase activity. The cofactors with greatest efficacy in enzyme assays using Arabidopsis IAA-conjugate hydrolases expressed in and purified from *E. coli* are Mn$^{2+}$ and Co$^{2+}$ (Davies et al., 1999; Tellez and Bartel, unpublished). Mutations in these amidohydrolase genes can lead to IAA-amino acid conjugate resistance (Bartel and Fink, 1995; Davies et al., 1999), so mutations that prevent the hydrolases from assembling with or retaining the proper cofactor might also cause this phenotype. I tested the response of the *iar1* mutant, as well as the response of *iar3 ilrl* mutants, to Mn$^{2+}$ alone or to IAA-Ala plus Mn$^{2+}$. There were no differences in response between wild type and the mutants to Mn$^{2+}$
Figure V-12: *iar1* is not resistant to JA, MeJA, or JA conjugates. *iar1* mutant and wild type seeds were plated on the indicated concentrations of JA, MeJA, or JA conjugate. JA-Ala < is the less polar form of JA-A; JA-A > is the more polar form. After eight days in yellow-filtered light at 22 °C, the longest root of each seedling was measured. Data were normalized to growth on unsupplemented media. No measurements were significantly different from wild type by Student's t-test, *p* < 0.00001. Error bars are standard deviations of the means (*n* = 14).
Figure V-13: Manganese suppresses the iar1 IAA-Ala resistant phenotype. Wild type, iar1 mutant, and iar3 ilr1 mutant seeds were plated on media containing the indicated concentrations of manganese with (A) or without (B) the addition of 30 μM IAA-Ala. Plates were placed under yellow-filtered light at 22 °C. After eight days, the seedlings' longest roots were measured. Data were normalized to growth on unsupplemented media. Asterisks indicate significant difference from wild type by Student's t test, \( p < 0.0001 \). Error bars are standard deviations of the mean (\( n = 12 \)).
alone (figure V-13B). However, addition of manganese to plates containing IAA-Ala partially suppressed the IAA-Ala resistant phenotype of the iar1 mutant (figure V-13A).

V.A.7. Identification of iar1 enhancers

Arabidopsis has a family of six IAA-conjugate hydrolase genes (Davies et al., 1999), but mutants defective in only two of these genes have been isolated (Bartel and Fink, 1995; Davies et al., 1999). To identify mutations in the remaining amidohydrolase-like genes or to reveal new genes involved in IAA conjugate sensing or metabolism, we screened for enhancers of the iar1 mutant phenotype. I screened 18,000 of the M₄ progeny of EMS-mutagenized iar1-1 seeds for resistance to root elongation inhibition by very high (80 µM) concentrations of IAA-Ala. I identified a putative iar1 enhancer, J56, that is resistant not only to high concentrations of IAA-Ala, but also to IAA-Leu and IAA-Phe, and slightly to free IAA (figure V-14). J56 may represent a mutation in a new component of the IAA conjugate metabolic pathway or a new mutant allele of an already identified component.

V.B. Positional cloning of IAR1

V.B.1. The iar1 mutation maps to the bottom of chromosome 1

The phenotypic characterization of the iar1 mutant shows that the IAR1 gene is involved in the response of Arabidopsis to IAA conjugates, either directly or indirectly. To determine how the iar1 mutation affects these processes, we cloned the IAR1 gene using a map-based approach. We determined the position of the iar1 mutation by recombination mapping. A mapping population was prepared by crossing iar1 mutant plants in the WS ecotype to wild type Col-0 plants. IAA-Ala resistant F₂ progeny were selected as the mapping population (Gibson and Somerville, 1992) and DNA isolated from these plants (Celenza et al., 1995) was analyzed using PCR-based markers polymorphic between ecotypes (Bell and Ecker, 1994; Konieczny and Ausubel, 1993). The iar1 mutation was
Figure V-14: *iar1* enhancer J56 is highly resistant to IAA-Ala and resistant to IAA-Leu and IAA-Phe. WS (wild type), *iar1-1*, *iar3-2* (Davies et al., 1999), and *ilr1-1* (Bartel and Fink, 1995) mutant, and J56 *iar1-1* enhancer seeds were plated on the indicated compound. After eight days under yellow filtered light at 22 °C, the longest root of each seedling was measured. Data were normalized against growth on unsupplemented media. Asterisks indicate measurement is significantly different from wild type by Student’s t test, $p < 0.0001$. Circles indicate significant difference from *iar1-1*. Error bars are standard deviations of the means ($n = 12$).
localized to the bottom of chromosome 1 between the SSLP markers nga280 and nga111 (Bell and Ecker, 1994). An 800 kb YAC (yeast artificial chromosome) contig had been described in this interval (Vijaraghavan et al., 1995). We obtained the YACs in this contig as well as others in the region from the Arabidopsis Biological Resource Center and used subclones to develop additional PCR-based markers for mapping (table V-2). We mapped the iar1 mutation to between the markers 9H12LE and abi13A11RE (figure V-15). Using a combination of PCR and DNA hybridization analysis, we determined that this interval is completely contained on the 116 kb BAC (bacterial artificial chromosome) T7E4 (figure V-15).

V.B.2. The iar1 phenotype is rescued by C37

We constructed a complementation library from T7E4 by partially digesting the BAC DNA and cloning resultant ~10 kb fragments into the plant transformation vector pBIN19 (Bevan, 1984). We used successive rounds of end hybridization to isolate a contiguous set of subclones from this library that spanned the region to which the iar1 mutation mapped, and transformed these subclones into the iar1 mutant by Agrobacterium-mediated transformation (Clough and Bent, 1998). T2 progeny of iar1-3 plants transformed with the various constructs were tested on 40 μM IAA-Ala for phenotypic rescue. One construct, C37 (figure V-15, 16B), was identified which resulted in a 3:1 ratio of phenotypically wild type (IAA-Ala sensitive) to mutant (IAA-Ala resistant) plants in the T2 generation (figure V-16A), which is the expected segregation ratio for a rescuing construct. I sequenced the 12 kb insert of C37 and identified three potential open reading frames (figure V-16B) contained on the construct. One of these, which has homology to annexins, was also contained in its entirety on a non-rescuing construct (figure V-16B), thus eliminating it as a candidate for IAR1. I PCR amplified and sequenced iar1-1 and WS
Figure V-15: iar1 maps to chromosome 1 and is contained on BAC T7E4. The iar1 mutation was initially localized to between the SSLP markers nga111 and nga280 (Bell and Ecker, 1994). We used subclones of YACs containing DNA from this region to design additional markers for further refining the map position. The position of iar1 was narrowed to the BAC T7E4 between the markers 9H12LE and abi13A11RE. A complementation library was constructed from the BAC, and subclones spanning the region to which iar1 mapped were tested for complementation of the IAA-Ala resistance phenotype. The construct C37, indicated in black, complemented the iar1 phenotype; other constructs did not.
Table V-2: Markers used to map the *iar1* mutation.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Type</th>
<th>Primers</th>
<th>Map position</th>
<th>Size</th>
<th>Enzyme</th>
<th>Polymorphism</th>
<th>Reference (made by)</th>
<th>Resolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>nga111</td>
<td>SSLP</td>
<td>nga111for and nga111rev</td>
<td>between <em>ADH</em> and <em>KNAT2</em> Col=128bp, Ler=162bp, WS=146bp</td>
<td>none</td>
<td>AluI</td>
<td>C≠L≠W</td>
<td>Bell and Ecker, 1994</td>
<td>4% gel</td>
</tr>
<tr>
<td>KNAT2</td>
<td>CAPS</td>
<td>KNAT2-6 and KNAT2-7</td>
<td>between nga111 and mi462 660 bp</td>
<td>MnII</td>
<td>C≠L≠W</td>
<td>(Lincoln et al., 1994)</td>
<td>2% gel</td>
<td></td>
</tr>
<tr>
<td>KNAT2</td>
<td>CAPS</td>
<td>KNAT2-6 and KNAT2-7</td>
<td>between nga111 and <em>AP1</em> 1.1 kb</td>
<td>MnII</td>
<td>C≠L≠W</td>
<td>(D. Pruess)</td>
<td>2% gel</td>
<td></td>
</tr>
<tr>
<td>mi462</td>
<td>CAPS</td>
<td>mi462-1 and mi462-2</td>
<td>between <em>KNAT2</em> and <em>AP1</em> 364 bp</td>
<td>RsaI</td>
<td>C≠L≠W</td>
<td>4% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mi462</td>
<td>CAPS</td>
<td>mi462-1 and mi462-2</td>
<td>between <em>KNAT2</em> and <em>AP1</em> 1.1 kb</td>
<td>AffIII</td>
<td>C≠L≠W</td>
<td>2% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11B6</td>
<td>CAPS</td>
<td>11B6-3 and 11B6-4</td>
<td>between nga111 and <em>AP1</em> 1.5 kb</td>
<td>EcoRI</td>
<td>C≠L≠W</td>
<td>2% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11B6</td>
<td>CAPS</td>
<td>11B6-3 and 11B6-4</td>
<td>between 11B6 and <em>AP1</em> 500 bp</td>
<td>XmnI</td>
<td>C≠L≠W</td>
<td>4% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14G4</td>
<td>CAPS</td>
<td>14G4-2 and 14G4-3</td>
<td>between 14G4 and 4-1C-19 144 bp</td>
<td>Tsp509I</td>
<td>C≠W</td>
<td>4% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>abi</td>
<td>CAPS</td>
<td>13A11-7</td>
<td>between 4-1C-2 and t7E4 160 bp</td>
<td>MseI</td>
<td>C≠W</td>
<td>2% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14G4</td>
<td>CAPS</td>
<td>13A11-11 and 13A11-11</td>
<td>between 4-1C-2 and 9H12 160 bp</td>
<td>MseI</td>
<td>C≠W</td>
<td>4% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-1C-</td>
<td>CAPS</td>
<td>4-1C-16 and 4-1C-17</td>
<td>between 4-1C-16 and 4-1C-17 160 bp</td>
<td>MseI</td>
<td>C≠W</td>
<td>sequencing w/ 4-1C-19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9H12</td>
<td>CAPS</td>
<td>9H12-2 and 9H12-3</td>
<td>between 4-1C-2 and 9H12 1.5 kb</td>
<td>HpaII</td>
<td>C≠L≠W</td>
<td>4% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mi185</td>
<td>CAPS</td>
<td>mi185-3 and mi185-4</td>
<td>between <em>ETRI</em> and 9H12 648 bp</td>
<td>HpaII</td>
<td>C≠L≠W</td>
<td>2% gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCIT</td>
<td>CAPS</td>
<td>f117-1 and f117-2</td>
<td>between 9H12 and <em>ETRI</em> Col, WS=1.7 kb</td>
<td>XbaI</td>
<td>C≠W</td>
<td>J. Sedbrook and P. Masson</td>
<td>1% gel</td>
<td></td>
</tr>
<tr>
<td>9H12</td>
<td>CAPS</td>
<td>9H12-2 and 9H12-3</td>
<td>between 9H12 and <em>ETRI</em> 1.3 kb</td>
<td>NcoI</td>
<td>C≠L≠W</td>
<td>D. Preuss</td>
<td>1% gel</td>
<td></td>
</tr>
<tr>
<td>nga280</td>
<td>SSLP</td>
<td>nga280for and nga280rev</td>
<td>between <em>ETRI</em> and ILL5 Col=105 bp, Ler=85 bp, WS=85 bp</td>
<td>NcoI</td>
<td>C≠L≠W</td>
<td>Bell and Ecker, 1994</td>
<td>4% gel</td>
<td></td>
</tr>
</tbody>
</table>

Oligo sequences are listed in Table III-2. SSLP is simple sequence length polymorphism; CAPS is cleaved amplified polymorphic sequence.
genomic DNA from one of the two other genes, which encodes a protein with homology to the predicted KE4 protein encoded in the mammalian MHC class II locus (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). I identified a G to A base change in the iar1-1 mutant, consistent with an EMS-induced mutation, that changes a glycine conserved in the predicted Arabidopsis, mouse and human proteins to an arginine. Sequence analysis of the gene in the remaining iar1 alleles (except iar1-4, which represents a deletion of the entire C37 region) revealed lesions in all alleles: the iar1-2 mutation alters a different conserved glycine to an arginine, the iar1-3 mutation causes a frameshift twelve amino acids from the end of the predicted protein, the iar1-5 mutation changes an arginine to a lysine, the iar1-6 mutation causes a premature stop codon at position 162 of the predicted protein, and iar1-7 alters the conserved splice acceptor site of the predicted first intron of the IARI gene (figures V-17, 19; table V-2). The identification of mutations in this reading frame from all iar1 alleles confirms its identity as IARI. Furthermore, a 5.5 kb genomic fragment containing only the putative IARI open reading frame (pBINIAR1g, table III-4) rescues the IAA-Ala resistance of the iar1 mutant (not shown).

V.B.3. IARI encodes a protein with putative transmembrane domains and histidine-rich clusters

Using a 1.2 kb genomic fragment containing the last five predicted introns of IARI, I probed an Arabidopsis cDNA library (Minet et al., 1992) and obtained a full-length IARI cDNA (GenBank accession number AF216524). Comparison of this cDNA to the genomic sequence revealed that IARI has eleven exons and ten introns and encodes a putative protein of 469 amino acids that is 28% identical to the predicted mouse KE4 and human HKE4 proteins encoded by genes within the major histocompatibility complex (MHC) class II locus (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). Examination of sequences in the GenBank database also revealed IARI
Figure V-16: A). C37 rescues the iar1 IAA-Ala resistant phenotype. Seeds from wild type Col-0, iar1-3 mutant, and an iar1-3 transgenic line segregating the C37 construct were plated on 40 μM IAA-Ala. After eight days growth under yellow-filtered light at 22 °C, the longest root of each seedling was measured to the nearest mm. B). The iar1-rescuing construct C37 contains three predicted open reading frames. Non-rescuing constructs are indicated as gray lines; the rescuing C37 construct is shown as a black line. Predicted open reading frames are shown in the positions in which they are located on each construct.
<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation in predicted IAR1 protein</th>
<th>PCR with oligos</th>
<th>PCR program</th>
<th>digest with enzyme</th>
<th>fragment sizes (bp)</th>
<th>other</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>iar1-1</em></td>
<td>Gly to Arg at 400</td>
<td>T7E4-31</td>
<td>SSLP</td>
<td>HpaII or MspI</td>
<td>WT: ~380, ~280</td>
<td>run on 2% gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ T7E4-32</td>
<td></td>
<td></td>
<td><em>iar1-1</em>: 654</td>
<td></td>
</tr>
<tr>
<td><em>iar1-2</em></td>
<td>Gly to Arg at 423</td>
<td>T7E4-31</td>
<td>SSLP</td>
<td>AcII or MspAI</td>
<td>WT: 521, 133</td>
<td>run on 2% gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ T7E4-32</td>
<td></td>
<td></td>
<td><em>iar1-2</em>: 654</td>
<td></td>
</tr>
<tr>
<td><em>iar1-3</em></td>
<td>frameshift at 458</td>
<td>T7E4-23</td>
<td>CAPS</td>
<td>HaeIII</td>
<td>WT: 865 <em>iar1-3</em>: 622, 70</td>
<td>run on 2% gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ T7E4-41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iar1-4</em></td>
<td>deletion of entire coding region</td>
<td>T7E4-32</td>
<td>SSLP</td>
<td>none</td>
<td>WT and <em>iar1-5</em>: 563</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ T7E4-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iar1-5</em></td>
<td>Arg to Lys at 357</td>
<td>T7E4-29</td>
<td>CAPS</td>
<td>AsuHPI or HphI</td>
<td>WT: 777, 180, 54, 957, 54</td>
<td>run on 1% gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ T7E4-34</td>
<td></td>
<td></td>
<td><em>iar1-6</em>:</td>
<td></td>
</tr>
<tr>
<td><em>iar1-6</em></td>
<td>Gln to stop at 162</td>
<td>T7E4-29</td>
<td>CAPS</td>
<td>AluI</td>
<td>WT: 261, 249, 153, 126, 112, 110, <em>iar1-7</em>: 261, 249, 153, 126, 110, 72, 40</td>
<td>run on 4% gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ T7E4-34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequences for oligos listed are in Table III-2, Materials and Methods.
Figure V-17: Schematic representation of the IAR1 coding region. Exons are indicated by filled rectangles; introns are represented by a line. Positions of the seven iar1 mutations in the coding region are also shown.
homologs in *Drosophila* (Catsup; 31% identical) (Stathakis et al., 1999), *C. elegans* (GenBank accession number 3878059), and zebrafish (GenBank accession number AAF05821) (figure V-18).

No function has been assigned to any of the IAR1 homologs with the exception of the *Drosophila* protein Catsup (catecholamines up). Loss-of-function mutations in the gene encoding *Catsup* are lethal and are associated with increased levels of catecholamine neurotransmitters (Stathakis et al., 1999). The rate-limiting step in catecholamine biosynthesis is the hydroxylation of L-tyrosine to form 3,4-dihydroxy-L-phenylalanine. This reaction is catalyzed by the enzyme tyrosine hydroxylase, or TH (Kumer and Vrana, 1996). TH activity is regulated at a number of levels, including transcriptional regulation, alternate mRNA processing, and stability of the TH message; translational control and protein stability; and feedback inhibition, allosteric modulation, and phosphorylational control of enzyme activity (Kumer and Vrana, 1996). *catsup* mutant flies exhibit higher than normal TH activity but do not have more TH protein than wild-type flies, indicating that Catsup normally functions as a negative regulator of TH enzyme activity (Stathakis et al., 1999). The mechanisms through which this inhibition is carried out have not been reported.

IAR1 and its homologs share seven or eight predicted transmembrane domains (figures V-19, V-20), an N terminal signal sequence, and three histidine-rich regions (figure V-20). These are characteristics also associated with the ZIP family of zinc transporters (Eng et al., 1998; Guerinot and Eide, 1999). Furthermore, IAR1 and its homologs have several potential metal binding domains of the type HXHXH (Eng et al., 1998), which are found in metal transporters (for example: HDHTHDE in the yeast zinc transporter ZRT1 (Zhao and Eide, 1996) and HGHGHG in the Arabidopsis iron transporter IRT1 (Korshunova et al., 1999)). Additionally, IAR1 has weak sequence similarity to conserved membrane spanning regions (transmembrane domains IV and V) thought to be essential for metal transport in the ZIPs and contains the ZIP family signature sequence (Eng et al., 1998), which is derived from this region (figures V-20, V-21).
Figure V-18: IAR1 homologs are present in other organisms. IAR1, *Drosophila melanogaster* (Dm) Catsup (Stathakis et al., 1999), *Homo sapiens* (Hs) KE4 (Janatipour et al., 1992; Ando et al., 1996), and *Mus musculus* (Mm) KE4 (Abe et al., 1988; St.-Jaques et al., 1990) protein sequences were aligned with the Megalign program (DNASTar, Madison, WI) using the Clustal method (Higgins and Sharp, 1989). Residues conserved in at least three of the four proteins are shaded in black. Triangles indicate positions of introns in the IAR1 coding sequence. Positions of *iar1* mutations are shown above the IAR1 sequence, except *iar1-4*, which is a deletion of the entire IAR1 gene.
The fifth putative transmembrane domain of the ZIPs is not predicted to be present in IAR1 using the PSORT motif prediction program (Nakai and Kanehisa, 1992) due to the presence of charged amino acids about every third or fourth residue in this region (figure V-20). However, this is a pattern associated with amphipathic helices (Lodish et al., 1995) and may indicate a transmembrane domain that is part of a membrane-spanning pore. IAR1 contains the fully conserved glyceryl and histadyl residues of the ZIP signature sequence and only deviates from the signature sequence at one residue, position 12 of the 15 amino acid signature. Although IAR1 and the ZIPs are only about 10% identical at the amino acid level, the similarity in molecular structure between IAR1 family members and ZIP family members suggests that IAR1 might be a metal transporter, or might bind a metal cofactor.
Figure V-19: IAR1 and its homologs encode potential transmembrane proteins. Hydropathy plots of IAR1 and the human homolog were made using the TM predict program (ulrec3.unil.ch/software/TMPRED_form.html). Amino acid positions are indicated along the bottom of each plot. Positive values on each graph indicate hydrophobic regions; negative values are hydrophilic regions. The predicted signal sequence for each protein is indicated by s and predicted transmembrane domains are numbered. The fifth putative transmembrane domain shares identity with a predicted transmembrane region of the ZIP family of proteins (Eng et al., 1998), but is not hydrophobic in IAR1 or HKE4 due to the presence of charged residues approximately every third or fourth amino acid.
Figure V-20: IAR1 is similar to the ZIP family of metal transporters. Sequences were aligned with the Megalign program (DNASTar) using the clustal method (Higgins and Sharp, 1989). Amino acid residues identical among IAR1, mouse KE4 (Mm KE4) and human KE4 (Hs KE4) are shaded in dark blue, residues identical in four of the six ZIP family members shown are shaded in light blue, and residues identical among five of the nine proteins are shaded in black. Histidine residues are shown in red. Transmembrane domain V is not predicted (Nakai and Kanehisa, 1992) in IAR1, Mm KE4, Hs KE4, or ZIP2. Sequences shown are from *Mus musculus* (Mm) KE4 (Abe et al., 1988; St.-Jaques et al., 1990); *Homo sapiens* (Hs) KE4 (Janatipour et al., 1992; Ando et al., 1996); *Arabidopsis thaliana* (At) IRT1 (Eide et al., 1996); ZIP1, ZIP2, and ZIP3 (Grotz et al., 1998); and *Saccharomyces cerevisiae* (Sc) ZRT1 (Zhao et al., 1996a) and ZRT2 (Zhao et al., 1996b).
Figure V-21: IAR1 contains the ZIP family signature sequence (Eng et al., 1998). A) Alignment of the ZIP family signature sequence region from IAR1, *Mus musculus* (Mm) KE4 (Abe et al., 1988; St.-Jaques et al., 1990), *Homo sapiens* (Hs) KE4 (Janatipour et al., 1992; Ando et al., 1996), Arabidopsis IRT1 (Korshunova et al., 1999), ZIP1, ZIP2, and ZIP3 (Grotz et al., 1998), and *S. cerevisiae* ZRT1 (Zhao and Eide, 1996) and ZRT2 (Zhao and Eide, 1996). Residues conserved in at least four of the sequences are shaded in black. B) Alternate residues possible in the fifteen amino acid ZIP motif. IAR1 residues are boxed.
CHAPTER VI: MOLECULAR CHARACTERIZATION OF IARI

Luise E. Rogg mounted and photographed the GUS-stained seedlings and photographed 3SsSmKE4 seedlings. Bonnie Bartel made JA and wound-induced RNA; root and stem RNA was prepared by Rosie Tellez. David C. Nelson assisted with protein gels and blots.

VI.A. IARI expression

I examined IARI expression using gel blot analysis of RNA prepared from various Arabidopsis organs. IARI mRNA was detected in all organs examined, with the highest levels in roots and stems and lowest levels in dry seeds (figure VI-1). To examine more specifically the sites of IARI expression, I examined wild-type plants transformed with an IARI promoter-β-glucuronidase (GUS) fusion. In seedlings expressing this construct, GUS activity is limited to the vascular tissue or vascular-associated tissue in the shoot apex (figure VI-2A). Staining associated with the vasculature is also observed in the hydathodes of adult plant leaves (figure VI-2B). Perhaps the most striking staining is observed in reproductive structures. This staining is apparent from the time of bud emergence from the inflorescence stem (figure VI-2C). In more mature flowers, GUS expression is associated with the anthers (figure VI-2D), and closer examination reveals that this expression is in the pollen (figure VI-2G,H). Prior to fertilization, staining is observed in both the pollen and the ovules (figure VI-2E); after fertilization occurs, pollen-associated staining disappears (figure VI-2F). Developing embryos exhibit staining associated with the vasculature (figure VI-2I,J), which disappears in mature seeds (not shown). Interestingly, no IAR1-GUS expression is observed in the root (not shown) even though the iar1 mutant was isolated based on a root elongation phenotype and a high level of IARI message is present in the root when examined by RNA blot.
Figure VI-1: *IAR1* message is highest in roots and stems. Total RNA (5 µg) isolated from dry seeds; roots and aerial tissues (rosettes) from 14-day-old plants; and leaves, inflorescence stems, flowers, and siliques of 29-day-old plants (all grown in continuous white light) were separated on a gel, transferred to a membrane, and probed with $^{32}$P-labeled antisense *IAR1* RNA probe to determine levels of *IAR1* message. Ethidium bromide-stained ribosomal RNA (rRNA) is shown as a loading control.
Figure VI-2: *IAR1*-GUS staining. A) Five-day-old *IAR1*-GUS seedlings grown under continuous yellow-filtered light show staining in or around the vasculature of the shoot apex; four-week-old T2 plants show staining in the hydathodes (B), emerging floral buds (C), pollen (D,G,H), ovules (E), and developing seeds (F, I, J). All panels show staining after 24 hours of incubation in substrate, except (A), which is after 48 hours.
*IAR1* is predicted to encode a protein with seven or eight potential transmembrane domains; therefore, I attempted to determine to which membrane the IAR1 protein localizes using several strategies. First, I designed a translational fusion of IAR1 with green fluorescent protein (GFP; Haseloff, 1999) under the control of the inducible *GAL1* promoter in a yeast expression vector. I transformed this construct (pRS314GALIAR1-GFP, table III-4) into yeast, along with cytoplasmic- and nuclear-targeted GFP controls. Upon induction with galactose, the control strains expressed GFP, and the GFP signal was localized to the expected subcellular compartment. However, the IAR1-GFP yeast strains did not have visible GFP expression (not shown). Furthermore, these yeast were small and did not divide after induction with galactose. Therefore, it is likely that the IAR1-GFP fusion protein is toxic to the yeast.

I also designed IAR1 fusions with both GFP (Haseloff, 1999) and the myc epitope (Kolodziej and Young, 1991) under the control of the *IAR1* promoter for expression in plants. I transformed both constructs into wild type and iar1 mutant plants, and obtained homozygous transgenic lines of IAR1-myc in Col-0, IAR1-GFP in Col-0, and IAR1-GFP in iar1-3. I tested the response of iar1-3 plants transformed with the IAR1-GFP construct to IAA-Ala, and found that this construct at least partially rescues the mutant phenotype (figure VI-3). This indicates that the fusion protein is functional and correctly localized. However, when I examined iar1-3 or wild type plants containing the IAR1-GFP construct using fluorescence confocal microscopy, I was unable to detect any GFP fluorescence. This suggests that IAR1 is present at very low levels and indicates that a more sensitive method is needed to determine its localization.

Therefore, I examined six independently-derived IAR1-myc transgenic lines for expression of this construct. I extracted protein from flowers and buds of the IAR1-myc transgenics based on GUS staining results that showed the highest level of IAR1 expression to be in the reproductive organs (figure VI-2). Gel blot analysis with an anti-
Figure VI-3: IAR1-GFP construct rescues the mutant phenotype. Col-0 wild type, iar1-3 mutant, and two independently derived iar1-3 lines transformed with the IAR1-GFP fusion construct (pBIN19IAR1c+gGFP) were plated on 40 μM IAA-Ala. After eight days under yellow-filtered light at 22 °C, the longest root of each seedling was measured. The transformed lines are T2 generation plants and are therefore segregating the IAR1-GFP construct.
myc antibody revealed that four of the six lines express a protein of the expected size of the IAR1-myc fusion (~50 kDa) that reacts with the anti-myc antibody (figure VI-4).

To determine to which membrane the IAR1-myc fusion localized, I isolated microsomal membranes from combined tissue samples of these four lines followed by two-phase partitioning (Larsson, 1985) to separate the plasma from the intracellular membranes. Preliminary analysis confirms the presence of IAR1-myc in purified membranes (not shown), although it remains to be determined to which membrane the protein localizes.

VI.B. IAR1 expression is induced by jasmonic acid and wounding but not by zinc

Expression of IAR3 is strongly induced by wounding and treatment with jasmonic acid (Titarenko et al., 1997). This suggests an interrelation of the jasmonic acid-mediated wound response and conjugate hydrolysis. Therefore, we tested the expression of IAR1 in response to methyl jasmonate, a jasmonic acid precursor, and wounding in wild type, the iar3 ilrl mutant (Davies et al., 1999), and the jasmonic acid-insensitive mutant coi1 (Feys et al., 1994). IAR1 expression is weakly induced in wild type in response to both stimuli and in the iar3 ilrl mutant in response to methyl jasmonate (figure VI-5). Interestingly, IAR1 expression appears more strongly induced in the iar3 ilrl mutant background in response to wounding (figure VI-5).

Expression of Arabidopsis genes in the ZIP family is induced by zinc deficiency (Grotz et al., 1998). The resemblance of IAR1 to ZIP family members and the connection between zinc nutrition and auxin (see Chapter I) suggested that IAR1 expression might also be regulated by zinc levels. I examined IAR1 expression in plants grown on medium containing no added zinc, normal amounts of zinc (1 µM) or 75 µM zinc in both wild type and iar1 mutants. IAR1 expression may be slightly decreased in wild type plants grown on 75 µM ZnSO₄ (figure VI-6). No other differences are apparent between wild-type and the IAR1 mutant or among the zinc concentrations tested (figure VI-6).
Figure VI-4: IAR1-myc lines express detectable levels of the fusion protein. 30 μg total protein isolated from buds and flowers of six independently derived IAR1-myc transgenic lines was electrophoresed on a 6% SDS-PAGE and probed with an anti-myc antibody. 15 μg of total protein from a transgenic plant expressing ILR1-myc (a gift from Sherry LeClere), which is approximately the same size as IAR1-myc, were loaded as a positive control.
Figure VI-5: *IAR1* is induced by wounding and jasmonic acid. Total RNA from rosette leaves of adult wild type, *iar3 ilr1* mutant, and *coil* mutant plants left untreated (control), wounded, or sprayed with 50 μM methyl jasmonate (JA treated) was separated on a gel, transferred to a membrane, and probed with 32P-labeled antisense *IAR1* RNA probe to determine levels of *IAR1* message. Ethidium bromide-stained ribosomal RNA is shown as a loading control.
Figure VI-6: *IAR1* expression may be down-regulated by high zinc. Total RNA from 14-day-old *iar1-3* and Col-0 (wild type) plants grown on 0, 1, or 75 μM ZnSO₄ was separated on a gel, transferred to a membrane, and probed with ³²P-labeled antisense *IAR1* RNA probe to determine levels of *IAR1* message. Ethidium bromide-stained ribosomal RNA is shown as a loading control.
VI.C. Functional studies of IAR1

VI.C.1. Plants transgenic for an IAR1 overexpression construct are not more sensitive than wild type to IAA-Ala

Because mutations in IAR1 and IAR3 lead to IAA-Ala resistance and overexpression of IAR3 causes IAA-Ala supersensitivity (Davies et al., 1999), I examined the responses of transgenic plants containing IAR1 driven by the constitutive Cauliflower Mosaic Virus (CaMV35S) promoter. This construct, pBARNIAR1 (table III-4), was made in the plant transformation vector 35SpBARN (LeCler and Bartel, unpublished) and infiltrated into Col-0 wild type and iar1-3 mutant plants. Transgenic lines were tested on a relatively low concentration of IAA-Ala to ascertain whether they were hypersensitive to inhibition of root elongation by the conjugate. Of the ten Col-0 derived lines tested, none were more sensitive than the wild type to the conjugate, and one showed resistance similar to that observed in the iar1-3 mutant (figure VI-7). Because the resistant line phenocopies the iar1 mutant, it may have cosuppressed the endogenous IAR1 gene. The levels of IAR1 message in these lines have not yet been determined, and it remains possible that none are actually overexpressing IAR1.

VI.C.2. IAR1 does not confer IAA-Ala transport activity to yeast microsomes

Arabidopsis encodes a family of conjugate hydrolase-like enzymes (Bartel and Fink, 1995; Davies et al., 1999). The proteins that hydrolyze IAA-amino acid conjugates in vitro contain C-terminal ER retention signals, suggesting that they function in the ER (Bartel and Fink, 1995; Davies et al., 1999). The IAA conjugate-resistant phenotype of the iar1 mutant and multiple predicted transmembrane domains of the IAR1 protein suggest that IAR1 might encode an IAA conjugate transporter localized to the ER. Loss of IAR1 function
Figure VI-7: Plants transgenic for an *IAR1* overexpression construct are not more sensitive than wild type to IAA-Ala. Col-0 wild type, *iar1-3*, and ten independently derived transgenic lines homozygous for the pBARNIAR1 construct were plated on 15 µM IAA-Ala. After 8 days in yellow-filtered light at 22 °C, the longest root of each seedling was measured. Asterisks indicate significant difference from wild type by Students t test, $p < 0.0001$. Error bars are standard deviations of the means ($n \geq 10$).
would prevent conjugates from reaching the hydrolases and therefore result in conjugate resistance.

To test this hypothesis, I transformed the \textit{IARl} cDNA under the control of the inducible \textit{GALI} promoter (pRSGALIAR1; table III-4) into yeast. Interestingly, although \textit{IAR1-GFP} appears to be toxic to yeast (section VI.A.), induction of \textit{IARl} expression with galactose caused no deleterious effects, suggesting that the previously observed toxicity is specific to the GFP fusion protein. Initially, I made yeast strains expressing both \textit{IARl} and either the \textit{ILRI} or \textit{IAR3} amidohydrolase gene. I isolated microsomes from these yeast and assayed them for ability to take up and hydrolyze IAA-Ala, IAA-Leu, and IAA-Phe, three conjugates to which the \textit{iarl} mutant is resistant (figures V-2 and V-3). Microsomes isolated from control yeast transformed with empty vector were able to hydrolyze these conjugates to some extent, and no additional hydrolysis attributable to the presence of \textit{IARl}, \textit{ILRI}, or \textit{IAR3} in the microsomes was observed (data not shown).

To eliminate complications involved in assaying for hydrolysis of conjugates, I isolated microsomes from yeast expressing only \textit{IARl}. I assayed these microsomes for their ability to take up IAA-Ala, IAA-Leu, or IAA-Phe by incubating the microsomes with the conjugates, then recovering the microsomes by ultracentrifugation and assaying by TLC to determine whether they contained any conjugate. I also tested for uptake of the jasmonic acid conjugates JA-Ala, JA-Leu, and JA-Phe. Microsomes from \textit{IARl}-expressing or control yeast showed no ability to take up any of the tested conjugates (data not shown).

\textbf{VI.C.3. The mouse homolog of \textit{IARl} rescues the \textit{iarl} mutant phenotype}

\textit{IAR1} homologs, KE4 and HKE4, have been described in mice and humans (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). The genes encoding these proteins are part of the MHC II locus, but their functions are unknown (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). \textit{IAR1} is
approximately 28% identical at the amino acid level to each of these proteins (figure V-19) and has very similar predicted membrane topology (figure V-20). To determine if the Arabidopsis IAR1 and mammalian KE4 proteins have the same function in vivo, I introduced the cDNA encoding the mouse KE4 protein under the control of the constitutive CaMV 35S promoter (35SmKE4) into iar1-3 mutant plants. I then examined the root elongation of resultant T2 plants on 40 μM IAA-Ala. iar1-3 (35SmKE4, table III-4) T2 lines displayed a 3:1 ratio of IAA-Ala sensitive to IAA-Ala resistant plants (figures VI-8, VI-9), indicating that the mouse KE4 gene can functionally substitute for IAR1. This suggests that IAR1 and KE4 have similar, if not identical, functions in Arabidopsis and mammals and that IAR1 is unlikely to be an IAA conjugate transporter.

VI.C.4. IAR1 does not rescue the phenotypes of the yeast pmr1 or zrt1 zrt2 mutants

The Arabidopsis IAA conjugate hydrolases characterized to date require a metal cofactor for optimal hydrolase activity. The cofactors with greatest efficacy in assays using recombinant hydrolase protein purified from E. coli are Mn2+ and Co2+ (Davies et al., 1999; Tellez and Bartel, unpublished). Mutations in the genes encoding the hydrolases can lead to IAA conjugate resistance in Arabidopsis (Bartel and Fink, 1995; Davies et al., 1999), so it is possible that mutations that prevent the enzymes from assembling with or retaining the proper cofactor might also cause an IAA conjugate-resistant phenotype. Because of the probable ER localization of the amidohydrolases and the resemblance of IAR1 to the ZIP family of metal transporters, we hypothesized that IAR1 might transport manganese into the ER as a cofactor for the hydrolases. To test this possibility, I transformed IAR1 into the yeast pmr1 mutant.

The PMR1 gene of S. cerevisae encodes a P-type ATPase (Rudolph et al., 1989) that transports manganese and calcium into the endoplasmic reticulum (Dürr et al., 1998). pmr1 mutants accumulate manganese in the cytoplasm (Lapinskas et al., 1995) and exhibit
Figure VI-8: The mouse KE4 gene can functionally substitute for IAR1. iar1-3 mutant plants were transformed with a construct containing the mouse KE4 cDNA (Abe et al., 1988; St.-Jaques et al. 1990) under the control of the CaMV 35S promoter. T2 progeny of transformed plants, as well as seeds from the wild type Col-0 and the parent mutant, were plated on media containing 40 μM IAA-Ala. After eight days, the longest root of each seedling was measured. Lines transformed with 35SmKE4 showed a 3:1 ratio of IAA-Ala sensitive to IAA-Ala resistant plants in the T2 generation, indicating that mouse KE4 can substitute for IAR1.
Figure VI-9: The mouse KE4 gene rescues the iar1 IAA-Ala resistant phenotype. Col-0 wild type, iar1-3 mutant, and iar1-3 (35SsmKE4) seeds were plated on either unsupplemented media or media containing 40 μM IAA-Ala and grown under continuous yellow-filtered light. Seedlings were photographed after eight days.
growth defects that are alleviated by high calcium or manganese concentrations in the
growth medium (Dürr et al., 1998; Rudolph et al., 1989). If IAR1 encodes an ER-localized
manganese transporter, expression of the gene might rescue the growth defect of the pmrl
mutant on low-manganese medium. However, pmrl yeast transformed with IAR1
(pRS314GALIAR1, table III-4) were not rescued (not shown).

The similarity of IAR1 to the ZIP family, most members of which are zinc
transporters, suggested that IAR1 might transport zinc. To examine this possibility, I
transformed IAR1 into the ztrl zrt2 yeast mutant, which is defective in both high- and low-
affinity plasma membrane-localized zinc transporters (Zhao and Eide, 1996). The ztrl zrt2
mutant is able to grow normally on media supplemented with 1 mM zinc, but grows poorly
on low-zinc media. If IAR1 is a plasma membrane-localized zinc transporter, then it might
confer ability to grow on low zinc to the ztrl zrt2 mutant. I transformed IAR1 into the
mutant and assayed for growth on low zinc. As a control, I used ZIP1, an Arabidopsis ZIP
that partially rescues the phenotype of ztrl zrt2 (Grotz et al., 1998). In contrast to ZIP1,
IAR1 failed to rescue the growth defect of ztrl zrt2 yeast (figure VI-10), suggesting that it
does not encode a plasma membrane zinc transporter.

VI.C.5. Atomic absorption studies

To determine if iar1 mutants and wild-type plants have differences in metal ion
concentrations at a whole plant level, I grew iar1 and wild type plants in a variety of
conditions, harvested, dried, and ground them, and sent the resulting powder to the
Department of Fruit and Vegetable Sciences at Cornell University for atomic absorption
analysis of metal ion concentrations. Initially, I examined metal concentrations in four-
week-old soil-grown plants. There were no significant differences between wild type and
iar1 in any metals tested except calcium and magnesium, which were slightly higher in iar1,
and zinc, which was slightly lower in iar1 (figure VI-11). I then grew plants in liquid
containing no zinc, normal levels of zinc (0.1667 μM), or 20 μM zinc. Plants were
Figure VI-10: *IA1* does not rescue the yeast *zrt1 zrt2* mutant. Wild-type and *zrt1 zrt2* mutant yeast were transformed with control plasmid (pFL61), *IA1* (pFL61IA1), or the Arabidopsis zinc transporter *ZIP1* (pFL61ZIP1; Grotz et al., 1998) and assayed for growth on low zinc or zinc supplemented medium. A) Growth on low zinc medium; B) growth on 1 mM ZnSO₄. (1) *zrt1 zrt2* transformed with *ZIP1*; (2) *zrt1 zrt2* transformed with *IA1*; (3) *zrt1 zrt2* transformed with control vector; (4) wild type transformed with *ZIP1*; (5) wild type transformed with *IA1*; (6) wild type transformed with control vector.
Figure VI-11: Results of atomic absorption analysis on Col-0 wild type and iar1 mutant plants. iar1-2 mutants used in this study had been outcrossed to Col-0 twelve times. Four week-old soil-grown plants were harvested and dried, ground to a powder, and subjected to the dry ash method of atomic absorption (Department of Fruit and Vegetable Sciences, Cornell University). Data are shown as the percentage of wild-type value.
harvested and sent for analysis after two weeks. While plants grown in high zinc did accumulate higher zinc concentrations than the no zinc or normal zinc plants, there was no significant difference between iarl and wild type in these conditions (not shown). When the soil-growth experiment was repeated with duplicate samples, it became apparent that there is a great deal of variability in results obtained from atomic absorption, which makes the results difficult to interpret. Therefore, the results of these experiments were uninformative.

VI.C.6. Effects of metal ions on the growth of Arabidopsis seedlings

The rescue of the iarl IAA-Ala resistant phenotype by addition of manganese to the growth medium (figure V-13) and the similarity in molecular structure of the IAR1 protein and the ZIP family of metal transporters (figure V-20) suggested that IAR1 might encode a metal transporter. To address this possibility, I examined the response of the mutant at a gross level to a variety of metals (cobalt, calcium, potassium, manganese, nickel, magnesium, sodium, iron, silver, copper, cadmium, molybdenum, zinc, and lithium). Mutant (iarl-3) and wild type (Col-0) seeds were sterilized and placed in 1 mL of 1/6 strength liquid media containing 0.1% sucrose and the metal to be tested. I tested each metal over a wide range of concentrations, visually determining which concentrations had no effect on seedling growth, slightly inhibited growth, severely inhibited growth, or completely inhibited seed germination of the wild type after ten days (figure VI-12). I also compared the growth of iarl-3 seedlings to that of wild type in the same concentrations of each metal. For all of the metals at all concentrations tested, iarl-3 and wild type exhibited similar responses except in 10 μM and 20 μM AgNO₃, in which wild type was unaffected but iarl-3 plants were severely inhibited (data not shown). Repetition of this liquid-based assay failed to confirm the increased sensitivity of iarl to silver.

In order to quantify more precisely the response of iarl to various metals and to reveal possible small differences in sensitivity not apparent in the previously described
Figure VI-12: Effects of metal ions on growth of Arabidopsis seedlings. Wild type Col-0 and mutant iar1-3 seeds were sterilized, and approximately 10 seeds were placed in each well of a sterile 24 well microculture plate containing 1 mL of 1/6-strength media supplemented with 0.1% sucrose and the indicated concentration of metal. Seedling growth was examined after 10 days in white light at 22 °C. Open circles indicate that there was no obvious effect on seedling growth; open squares indicate slight growth inhibition; filled squares indicate severe growth inhibition; and Xs indicate complete inhibition of seed germination at the indicated concentration. In all cases except 10 and 20 μM AgNO₃, wild type and iar1-3 had the same response. AgNO₃ responses indicated above are for wild type.
Figure VI-13: iar1 mutants are neither resistant nor supersensitive to zinc. iar1 and wild type seeds were plated on the indicated concentrations of ZnSO₄. After eight days under yellow-filtered light at 22 °C, the longest root of each seedling was measured. Data were standardized against growth on unsupplemented media. No measurements were significantly different from wild type by Student's t test, p < 0.0001. Error bars are standard deviations of the means (n = 10).
experiment, I plated wild type and iar1 seeds on solid media containing a variety of metals over a range of concentrations. Because of the similarity of IAR1 to zinc transporters (figure V-20) and the connection between auxin levels and zinc nutrition in plants (section I.D.4.a), I tested root elongation of iar1 on plates containing zinc. The mutant did not display either resistance or hypersensitivity to zinc in root elongation in concentrations from 100 μM to 500 μM, a range that encompasses slight to severe inhibition of wild type root elongation (figure VI-13). I also tested the response of iar1 to a variety of other metals in similar assays, including calcium, cobalt, magnesium, aluminum, and copper. I observed no differences between iar1 and wild type in sensitivity to these metals (not shown).

Finally, because of the inhibition of iar1 growth initially observed in AgNO3 concentrations that had no affect on wild type, I examined root and hypocotyl elongation of iar1 mutants on high concentrations of silver. I carried out this experiment in the dark because silver nitrate is light sensitive. Most concentrations of AgNO3 tested had no effect on the growth of either wild type or the mutant. At the highest concentration tested (5 mM), neither germinated. At 1 mM, however, there is slight inhibition of hypocotyl elongation in iar1 alleles in the Col-0 background (figure VI-14). This is consistent with the previous results from the liquid assay, but is not as dramatic. Examination of growth on AgNO3 concentrations between 1 and 5 mM might clarify the differences between wild type and the iar1 mutant in response to this metal.

VI.C.7. Copper and zinc inhibit the hydrolase activity of the IAR3 enzyme

As previously discussed, the Arabidopsis IAA conjugate hydrolases require a metal cofactor for optimal activity, and the most effective cofactors are manganese and cobalt. IAR1 does not rescue the yeast pmrl mutant, which is defective in an ER-localized manganese transporter, which suggests that IAR1 does not transport manganese into the ER for the hydrolases to use as a cofactor. Furthermore, an Arabidopsis protein capable of
Figure VI-14: *iarl* mutants may be hypersensitive to silver. *iarl* mutant and wild type seeds were sterilized and allowed to imbibe in 0.1% agar under yellow-filtered light for 24 hours before plating on 1 mM AgNO$_3$. Plates were placed in the dark at 22 °C. After 8 days, the hypocotyl and longest root of each seedling was measured. Data were normalized against growth on unsupplemented media. Asterisks indicate significant difference from wild type by Student's t test, $p < 0.0001$. Error bars are standard deviations of the means ($n = 15$).
transporting manganese into the ER, ECA1 (Liang et al., 1997), has already been identified. However, it is possible that IAR1 transports inhibitory metals out of the ER and away from the hydrolases rather than transporting a cofactor into the ER. To determine if this is a viable explanation for the IAA conjugate-resistant phenotype of the iar1 mutant, I examined the effects of various metals on the activity of the IAR3 IAA-Ala hydrolase (Davies et al., 1999).

IAR3 was isolated from E. coli following fusion to glutathione-S-transferase (GST; Davies et al., 1999). I incubated purified recombinant GST-IAR3 (a gift from R. Tellez) with 1 mM IAA-Ala and 1 mM of the metal being tested and assayed for IAA-Ala hydrolysis by TLC. Purified IAR3 alone was capable of hydrolyzing IAA-Ala, suggesting that IAR3 is able to assemble with the proper cofactor in E. coli (figure VI-15A,B,C). The addition of 1 mM calcium, magnesium, cadmium, nickel, cobalt, manganese, molybdenum, or EGTA (a calcium chelator) had little effect on enzyme activity assayed after five hours. Addition of EDTA (which chelates divalent cations) inhibited the reaction (figure VI-15B,C), suggesting that the metal cofactor present in IAR3 can be dissociated from the enzyme and that this dissociation causes loss of activity. Aluminum caused slight inhibition of the hydrolysis reaction, and copper and zinc almost completely inhibited IAA-Ala hydrolysis (figure VI-15A).

To determine if copper or zinc can compete with manganese to inhibit IAR3 activity, I incubated IAR3 with 1 mM IAA-Ala, 1 mM manganese, and various concentrations of copper or zinc. Inhibition of IAR3 activity is apparent at 0.5 mM concentrations of copper and zinc, and is almost complete when the competing metal is 1:1 with manganese. This result provides compelling evidence that the presence of copper or zinc can interfere with the function of IAR3.
Figure VI-15: Copper and zinc inhibit the IAA-Ala hydrolyzing activity of the IAR3 enzyme. A.) Purified recombinant GST-IAR3 protein (Davies et al., 1999) was incubated at room temperature with 1 mM IAA-Ala and 1 mM of the indicated metal cofactor (Ca = CaCl₂, Mg = MgSO₄, Cd = CdCl₂, Ni = NiSO₄, Cu = CuSO₄, Co = CoCl₂, (–) = no cofactor added, Mn = MnCl₂, Ag = AgNO₃, Zn = ZnSO₄, Mo = Na₂MoO₄, Al = AlCl₃, * = EDTA) for 5 hours. Three μL of each reaction was run on a TLC plate using a 49.5% hexane/49.5% ethyl acetate/1% acetic acid solvent and stained for indoles with Van Urk-Salkowski reagent (Ehmann, 1977). B, C.) Same as in A, except the indicated amount of each metal was added.
VI.D. Discussion

VI.D.1. \textit{iar1} mutants are resistant to the inhibitory effects of IAA-amino acid conjugates

It is apparent from the phenotypic analysis of \textit{iar1} (IAA-Ala resistant) mutants that the gene defective in these mutants is involved in IAA-conjugate metabolism or sensing. \textit{iar1} mutants were originally isolated as resistant to root elongation inhibition by the IAA-amino acid conjugate IAA-Ala, and were named for this resistance. However, \textit{iar1} is not only resistant to IAA-Ala (figures V-1, V-2) but to other conjugates as well (figures V-3, V-4). This suggests a general role for the \textit{IARI} gene in IAA-conjugate related processes, rather than an IAA-Ala - specific role.

\textit{iar1} mutant hypocotyls display wild-type sensitivity to exogenously supplied free IAA (figure V-7), elongate normally under conditions that increase endogenous free IAA (figure V-5), and respond normally to various light conditions (figure V-6). However, \textit{iar1} mutant hypocotyls are more resistant than wild type to inhibition by high concentrations of exogenous IAA-Ala (figure V-8). This reinforces the role of \textit{IARI} in conjugate metabolism and suggests that the \textit{IARI} gene product functions not only in the root, but in other plant organs as well.

VI.D.2. \textit{iar1} mutants may be early germinating

IAA conjugates may serve as a storage form of IAA in the seed and provide IAA to the germinating seedling (see section I.D.3.b.). Mutants defective in conjugate metabolism, then, might be expected to exhibit defects in germination. Surprisingly, \textit{iar1} mutants may germinate earlier than wild-type plants (figure V-9). The staining pattern of plants expressing an \textit{IARI} promoter-GUS fusion may provide some explanation of this result. GUS activity is observed in pollen, ovules, and developing embryos (figure VI-2D-J), implicating \textit{IARI} in fertilization and seed development. Developing seeds unable to
metabolize conjugates may store more conjugates and have larger conjugate pools available at germination. The fact that \textit{IAR1} promoter-GUS expression is extremely low in seedlings (figure VI-2A and data not shown) also suggests that the gene does not function in germination and early development, which could explain the lack of late germination in iar1 mutants.

However, the results of GUS fusion analysis to determine sites of gene expression must be interpreted with caution (Taylor, 1997). It is interesting to note that, although \textit{IAR1} message is detected by RNA gel blot analysis in roots and the iar1 mutant was isolated based on a root phenotype, no GUS staining is apparent in roots of \textit{IAR1} promoter-GUS transgenic plants. There are several possible explanations for this apparent discrepancy. First, \textit{IAR1} may be present at extremely low levels in most tissues, and GUS staining assays might not be sensitive enough to reveal such low levels of expression. Second, the regulatory elements needed for proper expression of \textit{IAR1} may not all be present in the \textit{IAR1} promoter-GUS fusion. This fusion contains approximately 2.5 kb 5' of the \textit{IAR1} ATG, and includes all of the DNA between \textit{IAR1} and the gene predicted to be 5' of \textit{IAR1}. However, it is possible that important regulatory regions for \textit{IAR1} expression are present in the introns or 3' of the coding sequence. Finally, it is possible that \textit{IAR1} expression is translationally controlled and the IAR1-GUS translational fusion is transcribed in roots but not translated.

\textbf{VI.D.3. The iar1 mutation partially suppresses the alf1 mutant phenotype}

The Arabidopsis mutant \textit{alf1} (Celenza et al., 1995), also isolated as \textit{surl, ivr, hls3, and rty}, (Boerjan et al., 1995; King et al., 1995; Lehman et al., 1996) overproduces both free and conjugated IAA. \textit{alf1} plants have shortened primary roots, display increased numbers of adventitious and lateral roots, and are completely sterile (Celenza et al., 1995). These phenotypes can be copied by addition of exogenous auxin to wild-type plants (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995; Lehman et al., 1996). The iar1 mutation
partially suppresses the *alf* phenotype, restoring normal root elongation (figure V-10) and some fertility (Bartel, unpublished) to *alf*. The fact that mutations in *IAR1* are able to alleviate auxin-related defects in the *alf* auxin overproducing mutant indicates that the *IAR1* gene product normally plays a role in increasing free IAA levels or IAA sensitivity *in vivo.*

**VI.D.4.** The *iar1* mutant responds normally to IBA, JA, MeJA, and JA conjugates

I tested the response of the *iar1* mutant to IBA, a naturally-occurring form of auxin, and to jasmonic acid (JA), the JA precursor methyl jasmonate (MeJA), and a variety of JA-amino acid conjugates. *iar1* responded like wild type to IBA (figure V-11), indicating that *IAR1* is not involved in IBA signaling or metabolism. *iar1* mutants are able to elongate roots normally on JA, MeJA, and JA-amino acid conjugates as well (figure V-12).

**VI.D.5.** *IAR1* may be involved in wound responses

Interestingly, *IAR3* was also cloned as *JR3*, a JA-induced gene identified by differential display (Titarenko et al., 1997). *IAR3/JR3* is also rapidly and transiently upregulated by wounding, and this wound- and JA-induced expression is blocked in the JA-insensitive mutant *coil* (Rojo et al., 1998; Titarenko et al., 1997), suggesting a role for IAA conjugates in wound responses. *IAR1* expression in wild type plants is induced weakly in response to MeJA and wounding, weakly in *iar3 ilr1* mutants in response to MeJA, and more strongly in *iar3 ilr1* mutants in response to wounding (figure VI-5). Therefore, *IAR1* may play a role in wound responses in a pathway that involves IAA conjugates rather than jasmonic acid, or may provide an important link between the two pathways. It will be interesting to examine *IAR1* transcript levels in response to other hormones, such as salicylic acid, which has been reported to block wounding responses (Raskin, 1995), and to examine in more detail the possible connections between wounding and IAA conjugates.
VI.D.6. *IAR1* encodes a protein with predicted transmembrane domains and histidine-rich regions that shares characteristics with the ZIP family of metal transporters

The phenotypic characterization of the *iarl* mutant shows that the *IAR1* gene is involved either directly or indirectly in the response of Arabidopsis to IAA conjugates. To determine how the *iarl* mutation affects these processes, we cloned the *IAR1* gene using a map-based approach. The mutation was mapped to a region on the bottom of chromosome 1 that was contained on a single BAC (figure V-15). We constructed a complementation library from this BAC and were able to rescue the *iarl* phenotype with a clone from the library that contained three potential open reading frames (figure V-16). Sequence analysis revealed mutations in one of these predicted genes in all *iarl* alleles (figure V-17, table V-3), indicating that the gene is *IAR1*.

The *IAR1* gene has eleven exons and ten introns and encodes a putative protein of 469 amino acids that is 28% identical to the predicted mouse KE4 and human HKE4 proteins encoded by genes within the major histocompatibility complex (MHC) class II locus (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). *IAR1* homologs are also present in *Drosophila* (Catsum; 31% identical) (Stathakis et al., 1999), *C. elegans* (GenBank accession number 3878059), and zebrafish (GenBank accession number AAF05821) (figure V-18). These proteins are all predicted to have cleavable N-terminal signal sequences and seven or eight transmembrane domains (figure V-21). In fact, the hydropathy plots of *IAR1* and human KE4 proteins are almost identical (figure V-19), indicating that the proteins likely have very similar membrane topology. Another intriguing feature of *IAR1* and its homologs is the presence of several extremely histidine-rich regions (figure V-18, V-20).

Interestingly, the ZIP family of metal transporters also have N-terminal signal sequences, seven or eight transmembrane domains, and histidine-rich regions (Eng et al., 1998; Guerinot and Eide, 1999). *IAR1* and its homologs have several potential metal
binding domains of the type HXHXH (Eng et al., 1998) within their histidine-rich regions. IAR1, for example, has four such domains. These domains are present in some members of the ZIP family of metal transporters including the yeast zinc transporter ZRT1 (HDHTHDE; Zhao and Eide, 1996) and the Arabidopsis iron transporter IRT1 (HGHGHGH; Korshunova et al., 1999). These potential metal-binding motifs are located between the third and fourth transmembrane domains of ZRT1, ZRT2, and IRT1 (Eng et al., 1998), as are two of the HXHXH repeats found in IAR1 (figure V-20).

Also of interest is the presence of the ZIP family signature sequence in the IAR1 protein (figure V-21). This signature was identified by Eng et al. (1998) in the mouse and human KE4 proteins, although they referred to these proteins as Mmu Orf1 and Hsa Orf1 rather than KE4. IAR1 contains the fully conserved glycyl and histadyl residues of the ZIP signature sequence and only deviates from the signature sequence at one residue, position 12 of the 15 amino acid signature (figure V-21).

The fifth putative transmembrane domain of the ZIPs is not predicted to be present in IAR1 using the PSORT motif prediction program (Nakai and Kanehisa, 1992) due to the presence of charged amino acids about every third of fourth residue in this region (figure V-20). However, this is a pattern associated with amphipathic helices (Lodish et al., 1995) and may indicate a transmembrane domain that is part of a membrane-spanning pore. The fourth and fifth transmembrane domains of the ZIPs are thought to be essential for metal transport, so it is reasonable to assume that the charged nature of the amphipathic fourth transmembrane domain of IAR1 would facilitate passage of an ion through the membrane. Although IAR1 and the ZIPs are only about 10% identical at the amino acid level, the similarity in molecular structure between IAR1 family members and ZIP family members suggests that IAR1 might be a metal transporter or might bind a metal cofactor.
VI.D.7. Models for IAR1 function in IAA conjugate metabolism

*IAR1* encodes a putative transmembrane protein with characteristics of a transporter. There are at least four possible models that explain how loss-of-function mutations in the *IAR1* gene result in IAA conjugate resistance. These models, along with evidence supporting or refuting them, are discussed below.

VI.D.7.a. Model 1: IAR1 transports IAA conjugates into the ER

Arabidopsis encodes a family of amidohydrolases, some of which hydrolyze IAA-amino acid conjugates (Bartel and Fink, 1995; Davies et al., 1999). These proteins (with the exceptions of ILL3 and GR1) contain C-terminal ER retention signals, suggesting that they function in the ER (Bartel and Fink, 1995; Davies et al., 1999). The IAA conjugate resistant phenotype of the iar1 mutant suggests that *IAR1* might encode an ER-localized IAA conjugate transporter. Loss of IAR1 function would prevent conjugate from reaching the hydrolases and therefore result in conjugate resistance.

To test this hypothesis, I transformed the *IAR1* cDNA under the control of the inducible *GAL1* promoter into yeast. I isolated microsomes from these yeast and assayed for conjugate uptake. I did not detect any uptake activity using a variety of amino acid conjugates and jasmonic acid conjugates, suggesting that IAR1 does not have conjugate transport activity (section VI.C.2.).

IAR1 homologs of unknown function have been described in mammals. These are the KE4 proteins, which are encoded in the mammalian MHC class II locus (Abe et al., 1988; Ando et al., 1996; Janatipour et al., 1992; St.-Jaques et al., 1990). They are approximately 28% identical at the amino acid level to IAR1 and have the same predicted membrane topology (figures V-18, V-19). To determine if the KE4 proteins can functionally substitute for IAR1, I transformed the mouse *KE4* cDNA under the control of the constitutive CaMV 35S promoter into iar1 mutant plants. This construct restored normal IAA-Ala sensitivity to iar1 plants (figures VI-8, VI-9), indicating that the KE4 can
indeed functionally substitute for IAR1. Therefore, IAR1 and KE4 are likely to have similar, if not identical, functions in plants and mammals. It is therefore unlikely that IAR1 is an IAA-conjugate transporter, as mammals have no obvious need for such a transporter.

VI.D.7.b. Model 2: IAR1 transports a conjugate hydrolase cofactor into the ER

The predicted IAR1 protein has many shared structural characteristics with the ZIP family of metal transporters (figure V-20, section VI.D.6.), including potential metal binding domains of the type HXHXH, histidine-rich regions, and a common number of transmembrane domains (Eng et al., 1998; Guerinot and Eide, 1999). I therefore compared the response of the iar1 mutant to that of wild type when exposed to various concentrations of different metals in liquid medium (figure VI-12). For all metals at all concentrations tested, wild-type and iar1 plants responded similarly, except in AgNO3 at 10 and 20 μM, where the wild type was unaffected but the iar1 mutant showed severe growth inhibition. iar1 mutants in the Col-0 background were slightly more sensitive than Col-0 to inhibition of hypocotyl elongation by 1 mM silver when grown on solid medium in the dark (figure VI-14). It will be interesting to examine the response of iar1 mutants to higher concentrations of silver.

The Arabidopsis amidohydrolases characterized to date likely use a metal cofactor, as their activities can be inhibited by EDTA (Bartel and Fink, 1995; figure VI-15B,C). Mutations in these amidohydrolase genes can lead to IAA-amino acid conjugate resistance (Bartel and Fink, 1995; Davies et al., 1999), so mutations that prevent the hydrolases from assembling or retaining the proper metal cofactor might also cause this phenotype. Supplying higher quantities of the required cofactor to the plant might alleviate an IAA-amino acid resistant phenotype due to such a mutation. One of the most effective cofactors in enzyme assays using purified recombinant amidohydrolase protein is manganese. Intriguingly, addition of manganese to plates containing IAA-Ala partially suppressed the IAA-Ala resistant phenotype of the iar1 mutant (figure V-13).
The suppression of the *iar1* phenotype by manganese suggested that IAR1 might be a manganese transporter. Because the amidohydrolase proteins likely function in the ER, I hypothesized that IAR1 might be transporting manganese into the ER for the hydrolases to use as a cofactor. However, *IAR1* failed to rescue the phenotype of the yeast *pmr1* mutant, which is defective in an ER-localized calcium and manganese transporter (Dürr et al., 1998; Rudolph et al., 1989). Furthermore, an Arabidopsis protein capable of transporting manganese into the ER and that does rescue the *pmr1* mutant phenotype, ECA1, has already been identified (Liang et al., 1997). This suggests that IAR1 does not function to transport manganese into the ER as a cofactor for the IAA conjugate hydrolases.

VI.D.7.c. Model 3: IAR1 transports a conjugate hydrolase inhibitor out of the ER

It is possible that IAR1 serves to transport inhibitory metals out of, rather than transporting metal cofactors into, the ER. To determine if the amidohydrolase enzymes could be inhibited by metals, I tested the response of the IAR3 amidohydrolase to various metals and found that its activity is inhibited by zinc and copper (figure VI-15A). Furthermore, zinc and copper inhibited IAR3 activity even in the presence of manganese (figure V-15B,C). This is intriguing because of the structural similarity of IAR1 to the ZIPs (figure V-20) and its possible role as a metal transporter. Most ZIP family members are zinc transporters (Grotz et al., 1998; Zhao and Eide, 1996; Zhao and Eide, 1996), although at least one member of the family can also transport iron, cadmium, and cobalt (Korshunova et al., 1999). Therefore, it is not unreasonable to propose a model in which IAR1 transports zinc and/or copper out of the ER and away from the hydrolases.

This model is also consistent with the manganese-mediated suppression of the *iar1* mutant phenotype: if there is sufficient manganese present, it may be able to out-compete inhibitory zinc or copper for the metal binding site of the amidohydrolase and restore activity to the enzyme.
VI.D.7.d. Model 4: IAR1 inhibits an IAA conjugate hydroxylase

A recent report of a *Drosophila* IAR1 homolog, Catsup (catecholamines up; Stathakis et al., 1999), suggests a possible role for IAR1 in responses to IAA conjugates. Catsup, like the mammalian KE4 proteins, is approximately 30% identical to IAR1 at the amino acid level (figure V-18). The rate-limiting step in catecholamine biosynthesis is the hydroxylation of L-tyrosine to form 3,4-dihydroxy-L-phenylalanine. This reaction is catalyzed by the enzyme tyrosine hydroxylase, or TH (Kumer and Vrana, 1996). TH activity is regulated by multiple mechanisms at a number of levels, including transcriptional, translational, and post-translational (Kumer and Vrana, 1996). *catsup* mutant flies have higher TH activity, but normal TH protein levels, suggesting that the Catsup protein is a negative regulator of the TH. The increased TH enzyme activity in the mutants leads to increased synthesis of catecholamine neurotransmitters in these flies, which is lethal (Stathakis et al., 1999).

Interestingly, IAA conjugates can be hydroxylated in plants (Östin et al., 1998; Östin et al., 1992). This may cause the conjugates to become permanently inactivated and therefore be a mechanism used by plants to detoxify excess auxin. Thus, if IAR1 regulates an auxin conjugate hydroxylase in the same manner that Catsup regulates TH, the *iar1* mutant would be expected to have higher conjugate hydroxylase activity and be IAA-conjugate resistant. While a model wherein IAR1 functions to inhibit the activity of a conjugate hydroxylase is an attractive one, the enzyme or enzymes responsible for conjugate hydroxylation in Arabidopsis have not yet been identified. Obvious homologs of the gene encoding TH or the related phenylalanine or tryptophan hydroxylases are not present in the sequenced portion of the Arabidopsis genome (89% complete as of April 2000).

The mammalian KE4 gene can functionally substitute for *IAR1* in Arabidopsis (figures VI-8,9). It is likely, then, that the encoded proteins have the same or very similar functions *in vivo*. *Drosophila* Catsup is more similar to IAR1 (31% identical to IAR1) than is KE4 (28% identical to IAR1), so it is reasonable to assume that Catsup has the same
function as well. This, coupled with the apparent lack of an Arabidopsis TH homolog, suggests a general, rather than TH-specific, role for these proteins.

Two important mechanisms for control of TH activity are the phosphorylation state of the enzyme and the availability of its cofactor, tetrahydobioppterin (BH$_4$; Kumer and Vrana, 1996; Zigmond et al., 1989). Increased phosphorylation of TH results in increased enzyme activity (Kaufman, 1997). The initial, and rate-limiting, step in BH$_4$ biosynthesis is catalyzed by GTP cyclohydrolase I (GTPCHI; Kaufman, 1997), and increased phosphorylation increases the activity of GTPCHI as well. Intriguingly, Burton, et al. recently reported that Catsup is capable of forming complexes with both TH and GTPCHI (Burton et al., 2000). Also, GTPCHI activity in catsup mutants is two to seven-fold higher than in wild-type flies, and TH phosphorylation levels are higher (Burton et al., 2000). This suggests that Catsup negatively regulates TH activity by inhibiting both phosphorylation and cofactor biosynthesis. Therefore, it is possible that IAR1 modulates phosphorylation of an IAA-amino acid conjugate hydroxylase, or perhaps a completely different type of enzyme, and thereby controls IAA conjugate metabolism.
CHAPTER VII: MUTANT CHARACTERIZATION AND CLONING OF THE FKF1 GENE

The work discussed in this chapter has been submitted for publication (Nelson et al., 2000). The fkf1 mutant was isolated as iar1-4 by Bonnie Bartel. Initial phenotypic characterization of the mutant was carried out by Mindy Anderson Cohen (Anderson, 1998). Additional phenotypic analysis was performed by David C. Nelson, including determination of flowering time in various day lengths and in response to vernalization and gibberellin. David C. Nelson and Luise E. Rogg were instrumental in the cloning of the FKF1 gene, David C. Nelson cloned the FKF1 and ZTL cDNAs and made the pBIN-FKF1 construct. Bonnie Bartel performed crosses of fkf1 to ft and fe, carried out initial mapping, and made the alignment shown in figure VII-6. Luise E. Rogg determined the size of the fkf1 deletion and of the BAC T7E4.

VII.A. Phenotype of the fkf1 mutant

VII.A.1. The fkf1 mutant is late flowering

fkf1 was originally isolated as an allele of iar1, iar1-4, in a screen for IAA-Ala resistant mutants (see Chapter V). This mutant, upon transfer to soil, was significantly later flowering than wild type. Additionally, the remaining six alleles of iar1 flowered normally. Because iar1-4 was isolated from a pool of fast-neutron mutagenized seeds, we reasoned that a gene closely linked to IAR1 may also have been mutagenized, as fast neutrons cause deletions in Arabidopsis (Bruggemann et al., 1996). We used recombination mapping to localize the iar1 mutation to the bottom of chromosome 1 (see Chapter V), near the ft and fe late-flowering mutants (Koornneef et al., 1991). F1 plants from crosses of iar1-4 to either of these mutants flowered normally and we could not separate the flowering defect and auxin conjugate resistance in iar1-4 by recombination (see Materials and Methods, III.B.).
indicating that we had identified a new locus required for normal flowering, which we named FKF1.

To quantitate the late-flowering phenotype of the fklf mutant, we compared fklf flowering time to that of wild type when grown in long days (LD; 16 hours light, 8 hours dark) and in short days (SD; 8 hours light, 16 hours dark). The fklf mutant flowers significantly later than wild type in LD as measured by either the number of days to flowering or the number of leaves at flowering (figures VII-1A, VII-2). In SD, the fklf mutant flowers more normally (figure VII-2B).

VII.A.2. fklf flowering defects are rescued by vernalization or gibberellin

There are a number of pathways that regulate flowering in Arabidopsis: the autonomous pathway, the photoperiod-dependent pathway, the vernalization pathway, and the gibberellin pathway (see Chapter II). Flowering time mutants can be placed in these pathways based on their responses to various stimuli. For example, mutants in the photoperiod-dependent pathway of floral promotion flower late only in inductive (LD) photoperiods and are generally non-responsive to vernalization treatment. Conversely, a separate group of mutants is late flowering regardless of photoperiod, and treatment of such mutants with vernalization restores normal flowering time. The genes defective in these vernalization-responsive mutants function in the autonomous pathway, rather than the daylength-dependent, or photoperiodic, pathway of floral promotion (Piñeiro and Coupland, 1998; Simpson et al., 1999). The phytohormone gibberellin (GA) also promotes flowering in both wild-type and autonomous pathway mutants, and this promotion likely occurs via a mechanism distinct from the autonomous, photoperiod-dependent, and vernalization-response pathways (Piñeiro and Coupland, 1998; Simpson et al., 1999).
Figure VII-2: The fkh1 mutant is late flowering in long days. Col-0 wild type and fkh1 mutant plants were grown in long days (16 hours light, 8 hours dark) and photographed after 37 days.
Figure VII-2: *fkl* mutants are late flowering in long days. A.) Col-0 wild type and *fkl* mutant seeds were sown on agar-based growth media and grown in LD conditions (16 hours white light: 8 hours dark). White light was ~200 µE m⁻² sec⁻¹. They were then transferred to soil and maintained in the same conditions. Days to flowering and leaf number at flowering were recorded on the day when the emerging bud was first visible in the shoot apex. B.) Flowering time of plants grown in SD (8 hours white light, 16 hours dark; white light was ~90 µE m⁻² sec⁻¹. Error bars are standard deviations of the means (*n* ≥ 33).
We examined the response of \textit{fkl} plants to treatment with vernalization or GA to help place \textit{FKFL} in a floral promotion pathway. Either treatment dramatically accelerates \textit{fkl} flowering to the rate of untreated wild-type plants (figure VII-3A,B), suggesting that \textit{FKFL} induces flowering through the autonomous pathway.

\textbf{VII.A.3. The \textit{fkl} mutant is defective in light-regulated hypocotyl elongation}

Plants suppress hypocotyl elongation in response to light, and this classical phenotype has been used to isolate numerous photoreceptor mutants (Neff et al., 2000). Because of the many connections between flowering time and light perception (Levy and Dean, 1998; Simpson et al., 1999; see Chapter II), we examined hypocotyl elongation in the \textit{fkl} mutant under various light conditions. \textit{fkl} mutant hypocotyls elongate normally in the dark (Figure VII-4) and are capable of elongation responses at high temperature (figure V-5) indicating that the mutant can carry out hypocotyl elongation growth. However, \textit{fkl} has short hypocotyls in several light wavelengths (Figure VII-4), suggesting that it is hypersensitive or hyperresponsive to light.

\textbf{VII.B. Cloning \textit{FKFL}}

\textbf{VII.B.1. \textit{FKFL} is a member of a gene family in Arabidopsis}

We cloned the \textit{FKFL} gene using the same map-based reagents employed in cloning \textit{IARL}. Using recombination mapping, we localized the \textit{iarl} mutation, which is closely linked to \textit{fkl}, to a then unsequenced region on the bottom of chromosome 1. Using markers developed for the map-based cloning of \textit{IARL} (table V-2), we narrowed the region that should contain the \textit{FKFL} and \textit{IARL} genes to a single bacterial artificial chromosome clone, T7E4 (figure VII-5). We used DNA from the 116 kb T7E4 clone to construct a genomic library (see section III.F), transformed individual clones from this library into the
Figure VII-3: The late-flowering phenotype of *fkl* is rescued by vernalization or gibberellin treatment. A.) *fkl* flowering time defects are rescued by gibberellin treatment. Plants grown in soil were left untreated or sprayed with 10 μM GA₃ seven and ten days after sowing (*n* ≥ 20). B.) *fkl* flowering time defects are rescued by cold treatment. Seeds were sown in moist soil and either placed at 22 °C under continuous illumination (untreated) or placed at 4 °C in dim white light for four weeks (vernalization) prior to incubation at 22 °C under continuous illumination. Day 0 represents the day that the plants were shifted to 22 °C (*n* ≥ 20).
Figure VII-4. *fkf1* has a short hypocotyl in the light. Hypocotyl lengths of six-day-old seedlings (wild type, *fkf1* mutants, and two homozygous T3 lines of *fkf1* mutants transformed with the *FKF1* genomic construct pBIN-**FKF1**) were measured after growth in the dark, under continuous white light, or under continuous red- or blue-filtered light. Asterisks indicate that measurements are significantly different than wild type; circles indicate that measurements are significantly different than *fkf1* (Students *t* test; *p* < 0.0005).
*fkfl* mutant, and looked for transgenic plants with normal flowering time (figure VII-5). We identified three overlapping subclones that restored normal flowering to the mutant and obtained the sequences of these subclones. We identified two putative open reading frames (ORFs) and found that one of these is entirely contained on a non-rescuing construct, ruling it out as *FKFI*. Therefore, the remaining ORF likely represents the *FKFI* gene. A construct containing only this open reading frame rescued the flowering time defect (figure VII-5B and data not shown) and partially rescued the short hypocotyl phenotypes (figure VII-4) of the *fkfl* mutant, but did not affect the auxin conjugate resistance (data not shown). This indicated that we had identified the *FKFI* gene and that *FKFI* and *IAI* do not overlap. We isolated and sequenced a full-length cDNA representing *FKFI* (GenBank accession number AF216523).

There are two close Arabidopsis homologs of *FKFI* in the GenBank database. We isolated and sequenced a full-length cDNA (GenBank accession number AF216525) for one of these genes, *ZTL* (Somers et al., 2000) which maps to the bottom of chromosome 5 on the P1 clone MSF19. The second (which we named *FKL1* for *FKFI*-like) maps to chromosome 2 on BAC F19F24. We did not detect an *FKL1* cDNA in two libraries (Minet et al., 1992; S. LeClere and B. Bartel, unpublished), suggesting that it might be less abundantly expressed than the other two family members. *FKFI*, *ZTL*, and *FKL1* each have a single intron and encode a ~600 amino acid protein. The encoded proteins are are similar along their lengths: *FKFI* is 62% identical to *ZTL* and 57% identical to *FKL1*, and *ZTL* and *FKL1* are 74% identical to one another (figure VII-6).

VII.B.2 *FKFI* encodes a putative flavin-binding protein with an F-box and Kelch repeats

*FKFI*, *ZTL*, and *FKL1* are similar to characterized proteins in three separate domains that suggest modes of action. A LOV (light, oxygen, voltage) domain is located
A

molecular markers:

predicted genes:
PIS1 HPR
FKF1
IAR1

complementation constructs:

■ rescues
□ does not rescue

B

Col-0 (wild type)
fkf1
fkf1 (pBIN19-FKF1) A

number at flowering

days

leaves
Figure VII-5. Positional cloning of *FKF1*. A.) Genetic and physical map of the genomic region containing *FKF1*. Initial mapping data placed the *iarl* and *fklf* mutations at the bottom of chromosome 1 between the SSLP markers nga280 and nga111 (Bell and Ecker, 1994). Additional markers from YAC CIC9H12 and abi14G4 end clones further refined the *iarl* interval to between 9H12 and 14G4. These latter markers both hybridized to the BAC clone T7E4, which was used to make a library for complementation. Constructs from this library that were introduced into the *fklf* mutant are indicated by rectangles below a schematic of the predicted coding sequences (arrows) in the region. At least 65 kb of this DNA is deleted in the *fklf* mutant, beginning at the point marked with a triangle. Filled rectangles indicate those constructs that restored normal flowering time to the *fklf* mutant; open rectangles failed to rescue. B.) Genomic DNA containing the *FKF1* gene restores normal flowering to the *fklf* mutant. Col-0 wild type, *fklf* mutants, and a transgenic *fklf* lines homozygous for the pBIN-*FKF1* construct (see panel A) were assayed for flowering time as described in the legend to figure VII-2. Asterisks indicate significant difference from wild type and diamonds indicate significant difference from *fklf* by Student’s t test, *p* < 0.0001. Error bars are standard deviations of the means (*n* ≥ 33).
near the N-terminus of each protein. LOV domains are a subfamily of the PAS domain superfamily and are found in a number of blue light receptors, where they bind a flavin chromophore (Briggs and Huala, 1999). Several eukaryotic photoreceptors have LOV domains 21-31% identical to those of the FKF1 family (figure VII-6). LOV domains in NPH1, a blue-light receptor kinase in Arabidopsis (Huala et al., 1997), and PHY3, a photoreceptor from the fern Adiantum (Nozue et al., 1998), stoichiometrically bind the chromophore flavin mononucleotide when heterologously expressed (Christie et al., 1998; Christie et al., 1999). Preliminary experiments with the FKF1 LOV domain expressed in E. coli suggest that FKF1 also binds flavin (Nelson and Bartel, unpublished), but the identity of the bound flavin has not been determined.

The FKF1, ZTL, and FKL1 proteins also each contain a 40 amino acid F-box motif in the central region (Figure VII-6). F-box proteins are thought to function as adapters that bring specific substrates to core ubiquitin protein ligase subunits for ubiquitination and subsequent degradation, suggesting that FKF1 and its homologs may target proteins for degradation.

Many F-box proteins also contain repetitive domains that function in protein-protein interactions (Bai et al., 1996; Patton et al., 1998). The C-terminal halves of FKF1, ZTL, and FKL1 consist of six kelch repeats (figure VII-6). This motif was originally identified as a six-fold tandem repeat of approximately 50 amino acids in the Drosophila kelch protein (Xue and Cooley, 1993), which promotes ring canal organization during Drosophila oogenesis (Robinson and Cooley, 1997). The seven kelch repeats in galactose oxidase fold to form a β-propeller, with each repeat forming a blade consisting of a four-stranded β-sheet (Ito et al., 1991). Other kelch motif proteins probably form similar structures which are though to mediate protein-protein interactions (Adams et al., 2000).

The FKF1-family kelch repeats have di-glycine and tryptophan residues (Figure VII-6B) that are conserved in 90% of kelch proteins (Adams et al., 2000), but a normally
Figure VII-6. *FKF1* encodes a putative flavin-binding, kelch domain, F-box protein (A) Schematic representation of the three domains of FKF1, ZTL, and FKL1 and other PAS (LOV)-domain containing proteins. WC-1 is a blue light receptor containing a zinc finger transcription factor motif from *Neurospora* (Ballario et al., 1996), PHY3 is photoreceptor from the fern *Adiantum* that contains features of both red and blue light receptors (Nozue et al., 1998), and NPH1 is an Arabidopsis blue-light receptor kinase that regulates phototropism (Christie et al., 1998; Christie et al., 1999; Huala et al., 1997). (B) Comparison of the predicted amino acid sequences of FKF1 and two Arabidopsis homologs, ZTL and FKL1, with relevant domains from other proteins. Sequences were aligned with the Megalign program (DNASTar) using the Clustal method (Higgins and Sharp, 1989). Identical residues among the PAS/LOV domains are shaded in green, identical residues in the F-box motif are shaded in blue, and residues conserved in five of the six FKF1 family kelch domains are shaded in magenta. Identities and conservation among FKF1 and its homologs in other regions are shaded in black and gray, respectively. Sequences in bold below the alignments are the residues conserved in >80% of PAS domains (in green; Zhulin and Taylor, 1997) or in >90% of kelch repeats (in magenta; Adams et al., 2000); h is a hydrophobic residue (FILMVWY). PAS domains shown are from *Neurospora* (*Nc*) WC-1 (Ballario et al., 1996), *Adiantum* (*Ac*) PHY3 (Nozue et al., 1998), Arabidopsis (*At*) NPH1 (Huala et al., 1997) and NPH2 (Jarillo et al., 1998), and *Oryza sativa* (*Os*) NPH1b (GenBank accession # AB018443). F-boxes shown are from Arabidopsis TIR1 (Ruegger et al., 1998), COI1 (Xie et al., 1998), and UFO (Samach et al., 1999); *Antirrhinum* (*Am*) FIM (Simon et al., 1994); human (*Hs*) SKP2 (Zhang et al., 1995); *Aspergillus* (*An*) SconB (Natorff et al., 1998); and *Saccharomyces* (*Sc*) MET30 (Thomas et al., 1995), CDC4 (Yochem and Byers, 1987), and GRR1 (Flick and Johnston, 1991).
conserved tyrosine is replaced by a leucine in the FKF1 family (figure VII-6B). Like galactose oxidase (Ito et al., 1991), FKF1 and its homologs terminate just prior to the fourth predicted β-sheet of the final kelch motif (figure VII-6B). This β-sheet is probably supplied by a predicted β-sheet just prior to the first kelch motif (containing the conserved tryptophan residue), providing a mechanism to hold the ring closed (Adams et al., 2000).

In summary, FKF1 and its two Arabidopsis homologs, ZTL and FKL1, are predicted to each encode a protein with an N-terminal PAS domain similar to the flavin-binding LOV domains of certain photoreceptors, an F-box motif found in proteins involved in targeting ubiquitin-mediated degradation, and six tandem kelch motifs predicted to fold into a β-propeller and mediate protein-protein interactions (figure VII-6).
CHAPTER VIII: MOLECULAR CHARACTERIZATION OF FKF1

The work discussed in this chapter is in press (Nelson et al., 2000). Luise E. Rogg performed the GUS staining and mounted and photographed GUS-stained seedlings. Root and shoot RNA was made by Rosie Tellez. David C. Nelson performed developmental timecourse RNA preparation and gel blot analysis, made the constructs and transgenic lines discussed in this chapter, and the remainder of the experiments were a cooperative effort between David C. Nelson and myself.

VIII.A. FKF1 is expressed in a variety of tissues

To determine where and when FKF1 is expressed, we examined FKF1 transcript levels using gel blot analysis of RNA prepared from various Arabidopsis organs and throughout plant development. FKF1 mRNA was detected in all organs examined, with the highest level in leaves (figure VIII-1A). In addition, FKF1 expression increases slightly throughout development, consistent with a role in floral promotion (figure VIII-1B). Although the FKF1 antisense RNA probe used in these experiments detects two mRNAs, the fkf1 mutant, which represents a deletion of the entire FKF1 gene, lacks only the upper band (figure VIII-1B), indicating that only this message derives from the FKF1 gene. We also examined transcript levels of the FKF1 homolog ZTL. Its message is distributed similarly to FKF1, except that ZTL transcripts are more abundant in seeds (figure VIII-1A) and do not appear to increase over the course of development (figure VIII-1B).

To examine more specifically the sites of FKF1 expression, we made a reporter construct consisting of the FKF1 promoter fused to β-glucuronidase (GUS). We transformed this construct into wild-type plants and analyzed the resulting expression pattern. The construct is expressed in a variety of tissues, including root tips, leaves, sepals, and anther filaments (figure VIII-1G-N). A number of stomata in leaves and cotyledons
Figure VIII-1: Specificity of FKF1 and ZTL expression. A.) Total RNA (5 \( \mu \)g) isolated from dry seeds; roots and aerial tissues (rosettes) from 14-day-old plants; and leaves, inflorescence stems, and siliques of 29-day-old plants (all grown in continuous white light) were separated on a gel, transferred to a membrane, and probed with \(^{32}\)P-labeled antisense FKF1 or ZTL RNA probes to determine mRNA levels and with 28S rDNA as a loading control. The FKF1 transcript, which is absent in the \( \textbf{f} \text{fl} \) deletion mutant, is indicated, and the asterisk indicates an unidentified cross-reacting transcript. The bars show normalization of the FKF1 and ZTL transcripts to 28S levels. B.) Levels of FKF1 and ZTL during development were monitored as described above by analyzing RNA prepared from rosettes of wild-type (Col-0) plants grown in continuous white light for the indicated number of days. A \(^{32}\)P-labeled antisense UBQ10 RNA was used as a loading control. The bars show normalization of the FKF1 and ZTL transcripts to UBQ10 levels. (C-N) FKF1-GUS expression. (C-F) Six-day-old T3 seedlings grown under white light show staining in the shoot (C), root/hypocotyl junction (D), and cotyledons (E), including stomata (F). (G-J) Eight-week-old T2 plants grown under continuous white light show staining in rosette (G) and cauline (H) leaves, sepals of immature flowers (I), and sepals and anther filaments of mature flowers (J). (K-N) Six-day-old T3 seedlings show root tip staining after growth in continuous white (K), blue (L), or red (M) light, or after incubation under white light for one day and in darkness for five days (N). All panels are after 20 hours incubation in X-Gluc, excluding (D), which was a six hour incubation. Scale bars for (C,G-J) represent 1 mm, all others represent 0.1 mm.
were clearly stained (figure VIII-1F). The GUS staining pattern also suggests light regulation of *FKF1* expression, as dark-grown seedlings had dramatically lower expression levels that were detectable only in root tips (figure VIII-1N and data not shown), and plants grown in red light (figure VIII-1M and data not shown) stained less than those grown in either white or blue light (figure VIII-1K,L and data not shown).

**VIII.B. Constitutive expression of the *FKF1* homolog *ZTL* leads to long hypocotyls and delayed flowering**

To explore how the *FKF1* homolog *ZTL* functions in plant development, we expressed a full-length *ZTL* cDNA behind the constitutively expressed Cauliflower Mosaic Virus 35S promoter and monitored hypocotyl elongation and flowering time in the resulting transformants. Flowering was delayed to varying degrees in some primary transformants expressing this construct (data not shown). We examined hypocotyl elongation in the progeny of two transformants with delayed flowering time and one transformant with normal flowering time. The transformants with delayed flowering time had dramatically longer hypocotyls when grown under red- or blue-filtered light (figure VIII-2).

Interestingly, this elongation was exaggerated in plants grown in light-dark cycles (figure VIII-2B) compared to plants grown in continuous light (figure VIII-2A). Growth in light-dark cycles also causes *ZTL* overexpressors to have longer hypocotyls than wild type in white light (figure VIII-2B). In contrast, the normally flowering transformant displayed nearly normal hypocotyl elongation (figure VIII-2). These results suggest that changes in the level of *ZTL* transcription disrupt two light-controlled processes, flowering time and hypocotyl elongation.
Figure VIII-2. Effects of constitutive expression of ZTL on hypocotyl elongation. A) T<sub>2</sub> progeny of wild-type (ecotype Col-0) plants transformed with a 35S-ZTL construct were grown in the dark, under continuous white light, or under continuous red- or blue-filtered light, after which hypocotyl lengths were measured. The G and H 35S-ZTL lines were from T<sub>1</sub> plants that were late flowering in continuous light, and the B line was from a T<sub>1</sub> plant that flowered similarly to wild type in continuous light. Asterisks indicate that measurements are significantly different than wild type (Students t test; P < 0.0005). B) Same as in (A), except that plants were grown in 12-hour photoperiods.
VIII.C. The *fkl1* mutation does not alter PhyA degradation rates

PhyA protein accumulates to high levels in dark-grown seedlings and is rapidly degraded upon exposure to activating wavelengths of light. This process is likely ubiquitin-mediated (Clough and Vierstra, 1997). Interestingly, *phyA* mutants exhibit long hypocotyls in far-red light (Reed et al., 1994) and respond less than wild type to night breaks (Reed et al., 1994), which normally promote flowering, indicating that *phyA* mutants are less sensitive to certain light stimuli. In addition, PhyA overexpressing plants have short hypocotyls in the light (Boylan and Quail, 1991).

The late-flowering and short hypocotyl phenotypes of the *fkl1* mutant are consistent with alterations in the ability of the plant to perceive light. These phenotypes, along with the presence of an FKF1 F-box, which may be involved in targeting proteins for degradation through the ubiquitin pathway, led us to explore the possibility that PhyA degradation rates might be altered in *fkl1* plants. We prepared protein from *fkl1* and wild-type seedlings grown in the dark for six days, then exposed to white light for various lengths of time. We analyzed PhyA levels in these samples by gel blot analysis using the O73D anti-PhyA antibody (Shanklin, 1988; kindly provided by Dr. Richard Vierstra, UW-Madison). No significant differences in the rate of PhyA degradation in response to light were observed between *fkl1* and wild type plants (figure VIII-3).

VIII.D. *FKF1* transcripts oscillate with a circadian rhythm

Because the *fkl1* mutant is defective in two light-regulated processes, flowering time and hypocotyl elongation, we examined *FKF1* transcript abundance in RNA prepared from plants grown in a 12 hour light:12 hour dark photoperiod. Consistent with the reporter gene expression (figure VIII-1N), we did not detect *FKF1* mRNA in the dark phase of the cycle. However, *FKF1* levels were strongly up-regulated (approximately five-fold) seven to ten hours after subjective dawn. This diurnal regulation of *FKF1* expression (data not shown) suggested that the circadian clock might control *FKF1* expression.
Figure VIII-3: PhyA degradation rates are not altered in the *fkl1* mutant. Col-0 and *fkl1* seedlings were grown in darkness for six days, then transferred to white light. Samples were harvested at the indicated time points, and protein prepared from these samples was subjected to gel blot analysis using the O73D anti-phyA antibody (see section III.P.2).
To test this possibility, we grew wild-type plants under a 12-hour photoperiod for ten days, then transferred them to continuous white light. RNA was prepared from plants harvested every three hours beginning during the last dark period. If *FKF1* expression is controlled by the clock, its transcripts should continue to cycle even after plants are removed from light/dark cycles as one characteristic of a circadian clock is the ability to maintain cycling in the absence of external stimuli. The cycling of *FKF1* transcript abundance continued with a period of approximately 24 hours even after the transfer to continuous light, indicating that *FKF1* expression is circadianly regulated (figure VIII-4).

**VIII.E. Deleting *FKF1* alters circadian transcript oscillation in continuous light**

Many genes involved in the transition to flowering in Arabidopsis have also been implicated in control of the circadian clock or clock-related processes. The clock is also involved in mediating hypocotyl elongation in Arabidopsis (see Chapter II). The *fkf1* mutant is late flowering (figures VII-1,2) and has abberant hypocotyl elongation in response to light (figure VII-4). These phenotypes and the fact that the circadian clock regulates *FKF1* transcript levels (figure VIII-4) led us to investigate whether the circadian clock itself is altered in the *fkf1* mutant. We compared the expression of several clock-controlled genes in the *fkf1* deletion mutant and in wild-type plants entrained in a 12-hour photoperiod and then transferred to constant white light. We analyzed mRNAs encoding chlorophyll a/b binding protein (CAB), which is part of the clock output (Millar and Kay, 1991) and CCA1, which may provide input to or comprise a component of the central oscillator (Wang and Tobin, 1998). Both transcripts oscillate with a normal period length in *fkf1* transferred to light. However, the waveform of transcript oscillation was altered, with delayed or slowed induction of both *CAB* and *CCA1* in *fkf1* (Figure VIII-5). These results suggest that *FKF1* is necessary for some aspects of normal circadian clock function.
Figure VIII-4. *FKF1* transcripts oscillate with a circadian rhythm. Arabidopsis plants were grown in a 12-hour light:12 hour dark photoperiod for ten days, then shifted to constant light at the end of the dark period on day ten. Plants were harvested for RNA preparation every three hours beginning in the final dark period. Total RNA (5 μg) was separated on an agarose gel, transferred to a nylon membrane, and hybridized to a $^{32}$P-labeled *FKF1* or *UBQ10* antisense RNA probes. The asterisk indicates an unidentified *FKF1*-cross-reacting transcript that, unlike the *FKF1* transcript, is present in the *fkf1* deletion mutant (see figure VIII-1B). The graph shows the ratio of *FKF1* to *UBQ10* hybridization following phosphorimager quantitation. The light and dark bars represent light and dark photoperiods, and the hatched bars represent subjective dark after the transfer to constant light.
Figure VIII-5. Loss of FKF1 alters oscillations of certain clock-controlled transcripts. CCA1 (A) and CAB (B) transcripts were monitored in wild type and fkl plants shifted to constant light after 11 days in 12-hour photoperiods. Graphs show normalization of cycling transcripts to UBQ10 following phosphorimager quantitation. Light and dark bars represent light and dark photoperiods and hatched bars represent subjective dark after transfer to constant light.
VIII.F. Discussion

VIII.F.1. The *fkl* mutation affects processes regulated by the circadian clock

Recent advances in dissecting the molecular nature of the circadian clock in various systems has led to the model of central oscillators that consist of autoregulatory negative feedback loops (Dunlap, 1999). Input pathways entrain the circadian oscillator to environmental cues. Clock-controlled genes may be components of the central oscillator or may act in output pathways that regulate rhythmic processes in the organism.

Hypocotyl elongation (Dowson-Day and Millar, 1999) and flowering time (Somers, 1999) are controlled by the circadian clock in Arabidopsis, and *fkl* has defects in both of these processes. This is consistent with a role for *FKF1* in clock function. The flowering time phenotype of *fkl* is completely rescued and the hypocotyl elongation phenotype at least partially rescued in plants transformed with an *FKF1* genomic construct (figures VII-4, VII-5), indicating that both result from loss of the same gene. Vernalization rescues the *fkl* flowering defect (figure VII-3), suggesting that *FKF1* acts in the autonomous pathway of floral promotion (Piñeiro and Coupland, 1998; Simpson et al., 1999), whereas the relatively normal *fkl* flowering in short days (figure VII-2B) suggests a photoperiodic pathway defect. Most previously characterized flowering time mutants with circadian defects are implicated in photoperiod responses, reflecting the role of the clock in day length perception. For example, *GI* promotes flowering in the photoperiod-responsive pathway and *gi* mutants have aberrant clock function (Fowler et al., 1999; Park et al., 1999).

However, the photoperiod-responsive and autonomous pathways interact, and autonomous pathway clock influences have been noted. For example, *ELF3*, which is involved in light input to the clock (Hicks et al., 1996), apparently functions upstream of genes in both pathways (Chou and Yang, 1999), and mutations in the autonomous pathway repressor *FLC* shorten circadian periods (Swarup et al., 1999). Furthermore, *cry1* mutants,
which have long periods in blue light (Somers et al., 1998a), flower late in both short and long days in Col-0 (Bagnall et al., 1996), suggesting autonomous pathway defects.

VIII.F.2. *FKFI* is expressed in a variety of tissues

RNA gel blot analysis reveals *FKFI* and *ZTL* messages in most plant organs, with high levels in leaves (figure VIII-1A). Consistent with these results, an *FKFI* promoter-GUS reporter fusion is expressed in cotyledons as well as rosette and cauline leaves (figure VIII-1C, E, G, H). Both leaves and cotyledons express *FKFI*-GUS in some stomata (figure VIII-1E, F). This is intriguing because blue light controls both stomatal opening (Assmann and Shimazaki, 1999; Briggs and Huala, 1999) and the circadian clock (Somers, 1999), but the responsible photoreceptor has not been identified (Lascève et al., 1999). However, a variety of evidence points to a zeaxanthin cofactor in this response (Frechilla et al., 1999; Zeiger and Zhu, 1998), and FKFI is more likely to bind a flavin cofactor.

*FKFI*-GUS staining was also reproducibly observed in the tips of primary (figure VIII-1K-N) and secondary roots, anther filaments (figure VIII-1J), and sepals (figure VIII-1I,J). Expression in the root seems unusual for a flowering time control gene. However, several genes functioning in the autonomous flowering promotion pathway are also expressed in roots, including *LD* (Aukerman et al., 1999), *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999) and *FCA* (Macknight et al., 1997).

Interestingly, light apparently modulates *FKFI*-GUS expression. Whereas seedlings grown in white or blue light stained strongly, seedlings grown in red light were only moderately stained, and dark-grown seedlings showed relatively weak root tip staining in strongly expressing lines and no staining in other tissues (figure VIII-1K-N and data not shown).
VIII.F.3. The *FKF1* transcript oscillates with a circadian rhythm

The *FKF1* transcript is diurnally regulated and this oscillation persists when photoperiod-entrained plants are shifted to constant light (figure VIII-4). *FKF1* transcript levels peak in the afternoon eight to ten hours after subjective dawn. This expression phase resembles that seen in the oscillation of transcripts of the Arabidopsis *GI* gene (Fowler et al., 1999; Park et al., 1999), a gene that also promotes the transition to flowering (Rédei, 1962). In contrast, transcripts of *LHY* and *CCA1*, which encode Myb-related transcription factors that may be part of the Arabidopsis central clock oscillator, peak at dawn and their levels decline as *FKF1* levels peak (Schaffer et al., 1998; Wang and Tobin, 1998).

Precise regulation of *FKF1* transcript levels may be necessary for its roles in floral promotion and hypocotyl elongation. Some of the *fkf1* mutant lines transformed with the rescuing *FKF1* genomic construct actually flower significantly earlier than wild type in long day photoperiods (16 hr light, 8 hr dark; figure VII-5B). This suggests that *FKF1* function is sensitive to gene dosage and the consequent expression level; the level of transgene expression can vary among transgenic plants depending on site of insertion and the number of copies of the insertion. Furthermore, the genomic *FKF1* construct only partially rescues the *fkf1* hypocotyl elongation defect in the lines analyzed (figure VII-4). Isolating and characterizing insertional mutations in *FKF1* should enable us to determine if the hypocotyl elongation defects observed in the *fkf1* mutant are due solely to loss of the *FKF1* gene, or if the deletion of another gene in the ~65 kb missing in *fkf1* (figure VII-5) contributes to the phenotype as well.

VIII.F.4 The flavin-binding domain suggests a role for FKF1 in light perception

FKF1 and its Arabidopsis homologs ZTL and FKL1 share three domains found in previously characterized proteins (figure VIII-6). One of these is a PAS domain, which is an approximately 100 amino acid motif that was originally identified (Nambu et al., 1991) as two direct repeats in the Drosophila clock protein PER (Jackson et al., 1986), the
mammalian transcription factor ARNT (Hoffman et al., 1991), and the Drosophila SIM protein (Crews et al., 1988). PAS domains can mediate protein dimerization (Huang et al., 1993) and have been identified in sensor domains of proteins from prokaryotes, eukaryotes, and archaeabacteria (Zhulin et al., 1997). For example, PAS domains, also known as S-boxes, mediate perception of oxygen and redox potential in the sensor modules of prokaryotic two-component regulatory systems (Zhulin et al., 1997).

Degenerate PAS domains recently have been described in several blue light photoreceptors, where they are known as LOV (light, oxygen, voltage) domains (Huala et al., 1997). NPH1 is an Arabidopsis blue light photoreceptor that is necessary for second-positive phototropism (Huala et al., 1997). NPH1 and its homologs share two N-terminal LOV domains and a C-terminal kinase domain (figure VIII-6A). The PHY3 photoreceptor from the fern Adiantum (Nozue et al., 1998) combines an N-terminal phytochrome (red light receptor) region with two central LOV domains and a C-terminal kinase (figure VIII-6A). Heterologously expressed NPH1 and PHY3 LOV domains bind the chromophore flavin mononucleotide (Christie et al., 1998; Christie et al., 1999), and even a single NPH1 LOV domain can bind flavin (Christie et al., 1999). The similarity to these photoreceptors in the flavin-binding domain (figure VIII-6B) suggests that FKF1 family members will also bind flavin and respond to light. Initial evidence suggests that the LOV domain of FKF1 also binds flavin (Nelson and Bartel, unpublished).

Neurospora WC-1 is a zinc finger-type transcription factor with a single LOV motif in addition to a canonical PAS domain (Ballario et al., 1996). wc-1 is a clock-associated gene required for inducing the frequency gene in response to light and for sustaining rhythmicity in the dark (Crosthwaite et al., 1997). Although LOV domains can mediate dimerization, certain WC-1 LOV domains with mutations that render Neurospora blind to blue light still dimerize normally, suggesting additional roles for the LOV motif (Ballario et al., 1998). The homology of FKF1 to WC-1 is particularly intriguing, as WC-1 is the circadian photoreceptor through which light entrains the Neurospora clock (Crosthwaite et
al., 1997). Interestingly, the WC-1, FKF1, ZTL, and FKL1 proteins share a conserved 11 amino acid insertion in the center of the domain that is absent in other LOV proteins (figure VIII-6B).

VIII.F.5. An F-Box suggests that FKF1 functions in ubiquitin-dependent degradation

The three members of the FKF1 protein family share a central F-box, suggesting that these proteins function in ubiquitininating proteins targeted for degradation. Ubiquitin-dependent degradation mediates the removal of short-lived cytoplasmic and nuclear proteins (Hochstrasser, 1995). Ubiquitin is attached to an E1 enzyme via a high energy thioester bond and then transferred to an E2 ubiquitin-conjugating enzyme. The E2 enzyme acts with an E3 ubiquitin protein ligase to covalently attach the C-terminal glycine residue of ubiquitin to the ε-amino group of a lysine residue on a target protein. Proteins with attached multiubiquitin chains are subsequently degraded by the 26S proteasome (Hochstrasser, 1995). One class of E3 enzymes consists of a protein complex termed the SCF ubiquitin protein ligase (Deshaiies, 1999; Patton et al., 1998). This complex contains Skp1p, Cdc53p, and a protein with a conserved Skp1p-interacting domain originally noted in cyclin F and Cdc4p (Bai et al., 1996) termed the F-box protein. The function of this complex was first elucidated during study of yeast cell cycle regulation, although similar components have been found in other eukaryotes (Deshaiies, 1999). The Cdc53p subunit docks Skp1p with an E2 enzyme, Skp1p binds the F-box protein through its F-box motif, and the F-box protein binds the substrate through a protein-protein interaction domain. F-box proteins thus function as adapters that bring specific substrates to core ubiquitin protein ligase subunits for ubiquitination and subsequent degradation. In several systems, substrate phosphorylation precedes ubiquitin-dependent degradation, and all substrates that have been examined must be phosphorylated to bind F-box proteins (Deshaiies, 1999). Several Arabidopsis mutants with defective F-box proteins have been isolated, including tir1
(Ruegger et al., 1998) and *coil* (Xie et al., 1998), which are disrupted in auxin and jasmonate responses, respectively, and *ufo*, which produces flowers with abnormal organ numbers (Samach et al., 1999). However, the substrates that these proteins target for degradation remain unidentified.

F-box proteins typically have central or N-terminal F-boxes and C-terminal repetitive motifs, such as WD40 or leucine-rich repeats, that function in substrate recognition (Bai et al., 1996; Patton et al., 1998). FKF1, ZTL, and FKL1 each contain six C-terminal kelch repeats (figure VIII-6B), which are predicted to fold into a β-propeller (Adams et al., 2000). Although kelch repeats have not been described in previously characterized F-box proteins, WD40 repeats, present in numerous F-box proteins (Bai et al., 1996; Patton et al., 1998) also form a β-propeller structure (Wall et al., 1995).

**VIII.F.6. Possible roles for FKF1 in the plant circadian clock**

There are several important roles for protein degradation in circadian oscillation. Rapid protein turnover is required for the oscillation of the protein components of the circadian clock, and this degradation is probably ubiquitin dependent in eukaryotes. In Drosophila, cycling of the *period* (*per*) and *timeless* (*tim*) gene products is required for circadian rhythmicity, and nuclear localization of PER-TIM heterodimers is required to down-regulate *per* and *tim* transcription (Young, 1998). Exposing flies to light results in rapid TIM degradation (Hunter-Ensor et al., 1996; Myers et al., 1996) in an apparently ubiquitin-dependent process (Naidoo et al., 1999). Prior to TIM degradation, the circadian photoreceptor CRY (Emery et al., 1998; Stanewsky et al., 1998) inactivates the PER-TIM heterodimer by light-dependent TIM binding (Ceriani et al., 1999).

The light receptors that provide clock input can also be short-lived. For example, the Arabidopsis CRY2 blue light receptor (Hoffman et al., 1996; Lin et al., 1996) accumulates in dim light, but is rapidly degraded when exposed to receptor-activating wavelengths (Ahmad and Cashmore, 1993; Lin et al., 1998). Similarly, photointerconversion of the
Arabidopsis PhyA photoreceptor from the red light absorbing (Pr) form to the far-red light absorbing (Pfr) form is accompanied by an ~100-fold decrease in the protein half-life, and this degradation appears to be ubiquitin-dependent (Clough and Vierstra, 1997). Because PhyA overexpression in transgenic Arabidopsis inhibits hypocotyl elongation in the light (Boylan and Quail, 1991), and the fkl1 mutant has a short hypocotyl in the light, we explored whether PhyA might be among the proteins that FKF1 targets for ubiquitin-dependent degradation. However, PhyA half-lives upon transfer of etiolated seedlings to light were similar in wild type and fkl1 (figure VIII-3), indicating that FKF1 is not necessary to efficiently degrade PhyA. This result does not preclude a redundant role for FKF1 and ZTL or FKLF1 in PhyA degradation; other members of the FKF1 family may be able to compensate for fkl1 deletion. Alternatively, FKF1 may be involved in the degradation of another protein that mediates light input to the clock, such as CRY2.

Although the period lengths of CCA1 and CAB circadian expression are unaltered in fkl1 in the tested conditions, the waveforms of both oscillations have an apparent induction delay (figure VIII-5). In Arabidopsis, the LHY and CCA1 transcription factors may be part of the central oscillator of the clock, or may link light signaling to the oscillator (Schaffer et al., 1998; Wang and Tobin, 1998). Constitutive expression of LHY or CCA1 results in flowering delays, elongated hypocotyls, and altered regulation of several clock-controlled genes (Schaffer et al., 1998; Wang and Tobin, 1998). CCA1 protein levels cycle (Wang and Tobin, 1998), indicating that CCA1 must be short-lived. Loss of CCA1 shortens the circadian period of several clock-controlled genes, but does not completely abolish rhythmic expression, indicating that if CCA1 is a component of the central oscillator, it is at least partially redundant (Green and Tobin, 1999). A similar short-period phenotype is seen in plants overexpressing CKB3, which encodes a regulatory subunit of the CK2 protein kinase that interacts with and phosphorylates CCA1 in vitro (Sugano et al., 1998).

An increase in CCA1 stability should delay CCA1 message accumulation, because CCA1 overexpression suppresses endogenous CCA1 transcription (Wang and Tobin,
1998). If CCA1 and LHY are central oscillators of the clock (McClung, 1998; Somers, 1999), or if the expression of the central oscillator is similarly phased, then the timing of FKF1 expression and the late flowering phenotype of the fkf1 mutant are consistent with a role in the negative feedback of clock activity. CCA1 was originally isolated as a factor binding to a phytochrome-responsive region of the CABI promoter (Wang et al., 1997), and the fkf1 lag in CAB induction (Figure VIII-5B) may reflect the lag in CCA1 induction. It will be interesting to determine whether FKF1 targets CCA1 for degradation following its phosphorylation by CK2. The central oscillators of the Arabidopsis clock have not yet been unequivocally identified (McClung, 1998; Somers, 1999), and other clock components, such as the proteins defective in the toc mutants (Millar et al., 1995; Somers et al., 1998), might also be FKF1 targets.

Although motif prediction programs (Nakai and Kanehisa, 1992) suggest that FKF1 and its homologs are cytoplasmic proteins, it will be interesting to determine the subcellular localization of FKF1 in response to various light regimens. If FKF1 is a photoreceptor, its subcellular localization or binding targets might change in response to light. A combination of circadian regulation of FKF1 transcription and light regulation of FKF1 activity or localization could render FKF1 active in a very narrow window of the circadian day.

VIII.F.7. Mutations in an FKF1 homolog, ZTL, also disrupt clock function

We have shown that constitutive expression of an FKF1 homolog, ZTL, results in late-flowering plants with elongated hypocotyls (figure VIII-2 and data not shown). Interestingly, point mutations in the ZTL kelch domains result in a late-flowering mutant with a lengthened circadian period, and this period lengthening is fluence-dependent, consistent with a role for ZTL in light perception (Somers et al., 2000). ztl mutants also have short hypocotyls in low-fluence red light (Somers et al., 2000). The opposite phenotypes of the ztl mutant and the ZTL overexpressing plants in hypocotyl elongation are expected. However, the fact that both are late flowering is a paradox. It is possible that the
35S-ZTL lines are overexpressing ZTL as seedlings, resulting in the long hypocotyl phenotype, and cosuppressing ZTL as adults, causing the late-flowering phenotype. This type of phenomenon is not unprecedented; Arabidopsis transgenic for rolB express the gene at high levels as seedlings but silence this expression to various degrees during the course of development (Dehio and Schell, 1994). This issue can easily be addressed by RNA blot analysis of ZTL levels from 35S-ZTL seedlings and adult plants.

The fact that both fkl and ztl mutants are late flowering and display clock defects indicates that the two genes are not fully redundant but have at least some specific roles. The generation and analysis of double and triple mutants among the members of the FKF1 family will reveal the level of redundancy present in this gene family. Furthermore, isolation of FKF1- and ZTL-interacting proteins may elucidate the role of these novel proteins in plant growth and development.
CHAPTER IX: CONCLUSIONS AND PERSPECTIVES

IX.A. IAR1

The primary goal of my work with iar1 was to identify and begin characterization of the gene defective in the mutant, with the ultimate aim of adding to the growing body of knowledge about how IAA conjugates and conjugate metabolism contribute to IAA regulation. Although the iar1 mutant was initially identified as an IAA-Ala resistant mutant, subsequent analysis revealed that it is resistant to several other IAA-amino acid conjugates. This is exciting as it suggests a general role for IAR1 in conjugate processing in Arabidopsis.

The sequence of the IAR1 gene has allowed me to develop several models for the function of IAR1 in conjugate metabolism. IAR1 encodes a protein with multiple predicted membrane-spanning domains and histidine-rich regions. There are relatively close IAR1 homologs present in other multicellular organisms such as C. elegans, Drosophila, and mammals, but none in bacteria or yeast. Interestingly, the cDNA encoding the mouse homolog of IAR1, KE4 (28% identical to IAR1), can rescue the iar1 mutant phenotype, suggesting that these proteins have similar functions. In Drosophila, the IAR1 homolog Catsup (31% identical to IAR1) is involved in the negative regulation of the enzyme tyrosine hydroxylase, or TH (Stathakis et al., 1999), which catalyzes the rate-limiting step in catecholamine neurotransmitter biosynthesis (Kumer and Vrana, 1996). Catsup is capable of forming complexes with TH and with another enzyme important in regulating TH activity (Burton et al., 2000). If the proteins in the IAR1 family function via similar mechanisms, it would be of great interest to determine whether IAR1 can form complexes with other proteins and, if so, to identify these proteins. Identification of these interactors would undoubtedly reveal a great deal about how Arabidopsis regulates its free auxin levels through IAA conjugates. For example, IAR1 might negatively regulate an IAA conjugate hydroxylase in the same manner that Catsup regulates TH.
IAR1, although not highly identical at the amino acid level, is strikingly similar in overall molecular structure to the ZIP family of metal transporters (Eng et al., 1998; Guerinot and Eide, 1999), suggesting a possible role for IAR1 in metal transport. For example, IAR1 might transport metals that inhibit the ILR1-like family of amidohydrolases out of the ER, the presumed site of amidohydrolase activity. I have shown that IAR3, an IAA-Ala specific amidohydrolase, can be inhibited by zinc and copper. Whether the remaining IAA-amino acid conjugate hydrolases are inhibited by these metals remains to be determined, but seems likely.

An important step in understanding the role of IAR1 will be the localization of the protein to a specific membrane. I have successfully made transgenic Arabidopsis lines expressing an IAR1-myc fusion protein and have preliminary evidence confirming membrane localization of IAR1. Localization to a specific membrane should provide significant clarification of how IAR1 contributes to conjugate metabolism in Arabidopsis.

IAR1 is a new and intriguing component of the complex and intricate network of molecules involved in regulation of auxin levels in Arabidopsis.

IX.B. FKF1

The FKF1 project was a fortuitous outgrowth of the iar1 project. The fkl1 mutant was originally isolated as a late-flowering allele of iar1, iar1-4. We have shown that the gene responsible for the late-flowering phenotype of the mutant is not IAR1, but a member of a new and exciting family of Arabidopsis genes involved in regulating the circadian clock. In addition to the late-flowering phenotype, fkl1 mutants exhibit hypersensitivity or hyperresponse to light and altered expression of circadianly-regulated genes.

The predicted sequence of the FKF1 protein is fascinating: the protein contains three domains that have each been separately characterized in other proteins, but not found together previously in the same protein. The first domain is an N-terminal LOV, or PAS domain, which likely binds flavin and may mediate light signaling. The central domain is an
F-box. F-box proteins give specificity to E3 ubiquitin-ligase complexes that target proteins for degradation. The final domain in FKF1 is a series of six Kelch repeats, predicted to fold into a β propeller and mediate protein-protein interactions.

Given the presence of these domains in FKF1, it is not difficult to envision a model in which FKF1 targets components of the Arabidopsis clock for degradation. The fact that FKF1 transcripts cycle with the circadian clock makes such a model even more attractive.

Homologs of FKF1 are present in the Arabidopsis genome. Mutations in one of these homologs, ZTL, also lead to late flowering and alterations in cycling of circadian transcripts (Somers et al., 2000). We have shown that overexpression of ZTL can lead to late flowering and elongated hypocotyls. Characterization of the FKF1 homolog FKL1 will yield additional information about how this family of genes functions.

Another exciting prospect is the identification of the targets of FKF1 and its homologs. The fact that mutations in a single gene in this family can lead to a phenotype indicates that they may have separate targets. Regardless of whether FKF1, ZTL, and FKL1 all target the same molecule for degradation or each have distinct targets, identification of these targets will be a significant step in understanding the function of these genes and in determining the nature of the elusive components of the Arabidopsis circadian clock.

**IX.C. Possible links between auxin and light perception**

At first glance, the two projects discussed in this thesis seem only tangentially related. However, a growing body of evidence points to connections between auxin and light perception.

The mutant shy2 was originally identified as a suppressor of the photomorphogenic mutant hy2, which is defective in phytochrome chromophore biosynthesis (Parks and Quail, 1991). The mutation can suppress hypocotyl elongation and flowering time defects of the hy2 mutant, and shy2 mutants are photomorphogenic in the dark (that is, they develop as if they were grown in the light; (Kim et al., 1996). Furthermore, two genes that are normally
only expressed in light-grown plants are expressed in dark-grown shy2 mutants (Kim et al., 1998). The cloning of SHY2 was recently reported. The gene encodes IAA3 (Tian and Reed, 1999), a member of the Aux/IAA family of auxin-responsive transcription factors that are thought to regulate auxin-mediated processes.

Interestingly, two additional Aux/IAA-related proteins have been reported in the GenBank database as phytochrome-associated proteins (GenBank accession numbers AF088281 and AF087936). Furthermore, the Aux/IAA gene IAA28 is not expressed in dark-grown seedlings, and the IAA28 promoter contains multiple elements associated with light regulation (Rogg and Bartel, unpublished).

Additional evidence connecting auxin and light perception is currently being uncovered. The connection is not entirely unexpected: auxin and light are two of the most important stimuli mediating plant growth. For example, the fact that light regulates hypocotyl elongation is well established (Neff, 2000) and auxin is also implicated in this process (Gray et al., 1998).

Exploration of the interrelation of these fields will undoubtedly be challenging, but will lead to a deeper understanding of the overall mechanisms used by plants to respond to their environments.
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