The nonprotein amino acid meta-tyrosine: Its biosynthesis, phytotoxicity, and application as a tool for research on aromatic amino acid metabolism in plants

by Tengfang Huang

THE NONPROTEIN AMINO ACID *META*-TYROSINE: ITS BIOSYNTHESIS, PHYTOTOXICITY, AND APPLICATION AS A TOOL FOR RESEARCH ON AROMATIC AMINO ACID METABOLISM IN PLANTS

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In addition to the twenty amino acids that are universally found as building blocks of proteins, nonprotein amino acids are also present in numerous plant species. One such nonprotein amino acid, *meta*-tyrosine, is abundant in the root exudates of Chewings fescue (*Festuca rubra* L. ssp. *commutata*). Consistent with the phytotoxic effects of *m*-tyrosine, prior studies showed that Chewings fescue has superior weed-suppressive capacity in field trials involving dozens of turf grass varieties. Further research with *m*-tyrosine demonstrates that this compound is stored in a different compartment than the protein amino acids, and can be easily washed off fescue roots with aqueous solutions. Chewings fescue roots have enzymatic activity, likely from a cytochrome P450, for synthesizing *m*-tyrosine from phenylalanine. An EST library was constructed from roots of Chewings fescue and four candidate cytochrome P450 genes are identified and cloned.

When added to *A. thaliana* growth medium, *m*-tyrosine can be misincorporated into *A. thaliana* proteins. Several protein amino acids, in particular phenylalanine, partially rescue the toxicity of *m*-tyrosine. Correspondingly, a screen for *m*-tyrosine resistance in *A. thaliana* identified a mutant that over-accumulates free phenylalanine. Map-based cloning showed that the genetic basis of this phenotype is *adt2-1D*, a

mutant allele of *ADT2,* which encodes arogenate dehydratase, the final enzyme of the phenylalanine biosynthesis pathway. Characterization of ADT2 and its mutant form revealed feed-back regulation of phenylalanine biosynthesis, which depends on a critical motif of the ADT2 protein. Overexpression of the feedback-insensitive *adt2-1D* produces even higher levels of free phenylalanine and has pleiotropic physiological consequences, including abnormal leaf development, resistance to 5-methyltryptophan, reduced growth of the generalist lepidopteran herbivore *Trichoplusia ni* (cabbage looper), and increased salt tolerance. Finally, several hypotheses for *m*-tyrosine modes of action were tested and discussed.

BIOGRAPHICAL SKETCH

Tengfang Huang grew up in Fujian Province on China's southeast coast. After finishing study at Guoguang High School, he went to Fudan Univsersity in Shanghai, China, where he received a Bachelor's degree in Biological Sciences in 2004. During his senior year at college, he participated in an academic exchange program at Hong Kong University of Science and Technology, and started the research on *Arabidopsis thaliana*.

In 2004, Tengfang Huang was admitted into the Ph.D. program at Department of Plant Biology, Cornell University. After a year of rotation, he joined Dr. Georg Jander's lab at Boyce Thompson Institute in 2005 and focused his research on plant amino acid metabolism. He was married to Ms. Jing Zhou, a fellow Cornell graduate student.

献给我的家人，特别是我的外祖父黄英标先生 (1921-2009)

Dedicated to my family, especially my grandpa Mr. Yingbiao Huang (1921-2009)

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OVERVIEW OF NONPROTEIN AMINO ACIDS IN PLANTS

Occurrence and biosynthesis of nonprotein amino acids

Other than the 20 common amino acids used in the genetic code for protein biosynthesis, plants also produce other nonprotein amino acids (Bell 1976). Some of these are common and important intermediates in primary metabolism, such as ornithine, homoserine and *S*-adenosylmethionine which can be detected in most plant species and are well understood. There are also uncommon amino acids found as plant secondary metabolites whose occurrence is often limited to a small number of families. More than 250 amino acids of this kind have been identified, with seeds of various legumes being very common sources (Fowden 1981). Several comprehensive reviews already covered thorough surveys of these amino acids (Rosenthal 1982; Barrett 1985) and will not be repeated here.

However, compared to the long list of nonprotein amino acids described, the biosynthesis of these compounds are largely unknown. Studies of this subject by isotope labeling and enzyme purifications suggest that these uncommon nonprotein amino acids can have three different origins: direct modification of existing amino acids, deviation of biosynthetic pathways used for common amino acids and *de novo* biosynthesis by novel enzymes (Barrett 1985). Understanding of these pathways has a practical value: genetic engineering of nonprotein amino acid metabolism is often desired in many circumstances, either to increase the production of these amino acids for their medicinal values, or to decrease the production of them to avoid the toxic effects to human and animals (Bell 2003; Dixon and Sumner 2003). Unfortunately, without sufficient genomic information available for most of the species producing uncommon nonprotein amino acids, enzymes involved in nonprotein amino acid

biosynthesis could not be easily identified and cloned, especially for enzymes of rate-limiting steps (Saito *et al.* 1997). Research of this field, however, could be accelerated with the rapid progresses of plant genome and RNA sequencing projects, thanks to the higher speed and lower cost brought to us by the next-generation sequencing techniques (Lister *et al.* 2009; Simon *et al.* 2009; Yonekura-Sakakibara and Saito 2009). With powerful tools of bioinformatics, a sequence genome would allow better identification of candidate genes, which could then be used for hypothesis generation and functional tests.

Functions of nonprotein amino acids in plant defense to insect attacks

Chemical defense is one of the most important strategies when sessile plants have to protect themselves from numerous herbivores and competitors during the entire life cycle (Levin 1976; Swain 1977; Wittstock and Gershenzon 2002). Most of these chemical toxins, repellents or barriers are plant secondary metabolites such as alkaloids, glucosinolates, terpenoids and phenolics (Schoonhoven *et al.* 2005). Amino acids, especially nonprotein amino acids, were also found to play important roles in this process (Rosenthal and Berenbaum 1991). Here I list three representative examples with recent updates to review the functional diversity of nonprotein amino acids in plant – insect interactions. Figure 1.1 illustrates the structures of amino acids discussed in this section.

Canavanine

L-Canavanine (L-2-amino-4-guanidinooxybutyric acid) is probably the most studied nonprotein amino acid in plant defense against insects (Rosenthal 2001). Initially isolated from jack bean, *Canavalia ensiformis* (L.) DC, L-canavanine is a major

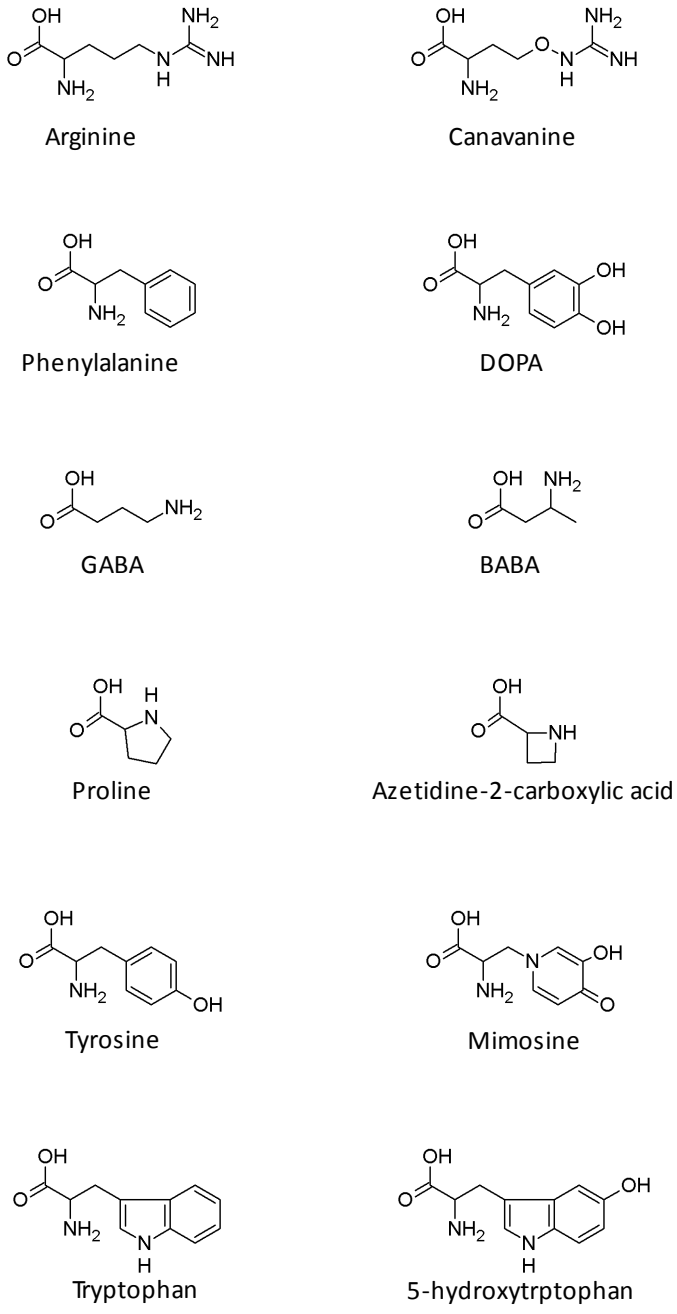

Figure 1.1. Comparison of amino acid structures. Amino acids on the left are more commonly found, whereas their less popular analogs are on the right.

nitrogen storage compound in the seeds of many plants from the Leguminosae family and constitutes up to 13% of the dry weight of seeds (Rosenthal 1972). As an analog and antimetabolite of L-arginine, L-canavanine is highly toxic to a wide range of organisms including bacteria, fungi, yeast, algae, plants, insects, and mammals (Rosenthal 1977; Nakajima *et al.* 2001). The misincorporation of L-canavanine into proteins in place of L-arginine is considered the major mode of action of its toxicity (Nakajima, Hiradate and Fujii 2001; Igloi and Schiefermayr 2009). On the other side, two insect species that are specialized in feeding on L-canavanine-containing plants have developed different mechanisms of tolerance: The tobacco budworm *Heliothis virescens* (Noctuidae) detoxifies L-canavanine by a novel canavanine hydrolase which converts canavanine to homoserine and guanidine (Melangeli *et al.* 1997). The bruchild beetle *Caryedes brasiliensis* deals with canavanine in another way by converting it first to canaline and urea, and then from the equally toxic canaline to homoserine and ammonia (Rosenthal *et al.* 1977; Rosenthal *et al.* 1978). In addition, *Caryedes brasiliensis* seems to have a more accurate arginyl-tRNA synthetase that can discriminate canavanine from arginine, resulting in a much lower rate of misincorporation into proteins (Rosenthal *et al.* 1976). Interestingly, both of these mechanisms possessed by *Caryedes brasiliensis* are also present in some legumes that produce canavanine, and might form the basis to avoid self-toxicity in these plants (Rosenthal 1990; Igloi and Schiefermayr 2009).

The potent toxicity of canavanine results in strong selective pressure on insects that feed on canavanine-containing plants. Other than the very few species that are resistant to canavanine, as mentioned above, some insects sensitive to canavanine have developed the ability to recognize and avoid consumption of this toxic amino acid. Using *Drosophila* as an insect model, it was found that L-canavanine is recognized by flies followed by a behavioral avoidance response via a chemosensory mechanism

(Mitri *et al.* 2009). Further study revealed that L-canavanine is detected by the DmX receptor, a G-protein coupled receptor that is different from the family of gustatory receptors (Grs), the most common taste-receptive sensors in *Drosophila* (Mitri *et al.,* 2009). It is still unknown whether if this mechanism is conserved among different insect species.

γ - Aminobutyric Acid (GABA)

GABA is a four carbon nonprotein amino acid present in free amino acid pools of most prokaryotic and eukaryotic organisms. In plants, GABA biosynthesis is catalyzed by glutamate decarboxylase (GAD). The activity of GAD is regulated in two ways depending on the pH range: at pH 7.0-7.5, GAD activity is stimulated by calcium/calmodulin; at acidic pH range, GAD activity is independent of calcium/calmodulin and is sensitive to pH with an optimum of 5.8 (Baum *et al.* 1993; Snedden *et al.* 1995). Correspondingly, insect attack causes increased plant GAD activity in two ways: Touch of insects induced Ca^{2+} influx whereas further damage by insects releases acidic solutions from vacuoles and decreases the pH in cytosol (Hall *et al.* 2004; Hilker and Meiners 2010). As a result, GABA level usually increases several-fold and rapidly in response to insect attacks (Wallace *et al.* 1984; Ramputh and Bown 1996; Bown *et al.* 2002). A recent review discussed the function of GABA as plant defense compound against insects (Bown *et al.* 2006). Several lines of evidence indicate that the increased level of GABA could be an effective mechanism of plant defense: Growth tests using GABA-containing artificial diet showed that physiological concentration of GABA in wounded plants reduces the growth and survival of insect larvae. Choice tests using GABA overproducing transgenic tobacco demonstrate that insects avoid feeding on high-GABA plants. The damage of GABA to insects could be a result of the inhibition of GABA-gated Cl^- channels that are

important in the peripheral nervous system in insects (Hosie *et al.* 1997). It should also be noted that rapid accumulation of GABA also occurs in response to other stresses such as flooding, cold, heat, drought, salt and other mechanical damages through the regulation of Ca^{2+} and pH (Kinnersley and Turano 2000). GABA also plays other roles in plant development and metabolism, including guidance for pollen tube growth and regulation for carbon/nitrogen metabolism in plants, or even a general signaling molecule mediating the communications between plants and other organisms (Shelp *et al.* 1999; Bouché *et al.* 2003; Bouché and Fromm 2004; Shelp *et al.* 2006; Fait *et al.* 2008).

β-Aminobutyric Acid (BABA)

Structurally related to GABA, BABA is however rare in nature. To date, it has only been reported in root exudates of tomato plants grown in solarized soils (Gamliel and Katan 1992). Interestingly, when applied exogenously to plants, BABA seems to play a broad role that increases plant defense against both biotic stresses such as invasion of various bacterial, viral and fungal pathogens, and abiotic stress such as drought, salt and heat shock (Cohen 2001; Jakab *et al.* 2001; Jakab *et al.* 2005; Zimmerli *et al.* 2008). It appears that BABA acts by priming plants into a state that respond faster and stronger to the subsequent stresses via the interplay of several hormones including SA, ABA and ethylene (Ton *et al.* 2005). Recently, it was also found that similar application of BABA to plants also reduces the performance of several insect species including aphids and Lepidoptera (Hodge *et al.* 2006). Despite its versatile function in stress response, the physiological significance of natural occurring BABA still needs further investigation. Table 1.1 summarizes these three representative nonprotein amino acids.

Table 1.1.: Comparisons of three representative nonprotein amino acids

Compound	L-Canavanine	GABA	BABA
Occurrence	Massive accumulation in limited number of families	Readily detectable in almost every species	Rarely found in nature
Toxicity	Direct	Direct	Indirect
Mode of Action	Compete with protein amino acid analogs	Inhibits neurotransmission	Enhances existing defense response
Target	Herbivore and competitor	Herbivore with neural systems	General biotic and abiotic stresses
Inducibility	No	Yes	Unknown
Other functions	Storage	Development and metabolism	Unknown

Other Nonprotein Amino Acids and Their Common Themes

Similar to canavanine, L-3,4-dihydroxyphenylalanine (L-DOPA), 5-hydroxytryptophan, mimosine, and azetidine-2-carboxylic acid are other nonprotein amino acids found in large quantities in certain species, especially legume seeds (Fellows 1970; Ishaaya *et al.* 1991). These amino acids all inhibit the growth of tested insect species when blended into insect diets (Rehr *et al.* 1973; Birch *et al.* 1986; Adeyeye and Blum 1989). Their effects are not limited to insects only, since some of them are likely to target *de novo* protein biosynthesis, a fundamental processed conserved across different organisms (Rodgers *et al.* 2002; Ozawa *et al.* 2005; Bessonov *et al.* 2010). Furthermore, similar to GABA, L-DOPA and 5-hydroxytryptophan are precursors of neurochemicals (dopamine and serotonin, respectively) that regulate neural systems in insects and mammals, and could therefore

have additional effects on toxicity to these species (Osborne 1996).

Compared to carbohydrates which plants produce from photosynthetic carbon, nitrogen is a more limited resource for plants in most environments. Using nonprotein amino acids, which are nitrogen dense compounds, as defensive agents could be a risky strategy and should be used in an efficient way. As we can find from the examples, three strategies were used to improve the efficiency of these nitrogen compounds: Firstly, some of these massively accumulated nonprotein amino acids have double duties and often serve as seed storage compounds. The relatively simple structure of nonprotein amino acids and their similarity to protein amino acids make these nitrogen and carbon resources acids easier to be reused and integrated into primary metabolism when needed. Plants have also evolved the corresponding changes at the genetic and molecular levels to prevent self-toxicity; Secondly, several nonprotein amino acids target the sensitive nervous systems of invertebrate and vertebrate and can therefore minimize the dosage needed to interfere with the growth and development of these herbivores. Since these neural receptors involved are usually not present in plants, self-toxicity can be largely avoided. Last but not the least, although not fully investigated yet, plants could also use nonprotein amino acids as signaling molecules to assist with systematic defense response – another example that these compounds can be used in a highly efficient way. We should also notice that the introduction of model organisms such as *D. melanogaster* and *A. thaliana* to the research on nonprotein amino acid function has made significant contributions to our understanding of the mechanisms at the molecular level that would be otherwise almost impossible to reach by traditional approaches. The rich resources and research tools available to model organisms should greatly facilitate bringing research on nonprotein amino acids to the next level where their functions can be better understood.

Studies of amino acid metabolism using nonprotein amino acids

Most of the knowledge of amino acid biosynthesis pathways in plants was learned from bacteria and yeast, and verified *in planta* (Coruzzi and Last 2000). However, other approaches have been developed to investigate amino acid metabolism directly in plants. As mentioned above, many nonprotein amino acids display toxicity to a wide spectrum of organisms, including other plant species. As a consequence, biochemical analysis using toxic amino acid analogs has become a very popular way to gain insights into plant amino acid metabolism (Lea and Norris 1976). In the post-genomic era, genetic screens for plants resistant to toxic amino acids and the subsequent gene cloning has also been very fruitful.

When certain toxic nonprotein amino acids were used as for genetic screens, the resulting resistant mutants usually fell into two categories: mutants with defects in amino acid transporters or mutants overaccumulating amino acids that acts as antagonists to the toxic nonprotein amino acids. Here are some examples from previous studies:

Ethionine is a toxic analog of methionine. A genetic screen for ethionine-resistant *A. thaliana* identified three different mutants. Mutations in genes encoding cystathionine gamma synthase, threonine synthase and *S*-adenosyl-L-methionine synthetase 3 were identified to be responsible for the *mto1, mto2, mto3* phenotypes, respectively. All three of these enzymes are directly involved in biosynthesis and metabolism of methionine in *A. thaliana*. Subsequent research with these mutants provided new insights into both the metabolic pathways and feedback regulation of free methionine in plants (Inaba *et al.* 1994; Bartlem *et al.* 2000; Shen *et al.* 2002). The semidominant mutation in the *mto1* mutant also lead to the discovery of a novel mechanism controlling mRNA stability by amino acid intermediates (Chiba *et al.* 1999).

A comprehensive screen for D-alanine resistant *A. thaliana* mutants resulted in the identification of LHT1 transporter involved in plant uptake of amino acids (Svennerstam *et al.* 2007). Another transporter, AAP1, was found to transport uncharged amino acids into roots in a mutant screen for phenylalanine-resistant plants (Voll *et al.* 2004; Lee *et al.* 2007).

A dominant mutant of *A. thaliana*, *amt-1*, was previously selected for resistance to growth inhibition by the tryptophan analog α-methyltryptophan. This mutant had elevated tryptophan levels and exhibited higher anthranilate synthase (AS) activity that showed increased resistance to feedback inhibition by tryptophan. Cloning of the mutant gene revealed a critical amino acid residue involved in the allosteric feedback inhibition of tryptophan biosynthesis (Kreps *et al.* 1996; Li and Last 1996).

Overall, genetic screens for *A. thaliana* mutants resistant to toxic nonprotein amino acids has several advantages: the screen takes place in germination or seedling stages and can be done in a large scale; the phenotyping process is usually based on viability of seeds and does not require additional measurements or assays; the difference between mutant and wildtype plants is clear, which is critical for map-based cloning; and since most mutant genes are involved in amino acid metabolism, candidate genes can be apparent in the final stages of mapping, making the cloning of a mutant gene an easier process.

***meta*-Tyrosine and this thesis**

meta-Tyrosine (*m*-tyrosine or *m*-tyr) is a nonprotein amino acid previously found in donkey-tail spurge (*Euphorbia myrsinites*) and recently identified in Chewings fescue (*Festuca rubra* L. ssp *commutate*) (Bertin 2005; Mothes *et al.* 1964). As an analog and likely antimetabolite of the protein amino acids phenylalanine and tyrosine, this uncommon nonprotein amino acid display toxicity to a wide array of plant species.

The following three chapters investigate and explore *m*-tysosine from three different perspectives. In Chapter 2, the biosynthesis of this natural nonprotein amino acid is studied. In Chapter 3, *m*-tyrosine is used as a tool to study plant aromatic amino acid biosynthesis, which provides some interesting findings for phenylalanine biosynthesis and metabolism in *A. thaliana*. In Chapter 4, different hypotheses of the modes of action of *m*-tyrosine are tested and discussed.

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CHAPTER TWO:

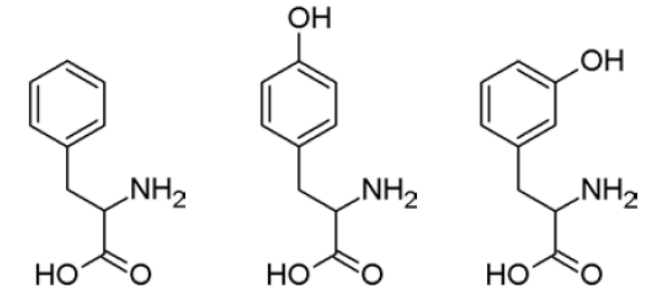
META-TYROSINE IN *FESTUCA RUBRA* SSP. *COMMUTATA* (CHEWINGS FESCUE) IS SYNTHESIZED BY HYDROXYLATION OF PHENYLALANINE

Introduction

Similar to many other nonprotein amino acids reviewed in Chapter 1, *meta*-Tyrosine (*m*-tyrosine) is an analog of the common protein amino acid *p*-tyrosine (Figure 2.1a). It was identified in Chewings fescue (*Festuca rubra* L. ssp. *commutata*) and donkey-tail spurge (*Euphorbia myrsinites*) (Bertin *et al.* 2007; Mothes *et al.* 1964). *In vitro* assays show that *m*-tyrosine is toxic to a wide variety of prokaryotic and eukaryotic species, including *Bacillus* species and cultured Chinese-hamster ovary cells (Aronson and Wermus 1965; Gurer-Orhan *et al.* 2006). In mammalian systems, misincorporation of this amino acid into proteins by phenylalanyl-tRNA synthases was found, (Gurer-Orhan *et al.* 2006; Klipcan *et al.* 2009), although the causal relationship between this misincorporation and its toxicity is not established. Recently, it is found that *m*-tyrosine also inhibits the growth of a wide array of plant species, making it an attractive candidates as an allelochemical (Bertin 2005). Although the exact role of *m*-tyrosine is not yet proven, the large amount of *m*-tyrosine in the roots of some fescue cultivars (> 1% of dry weight) suggests that this amino acid has an important function in the biology of these grasses.

Precursor feeding experiments with *E. myrsinitis* showed that the *m*-tyrosine found in this plant is produced from the shikimate pathway via transamination of *m*-hydroxyphenylpyruvate (Muller and Schutte 1967) (Figure 2.1b). In animal cells, *m*-tyrosine accumulates during oxidative stress through non-enzymatic oxidation of phenylalanine (Fell *et al.* 1979; Ishimitsu *et al.* 1986). Abundance of *m*-tyrosine in

a



Phenylalanine | Tyrosine "*p*-Tyrosine" | *m*-Tyrosine

b

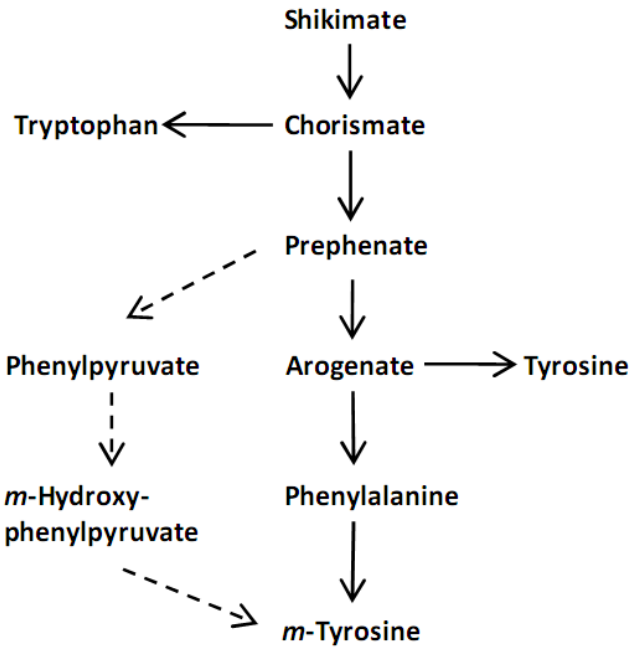


Figure 2.1. Structure and biosynthesis of *m*-tyrosine.
(a) Structures of *m*-tyrosine, tyrosine and phenylalanine. The common tyrosine refers to "*p*-tyrosine" and has the same chemical composition as *m*-tysoine.
(b) Biosynthetic pathways of *m*-tyrosine in different species. Dash line represents the proposed pathway in *Euphorbia myrsinites.* The alternative pathway is suggested by this study.

mammalian tissue has been used as an indicator of oxidative stress and the aging process (Matayatsuk *et al.* 2007; Wells-Knecht *et al.* 1993). In the present chapter, we study the biosynthesis of *m*-tysoine in Chewings fescue.

Results and discussion

Distribution of *m*-tyrosine in *F. rubra* roots

Although *m*-tyrosine is also found in the seeds and leaves of *F. rubra*, it is most abundant in roots (Bertin *et al*., 2007). To determine the distribution more precisely, roots from one-week-old *F. rubra* cultivar "Intrigue" seedlings (~ 4 cm long) were dissected into four parts of equal length, with section 1 including the root tip and section 4 being closest to the seed. Analysis of free amino acids from these root sections showed a gradient of *m*-tyrosine distribution, with the highest concentration in the root tips (Figure 2.2a), suggesting that *m*-tyrosine is either actively synthesized in lower parts of the roots or transported there from other plant parts.

To confirm that *m*-tyrosine is secreted into the rhizosphere, *F. rubra* root extracts obtained by two different methods were compared. Extraction buffer was used to rinse either a 5 mm long root tip cut off from a seedling or an equal length of an undamaged root. HPLC chromatography shows that, whereas the extracts from damaged (cut off from seedlings) roots contain a mixture of different amino acids, the surface wash of undamaged roots is dominated by a single amino acid, *m*-tyrosine (Figure 2.2b). Therefore, it appears that *m*-tyrosine is located at the root surface and has a different distribution than the common protein amino acids.

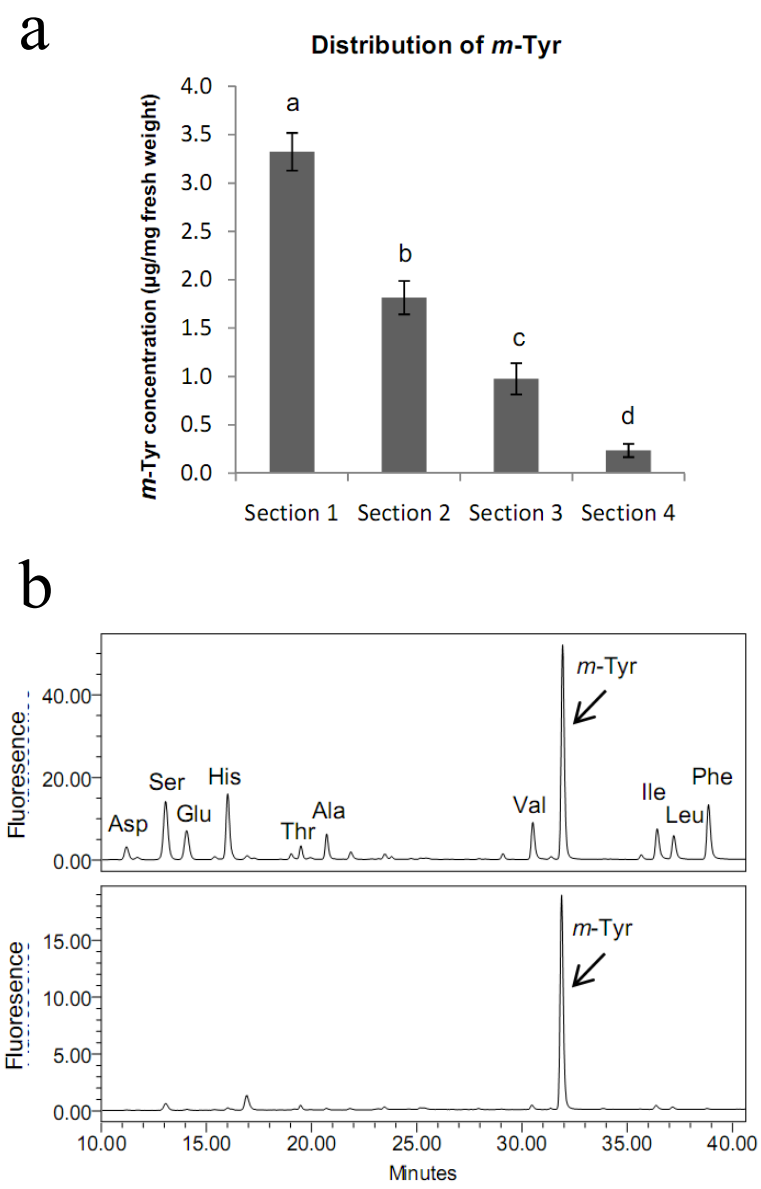


Figure 2.2. Distribution of *m*-tyrosine.
(a) Distribution of m-tyrosine in roots of Chewing's fescue cultivar "Intrigue". Section 1 – 4 refers to 4 root sections of equal length, with Section 1 containing the root tips and Section 4 closest to hypocotyl. Concentration of m-tyrosine is highest in lower parts of root and decreases when moved up. Mean ± S.E. n=3
(b) Amino acid profile of damaged roots (top) and undamaged root surface wash (bottom). Damaged root extract contains a mixture of different amino acids whereas undamaged surface wash has only m-tyrosine as the major amino acid.

Phenylalanine is a direct precursor for *m*-tyrosine biosynthesis

Although the structural similarity between *m*-tyrosine, phenylalanine and tyrosine indicates that they could share similar metabolic origin from the shikimate pathway, there is more than one possible pathway for the biosynthesis of *m*-tyrosine (Figure 2.1b). To identify possible metabolic precursors, six-day-old *F. rubra* seedlings were transferred to medium containing 100 µM shikimate, tyrosine, phenylalanine, or glucose. Measurement of *m*-tyrosine after two days showed that only feeding with phenylalanine significantly increases the concentration of *m*-tyrosine when compared to the control samples (Figure 2.3).

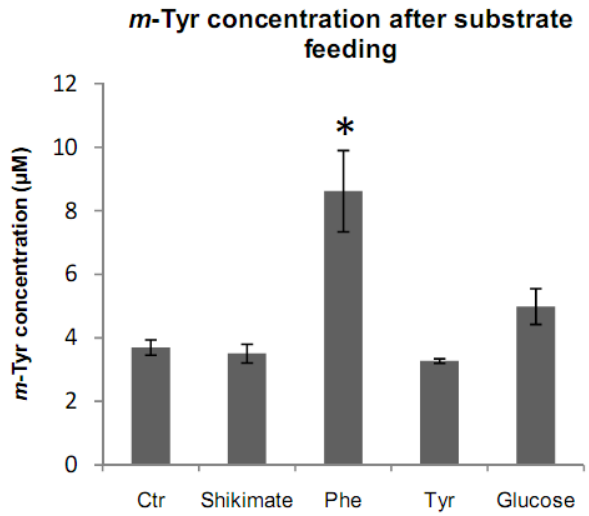


Figure 2.3. Feeding of phenylalanine increase production of *m*-tyrosine. Supply of exogenous phenylalanine, but not glucose, shikimic acid or tyrosine, increased the concentration of *m*-tyrosine in fescue roots. * $p < 0.05$, Student's t-test. Mean ± S.E. n=3

To further investigate the conversion of phenylalanine to *m*-tyrosine, *F. rubra* roots were labeled with [U-$^{13}C_9$, ^{15}N] phenylalanine. GC-MS analysis of extracted amino acids showed that approximately one third of the *m*-tyrosine was in the form of [U-$^{13}C_9$, ^{15}N] *m*-tyrosine (Figure 2.4). This provides evidence that *m*-tyrosine in *F. rubra* is produced through direct hydroxylation for phenylalanine, a pathway that is different from the one observed in *E. myrsinitis* (Muller and Schutte 1967) (Figure 2.1b) If *m*-hydroxyphenlypyruvate were an intermediate in the biosynthesis of *m*-tyrosine, then the ^{15}N label would have been lost during the biosynthesis. Other possible biosynthetic pathways (dash lines in Figure 2.1b) would result in the loss or one or more ^{13}C atoms.

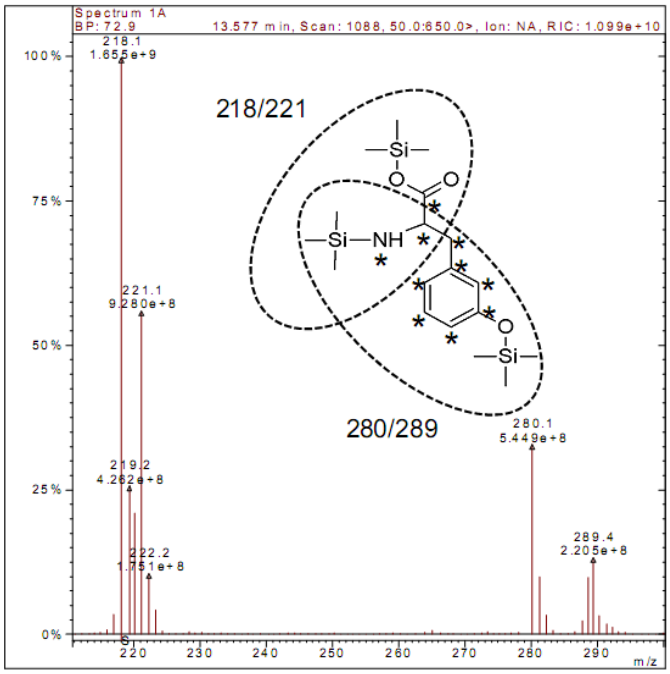


Figure 2.4. Biosynthesis of *m*-tyrosine from phenylalanine.
Major molecular fractions of 3TMS-*m*-tyrosine, the product of *m*-tyrosine derivatised with MSTFA used in GC-MS analysis in shown in center. In unlabeled samples, two major fractions (as indicated by eclipses) are of molecular mass 218 and 280, respectively. If every carbon and nitrogen atom in m-tyrosine derives from [U-13C9, 15N] phenylalanine, the molecular mass of these two fractions should be 221 and 289, respectively. These two peaks are readily detected in mass spectrum analysis of *m*-tyrosine after feeding fescue roots with [U-13C9, 15N] phenylalanine, as shown in the background.

Conversion of phenylalanine to *m*-tyrosine is independent of fescue endophytes and is highest in the roots

Fescue plants are often infected by fungal endophytes and it is therefore possible that *m*-tyrosine is produced by endophytes associated with Chewings fescue (Shelby and Dalrymple 1987). To test this hypothesis, we compared the concentrations of *m*-tyrosine in infected and uninfected Chewings fescue (Bonos *et al.* 2005). Chewings fescue 3188-1 DL1 E+ (containing the Delaware 1 endophyte) has no significant differences in *m*-tyrosine concentration compared to its endophyte-free counterpart 3188-1 E-. Similarly, *m*-tyrosine levels in Chewings fescue 1117 PA E+ (containing the *Poa ampla* endophyte) are not significantly different from the endophyte free 1117 E-. Concentrations of *m*-tyrosine found in these cultivars are similar to those found in the Intrigue cultivar used for all other experiments (Figure 2.5). Therefore, the large amount of *m*-tyrosine observed in fescue roots is produced by the plants themselves rather than by fungal endophytes.

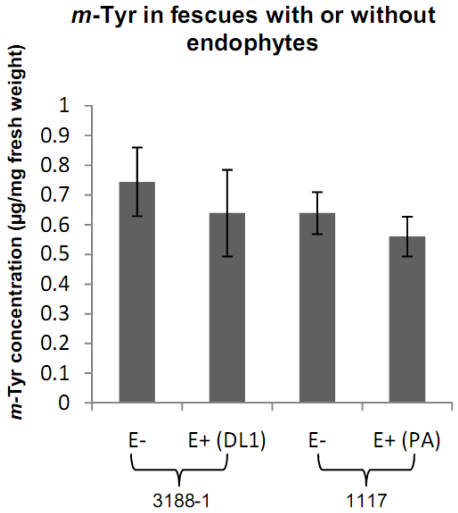


Figure 2.5. *m*-Tyr is made in endophyte free fescue.
m-Tyrosine biosynthesis in fescue is independent of endophytes. No significant difference is found in fescue with (E+) or without (E-) endophytes. Mean ± S.E. n=3. $P > 0.05$.

Although *m*-tyrosine is most abundant in roots, it could still be a result of transport rather than local synthesis. To test if fescue roots possess the enzymatic activity to synthesize *m*-tyrosine, fescue seedlings were dissected into three sections: leaves, midsections and roots. All sections were then floated on nutrient solution. After feeding with [U-$^{13}C_9$, ^{15}N] phenylalanine individually, peak area of isotope labeled *m/z* 221 fragments of *m*-tyrosine were quantified in all three sections. The results show that the fescue roots produced the most labeled *m*-tyrosine among all three sections (Figure 2.6). This could partly due to the higher substrate uptake efficiency from the roots but also confirms that roots of Chewings fescue contain the enzyme active in *m*-tyrosine biosynthesis from phenylalanine.

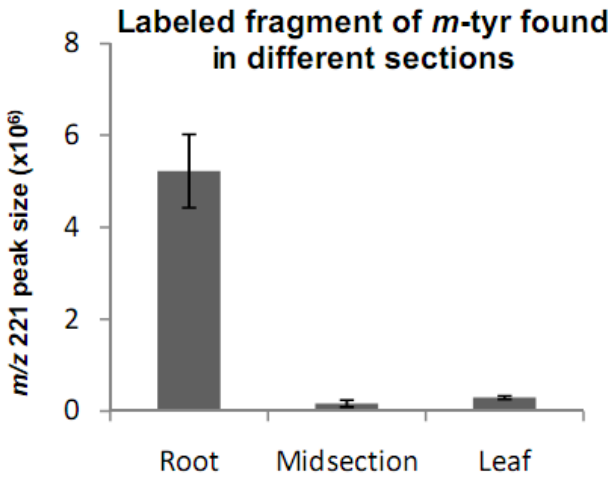


Figure 2.6. Fescue root is active in *m*-tyrosine biosyntheis.
The root section of fescue synthesized most *m*-tyrosine from isotope labeled phenylalanine compared to other parts of seedlings. *P < 0.05, Student's *t*-test. Mean ± SE n=4

Conversion of phenylalanine to *m*-tyrosine is reduced by cytochrome P450 inhibitors

The enzymatic hydoxylation of phenylalanine to *m*-tyrosine would require a monooxygenase. In plants, cytochrome P450s are the largest family of monooxygenases, with 273 known cytochrome P450 genes in *A. thaliana* and 489 in rice (Nelson 2009). To test the hypothesis that *m*-tyrosine is produced by a cytochrome P450, we measured the conversion of [U-$^{13}C_9$, ^{15}N] phenylalanine into *m*-tyrosine in the presence of two cytochrome P450 inhibitors, α-cyclopropyl-α-[*p*-methoxyphenyl]-5-pyrimidine methyl alcohol (Ancymidol) and 1-aminobenzotriazole (ABT). Ancymidol is a plant growth regulator that inhibits some cytochrome P450s, including several important enzymes in gibberellin biosynthesis (Coolbaugh *et al.* 1978). ABT, on the other hand, has a stronger effect and a wide spectrum of inhibition by autocatalytic destruction of cytochrome P450s (De Montellano *et al.* 1984; Reichhart *et al.* 1982). Eight-day-old *F. rubra* seedlings growing in 100 μM [U-$^{13}C_9$, ^{15}N] phenylalanine solutions were treated with ABT, Ancymidol, or dimythylsulfoxide (DMSO, as a control) for 24 hours. In contrast to the control experiment, where 43% of *m*-tyrosine was labeled, only 31% of *m*-tyrosine was labeled in the presence of Ancymidol and more significantly, only 3% was labeled in the presence of ABT (Figure 2.7). Therefore, it is likely that the function of a cytochrome P450 is pivotal during the biosynthesis of *m*-tyrosine from phenylalanine.

Identification of *F. rubra* cytrochrome P450 genes

The above experiments strongly suggest that *m*-tyrosine is synthesized from phenylalanine by a cytochrome P450 enzyme in fescue roots. Therefore, we made an *F. rubra* cDNA library as a genomic resource for the exploration of such cytochrome P450s. RNA from the lower half of the roots was used for construction of a non-

normalized cDNA library. Sequencing 2300 individual cDNA clones produced 1767 high-quality EST sequences, with an average length of 672 bp after sequence cleaning and trimming. These sequences are deposited to Genbank (HO059988 – HO061754). Assembly of these sequences produced 1380 individual clusters (unigenes). Sequence annotation by BLAST identified five candidate cytochrome P450s: 02D11(HO061371), 04C06 (HO060904), 22C02 (HO060621), 25H03(HO060459), and 29A06 (HO061625), all of which are single copy in the cDNA library.

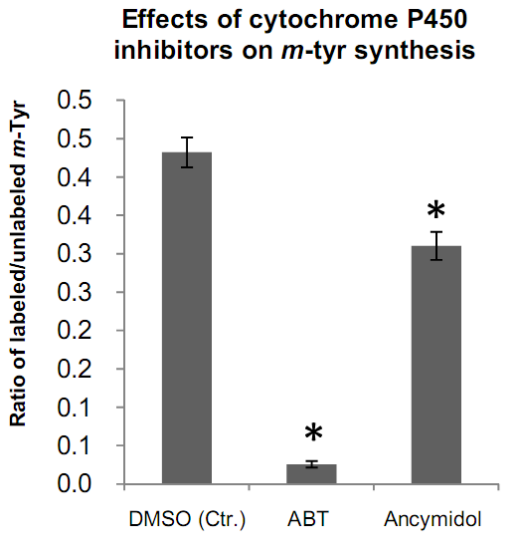


Figure 2.7. *m*-Tyrosine biosynthesis from phenylalanine is inhibited by cytochrome P450 inhibitors.
The root section of fescue synthesized most *m*-tyrosine from isotope labeled phenylalanine compared to other parts of seedlings. * $p < 0.05$, Student's *t*-test. Mean ± Stdev. n=4

Expression of the candidate cytochrome P450 genes in leaves and root sections was studied using RT-PCR (Figure 2.8). Four of the genes (02D11, 22C02, 25H03 and 29A06) are specifically expressed in the roots and have very low or virtually no expression in the leaves. In contrast, gene 04C06 has higher expression in the leaves and is thus less likely to be involved in *m*-tyrosine biosynthesis.

Figure 2.8. Expression of 5 candidate cytochrome P450s in different parts of Chewing's fescue.
RT-PCR analysis of gene expression in lower sector of root, upper section of root and leaf shows root specific or enriched expression of gene 02D11, 29A06, 25H03 and 22C02. In contrast, gene 04C06 has higher expression in leaves.

Full-length cDNA sequences of the four candidate genes were obtained by RACE (Rapid Amplification of cDNA Ends) or by full-length sequencing of the library clones and are designated as *CYP81A20* (02D11), *CYP75A47* (22C02), *CYP92A44* (25H03) and *CYP73A91* (29A06). To test the functions of these genes, three independent isolates of each gene were cloned into *Agrobacterium tumefaciens* for transient expression in *Nicotiana benthamiana* (Sequence of all clones are in Appendix of this thesis). Although expression of the *F. rubra* genes was detected by

RT-PCR, no *m*-tyrosine synthesis could be found in *N. benthamiana*. It is possible that this *N. benthamiana* transient expression system lacks sufficient enzymatic partners such as cytochrome P450 reductases that are required for the full function of these cytochrome P450s. A yeast strain WAT11 is engineered to express the *Arabidopsis* cytochrome P450 reductase *ATR1* and could be useful for this study (Urban *et al.* 1997). It could also be that the cloned sequences were mutated during PCR and lost the function. Alternatively, these four candidate genes might not be the right enzymes.

Concluding remarks

The biosynthesis of *m*-tyrosine in *F. rubra* has been of interest since the report of the presence and potential functions of this molecule (Duke 2007). Our study on the precise distribution of *m*-tyrosine supports its unique roles in plant-environment interactions. The difference in biosynthetic pathways in *F. rubra* and *E. myrsinites* might reflect their independent history of emergence and evolution. Our EST collection from the root tissue of *F. rubra* L. ssp. *commutata* is the first *F. rubra* EST collection in GenBank and provides a useful resource not only for further exploration of these enzymes but also for diverse interests of the turf grass research community. This study also identified and cloned four root specific or enriched cytochrome P450 enzymes that might play interesting roles, including *m*-tyrosine biosynthesis.

Experimental

Material and growth conditions

Seeds of Chewings fescue cultivar "Intrigue" were purchased from Summit Seed (www.summitseed.com). Seed germination pouches (www.mega-international.com) were used to grow fescue seedlings in growth chamber at 23 °C, 180 μmols $m^{-2}s^{-1}$ photosynthetic photon flux density, and a 16:8 h light-dark cycle. Seeds were

sterilized by shaking in 50% ethanol for 1 min and then in 50% bleach for 20 minutes, before being rinsed five times with sterile H_20. Each germination pouch was filled by 20 ml of H_20 and placed on racks.

For stable isotope feeding experiments, one week old intact seedlings or sections of seedlings were removed from germination pouches and placed in respective solutions in Petri dishes until harvested for measurements.

[U-$^{13}C_9$, ^{15}N] phenylalanine was purchased from Cambridge Isotope Laboratories (www.isotope.com), ABT from Acros Organics (www.acros.com) and ancymidol from Chem Service (www.chemservice.com). Other chemicals, if not specified, were purchased from Sigma-Aldrich (www.sigmaaldrich.com).

Analysis of free amino acids by HPLC

Extraction and HPLC analysis of free amino acids were performed as described (Joshi *et al.* 2006) with slight modifications, where 200 µl of extraction buffer were used for each sample. Data were normalized relative to the tissue fresh weight.

Analysis of isotope-labeled free amino acids by GC-MS

GC-MS samples were prepared and derivatized by MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide) according to (Lisec *et al.* 2006). One µl of derivatized sample was injected into a Varian FactorFour VF-17ms column in a Varian CP-3800 GC. Nitrogen gas flow was set to 1 ml/min. The column temperature gradient was: 70 °C for 5 minutes, ramped to 170 °C at a rate of 25 °C /min, increased to 205 °C at a rate of 5 °C/min, increased to 300 °C at a rate of 25 °C /min, and hold at 300 °C for 1 minute. Compounds after separation were detected by Varian 1200L mass spectrometer with electron impact ionization set -20 eV.

Fescue root cDNA library construction

RNA was extracted using Qiagen RNeasy Plant Mini Kit from the lowest 1 cm of roots from 8-day-old Chewings fescue cultivar "Intrigue" grown in seed germination pouches. The cDNA library was made following the LD PCR protocol from the Creator SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA). cDNA generated by reverse transcription was amplified, digested with *Sfi* 1A and *Sfi* 1B, and size fractionated. Double-stranded cDNA was directionally cloned into the pDNR-LIB plasmid vector, and transformed into DH10B competent cells (Invitrogen, Carlsbad, CA, USA).

Sequence analysis

Library aliquots were spread onto selective media and grown overnight at 37°C. Colonies were picked manually into 384 well plates (Genetix, New Milton, Hampshire, UK) containing selective media and grown overnight at 37°C. One µL of liquid culture was used as a template for colony PCR. Colony PCR products were analyzed by gel electrophoresis to confirm the presence of an insert. PCR products were purified using AMpure (Agencourt Biosciences, Beverly, MA, USA). Sequencing reactions were carried out using ABI PRISM BigDye technology, and sequences were analyzed on the ABI 3730XL automated multicapillary sequencer (Applied Biosystems, Foster City, CA, USA).

Phred (Ewing *et al.* 1998) was used to make base calls from the sequence traces. Raw sequences were trimmed and cleaned to eliminate poly-A tails and vector sequences by programs Lucy (Chou and Holmes 2001) and Seqclean (Chen *et al.* 2007). Sequences less than 100 bp were discarded. Processed sequences were

assembled to clusters using iAssembler (http://bioinfo.bti.cornell.edu/tool/iAssembler/) and annotated by Blast2GO (Conesa *et al.* 2005).

RT-PCR

RNA was prepared from leaves and both lower and upper halves of roots from 8-day-old Chewings fescue cultivar "Intrigue" using Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) followed by reverse transcription by SMART MMLV RNA transcriptase. PCR was conducted with 1 μL of reverse transcriptase reaction product at 94°C for 30 s, 48°C for 40 s, and 72°C for 50 s, for a total of 34 cycles. The actin sequence from fescue was identified from our library and used as an internal control. The primer sequences used for each gene are: (From 5' to 3')

02D11 F	TCATCTTCCTGCTCCACCAC
02D11 R	GCTCGTGGCGATCATTGAG
22C02 F	ATGTCCCTCCTCACCGG
22C02 R	GTGCTCCGCCAGAAGCTT
25H03 F	ATGGAGTTTCCTCAGTGGGC
25H03 R	CCTCACCTCCTTCTCCAGGTA
29A06 F	ATGGACGTCAACCTCCTGGAG
NbActin F	ATGGCAGATGGAGAGGATATTC
NbActin R	CCTGCCCATCCGGTAGCTCAT

Cloning and expression of candidate cytochrome P450 cDNAs

Full length sequences of candidate genes were obtained by Clontech SMART RACE cDNA amplification kit and cloned into vector pMDC32 using Invitrogen Gateway technology. For each candidate gene, three independent clones were sequenced and transformed to *Agrobacterium tumefaciens* strain GV3101. Cultures of these transformed strains were infiltrated into young leaves of *Nicotiana benthamiana*

together with RNA silencing suppressor p19 (Voinnet *et al.* 1999) and 100 μM phenylalanine.

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CHAPTER THREE:

PLEIOTROPIC PHYSIOLOGICAL CONSEQUENCES OF FEEDBACK-INSENSITIVE PHENYLALANINE BIOSYNTHESIS IN *ARABIDOPSIS THALIANA*[*]

Summary

A large portion of plant carbon flow passes through the shikimate pathway to phenylalanine, which serves as a precursor for numerous secondary metabolites. To identify new regulatory mechanisms affecting phenylalanine metabolism, we isolated *Arabidopsis thaliana* mutants resistant to the phytotoxic amino acid *m*-tyrosine, a structural analog of phenylalanine. Map-based cloning identified *adt2-1D,* a dominant point mutation causing a predicted serine to alanine change in the regulatory domain of ADT2 (arogenate dehydratase 2). Relaxed feedback inhibition and increased expression of the mutant enzyme causes up to 160-fold higher accumulation of free phenylalanine in rosette leaves, as well as altered accumulation of several other primary and secondary metabolites. In particular, abundance of 2-phenylethylglucosinolate, which is normally almost undetectable in leaves of the *A. thaliana* Columbia-0 accession, is increased more than thirty-fold. Other observed phenotypes of the *adt2-1D* mutant include abnormal leaf development, resistance to 5-methyltryptophan, reduced growth of the generalist lepidopteran herbivore *Trichoplusia ni* (cabbage looper), and increased salt tolerance.

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Introduction

The aromatic amino acids, phenylalanine, tyrosine, and tryptophan, are produced via a branched pathway from chorismate in plants (Figure 3.1). As is observed in many other biosynthetic pathways, aromatic amino acid biosynthesis is subject to feedback regulation. Isoforms of the committing enzyme, chorismate mutase, are allosterically inhibited by phenylalanine and tyrosine, and stimulated by tryptophan. (Coruzzi and Last, 2000; Eberhard *et al.*, 1996). Anthranilate synthase, the committing enzyme for tryptophan biosynthesis is inhibited by tryptophan (Belser *et al.*, 1971). Similarly, arogenate dehydrogenase is feedback inhibited by tyrosine (Rippert and Matringe, 2002). Expression of mutant enzymes with relaxed feedback inhibition often causes increased product accumulation. For instance, overexpression of a feedback-insensitive anthranilate synthase α-subunit dramatically increased free tryptophan accumulation in rice (Tozawa *et al*., 2001; Wakasa *et al*., 2006). Research with both monocots and dicots has revealed activation of arogenate dehydratese by tyrosine and inhibition by phenylalanine (Jung *et al.*, 1986; Siehl and Conn, 1988). Characterization of the rice *mtr1*mutant revealed that a mutation in the conserved ESRP peptide motif in the ACT regulatory domain of the arogenate dehydratase 2 gene causes overaccumulation of free phenylalanine (Yamada *et al*., 2008). This suggests that, similar to the mechanism found in the bacterial P-protein, feedback inhibition in arogenate dehydratase depends upon the ESRP motif (Chipman and Shaanan, 2001; Pohnert *et al*., 1999).

The relative importance of phenylpyruvate and arogenate as precursors for phenylalanine biosynthesis in plants has not been fully investigated. Biosynthesis of tyrosine by arogenate dehydrogenase has been demonstrated (Rippert and Matringe, 2002), and arogenate dehydratases catalyzing the final phenylalanine biosynthesis step

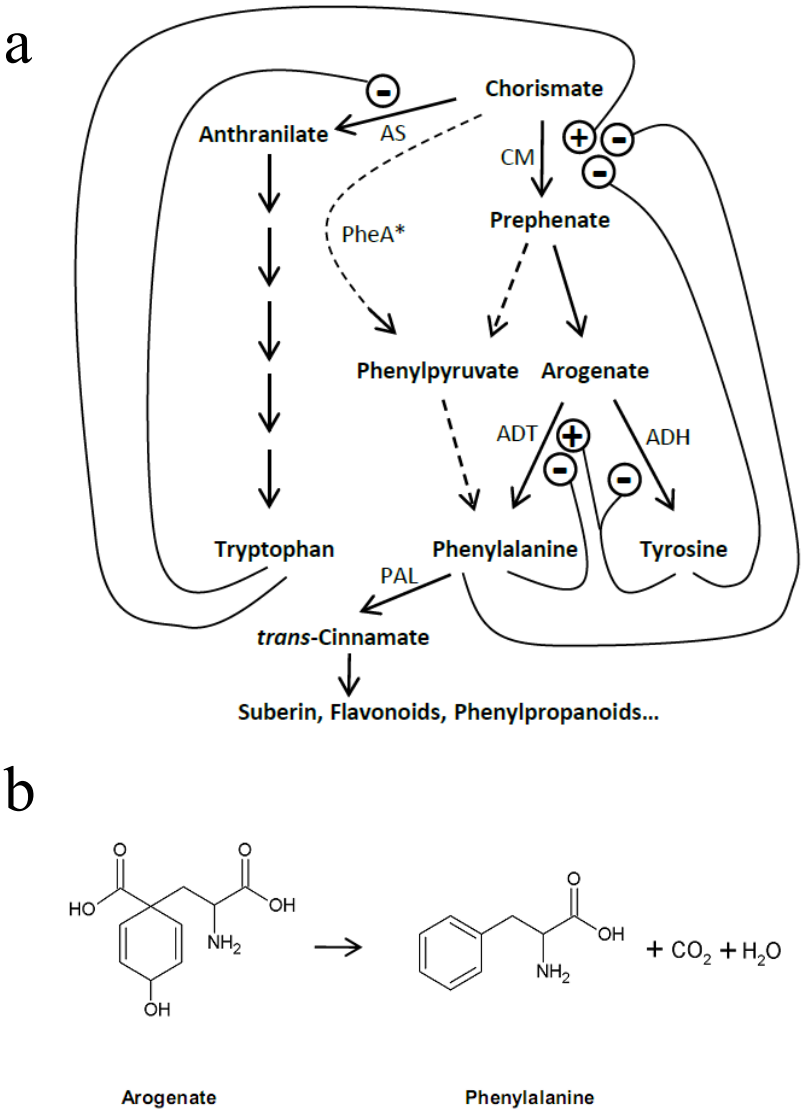


Figure 3.1. Metabolic pathways of aromatic amino acid biosynthesis.
(a) Known pathways and mechanisms of allosteric regulation are shown, inhibition with a line and a minus sign, activation with a plus sign. Also shown is a reaction catalyzed by overexpression of *E. coli* PheA*, which synthesizes phenylpyruvate from chorismate and leads to overaccumulation of phenylalanine in *A. thaliana* (Tzin *et al.*, 2009). AS, anthranilate synthase; CM, chorismate mutase; ADT, arogenate dehydratase; ADH, arogenate dehydrogenase; PAL, phenylalanine ammonia lyase; PheA*, modified *E. coli* chorismate mutase/prephenate dehydratase.
(b) ADT catalyzes the reaction from arogenate to phenylalanine, the last step of phenylalanine biosynthesis in *A. thaliana*.

were identified in rice and *A. thaliana* (Cho *et al.*, 2007). *In vitro* enzyme assays show a strong preference for arogenate over prephenate as the substrate for all six *A. thaliana* ADT enzymes, suggesting that the majority of prephenate is first converted to arogenate during phenylalanine biosynthesis in *A. thaliana* (Cho *et al.*, 2007). However, *pPheA** transgenic *A. thaliana* plants, which express a truncated *E. coli* feedback-insensitive *PheA* gene (Tzin *et al.*, 2009) and thereby overproduce phenylpyruvate (Figure 3.1), also accumulate elevated levels of phenylalanine.

Most of the phenylalanine produced in plants is not used for protein biosynthesis, but rather enters the phenylpropanoid pathway, leading to the production of secondary metabolites, including lignin, flavonoids, and many other small phenolic compounds (Coruzzi and Last, 2000). In *A. thaliana* and other crucifers, phenylalanine also serves as a precursor for the production of glucosinolates, a class of insect-defensive secondary metabolites (Graser *et al.*, 2001). Phenylalanine ammonia-lyase, the committing enzyme for the pathway (Figure 3.1), converts phenylalanine to *trans*-cinnamate and can account for more than 30% of the carbon flow in plants (Razal *et al.*, 1996).

Previous research has demonstrated phytotoxic effects of non-protein amino acids, most of them synthetic compounds such as 5-methyltryptophan, α-methyltryptophan, ethionine, and D-alanine (Kreps and Town, 1992; Wakasa and Widholm, 1987; Widholm, 1972a; Widholm, 1976). Many of these amino acid analogs compete with their structurally similar common protein amino acids for an array of metabolic reactions. Toxic effects can often be rescued at least partially by exogenous supplementation or increased biosynthesis of the competing common amino acids. The latter effect has been utilized as a tool to study amino acid metabolism *in planta*, especially by means of genetic screens that have identified *A. thaliana* mutants with increased amino acid biosynthesis, deficiencies in amino acid transporters, and altered

regulatory mechanisms. Examples of such *A. thaliana* mutations that have led to new insights into plant metabolism include *mto1, trp5-1, amt-1,* and *lht1* (Bartlem *et al.*, 2000; Inaba *et al.*, 1994; Kreps *et al.*, 1996; Li and Last, 1996; Shen *et al.*, 2002; Svennerstam *et al.*, 2007).

m-Tyrosine, an analog of phenylalanine and an isomer of the common protein amino acid tyrosine (*p*-tyrosine), has been found in *Euphorbia myrsinites* (donkey tail spurge) and some fescue species (Bertin *et al.*, 2007; Mothes *et al.*, 1964). Despite its plant origin, *m*-tyrosine is phytotoxic to a broad spectrum of species (Bertin *et al.*, 2003). *m*-Tyrosine added to agar medium at a 2 μM concentration inhibits *A. thaliana* root growth by 50%, and at 50 μM concentration it completely prevents seed germination (Bertin *et al.*, 2007). Higher concentrations of *m*-tyrosine also inhibit the growth of bacteria and mammalian cells (Aronson and Wermus, 1965; Gurer-Orhan *et al.*, 2006). Recent studies suggest that *m*-tyrosine can be misincorporated into proteins in place of phenylalanine by eukaryotic phenylalanyl-tRNA synthetases (Gurer-Orhan *et al.*, 2006; Klipcan *et al.*, 2009). *m*-Tyrosine toxicity in *A. thaliana* can be partially rescued by exogenous supply of several amino acids, with phenylalanine being the most effective (Bertin *et al.*, 2007). This suggested the possibility of gaining new insight into aromatic amino acid metabolism by isolating and cloning *A. thaliana* mutations that confer resistance to exogenously added *m*-tyrosine.

Results

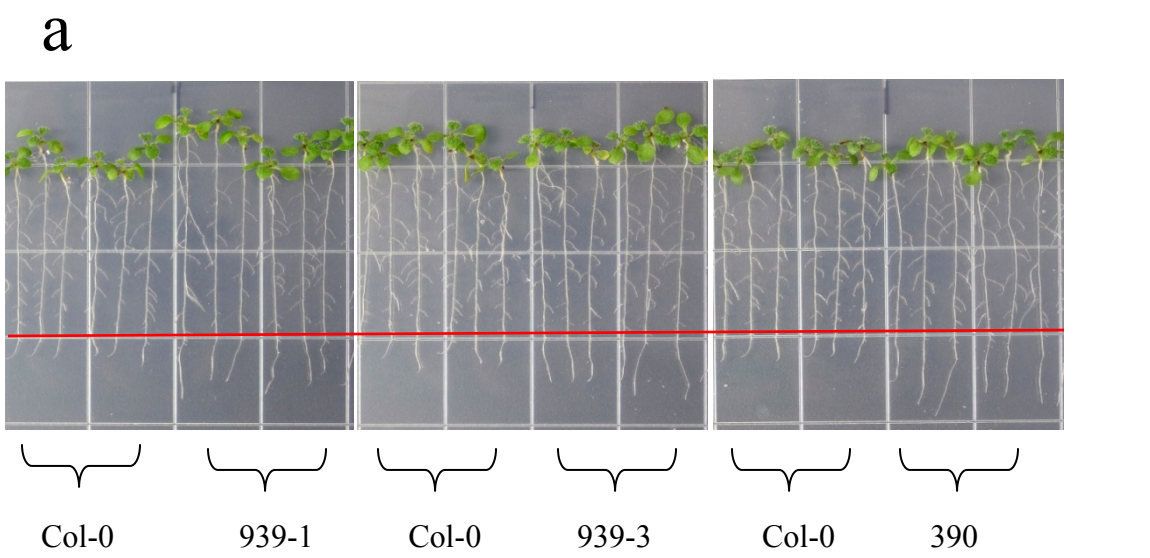
Selection for *A. thaliana* mutants resistant to *m*-tyrosine

Based on the observation that *m*-tyrosine inhibits root growth of *A. thaliana* accession Columbia-0 (Col-0) (Bertin *et al.*, 2007), several mutant collections, including fast neutron mutated lines, T-DNA activation tagged lines and EMS (ethyl

methanesulfonate) mutated lines were screened for elevated *m*-tyrosine resistance. No fast neutron mutants showed heritable resistance to *m*-tyrosine. Three T-DNA activation tagged lines showed improved root growth in presence of 3 µM but not 40 µM *m*-tyrosine (Figure 3.2a). One EMS mutant showed improved root growth on both 3 and 40 µM *m*-tyrosine (Figure 3.2b, *adt2-1D*). Due to the stronger phenotype of the EMS mutant and because crosses showed that the three T-DNA insertions were not genetically linked to the respective mutant phenotypes, all subsequent research was focused on the *m*-tyrosine-resistant EMS mutant line.

Identification of a point mutation in the *ADT2* gene

The genetic basis of *m*-tyrosine resistance was revealed by map-based cloning of the EMS-induced mutation. An F2 population from a cross to Landsberg *erecta* (L*er*) showed a 366:127 resistant:sensitive segregation ratio, which is not significantly different from the 3:1 ratio expected for a single dominant mutation (Chi-squared test, 95% confidence level). Map-based cloning using ~ 400 F2 lines identified a 260 kb interval containing 83 predicted genes between markers CER470676 and CER469707 (Jander *et al.*, 2002). Among these genes, *ADT2* (arogenate dehydratase 2; AT3G07630) was of particular interest, because it is directly involved in phenylalanine biosynthesis and a mutation in *ADT2* might cause a change in phenylalanine metabolism (Figure 3.1a) (Cho *et al.*, 2007; Hruz *et al.*, 2008; Yamada *et al.*, 2008). Consistent with our agar plate-based mutant screen, *ADT2* is the most highly expressed member of the *ADT* gene family in imbibed seeds and root tips of *A. thaliana* (Figure 3.3) (Hruz *et al.*, 2008). Sequencing the coding region of *ADT2* in the EMS mutant identified a single-nucleotide G959A mutation. This mutation is predicted to cause a S320A amino acid change in ESPR motif in the ACT regulatory



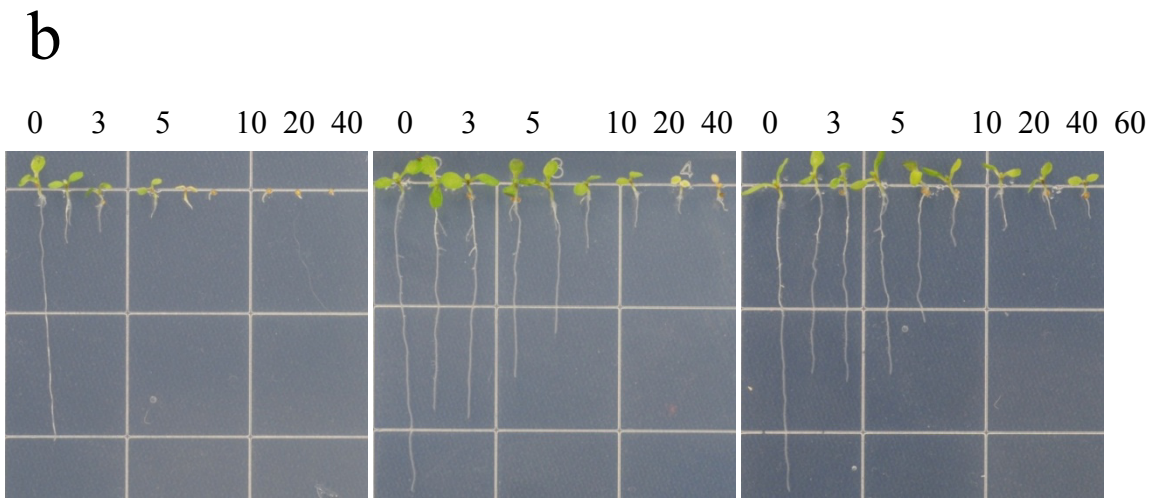


Figure 3.2. *A. thaliana* mutants resistant to *m*-tyrosine.
(a) Three T-DNA activation tagged lines showed better root growth than wildtype plants when transferred from normal MS agar plates to plates containing 10 μM *m*-tyrosine. Root tips were aligned to the orange line when the seedlings were transferred and the picture was taken 2 days later.
(b) Root growth of Col-0, *adt2-1D* and *padt2-1D* grown at different concentrations of *m*-tyrosine.

domain of the ADT2 protein, which is thought to mediate feedback inhibition of enzyme activity (Chipman and Shaanan, 2001; Pohnert *et al.*, 1999; Yamada *et al*., 2008). The identified mutation has, therefore, been named *adt2-1D.*

To confirm that the S320A change in ADT2 causes the observed dominant phenotype, genomic DNA encoding ADT2 and adt2-1D was cloned with the native promoter into vector pMDC123 (Curtis and Grossniklaus, 2003), and transformed into wildtype Col-0 *A. thaliana*. Several independent transgenic lines were collected and their resistance to *m*-tyrosine was analyzed. Only one homozygous transgenic line carrying the mutant gene, *padt2-1D-1*, was obtained. Three other lines, *padt2-1D-2, padt2-1D-7,* and *padt2-1D-3,* were only viable as heterozygotes. All of the lines carrying homozygous or heterozygous *adt2-1D* constructs show resistance to *m*-tyrosine at a level comparable to the original *adt2-1D* mutant (Figure 3.2b). In contrast, plants transformed with *ADT2* or the empty vector are as sensitive to *m*-tyrosine as wildtype Col-0. These results confirm that the G959A mutation in *ADT2* confers *m*-tyrosine resistance to *A. thaliana.*

In addition to their *m*-tyrosine resistance, transgenic *padt2-1D-1* plants have altered rosette leaf morphologies (Figure 3.4a). The leaves, especially when young, are narrower than wildtype leaves and show hyponastic growth, with the edges curled upward as opposed to downward. The serration of the edges is also more apparent than in wildtype leaves (Figure 3.4b). In the segregating populations of *padt2-1D-3*, three different categories of plants with different morphologies were observed: wildtype, similar to *padt2-1D-1*, and very dwarfed plants (Figure 3.4a, V-VII). Plants looking like *padt2-1D-1* were heterozygous based on progeny tests. The dwarfed plants always died without setting seeds, which may explain why homozygous transgenic lines were never obtained. Two other heterozygous lines, *padt2-1D-2* and *padt2-1D-7,* display

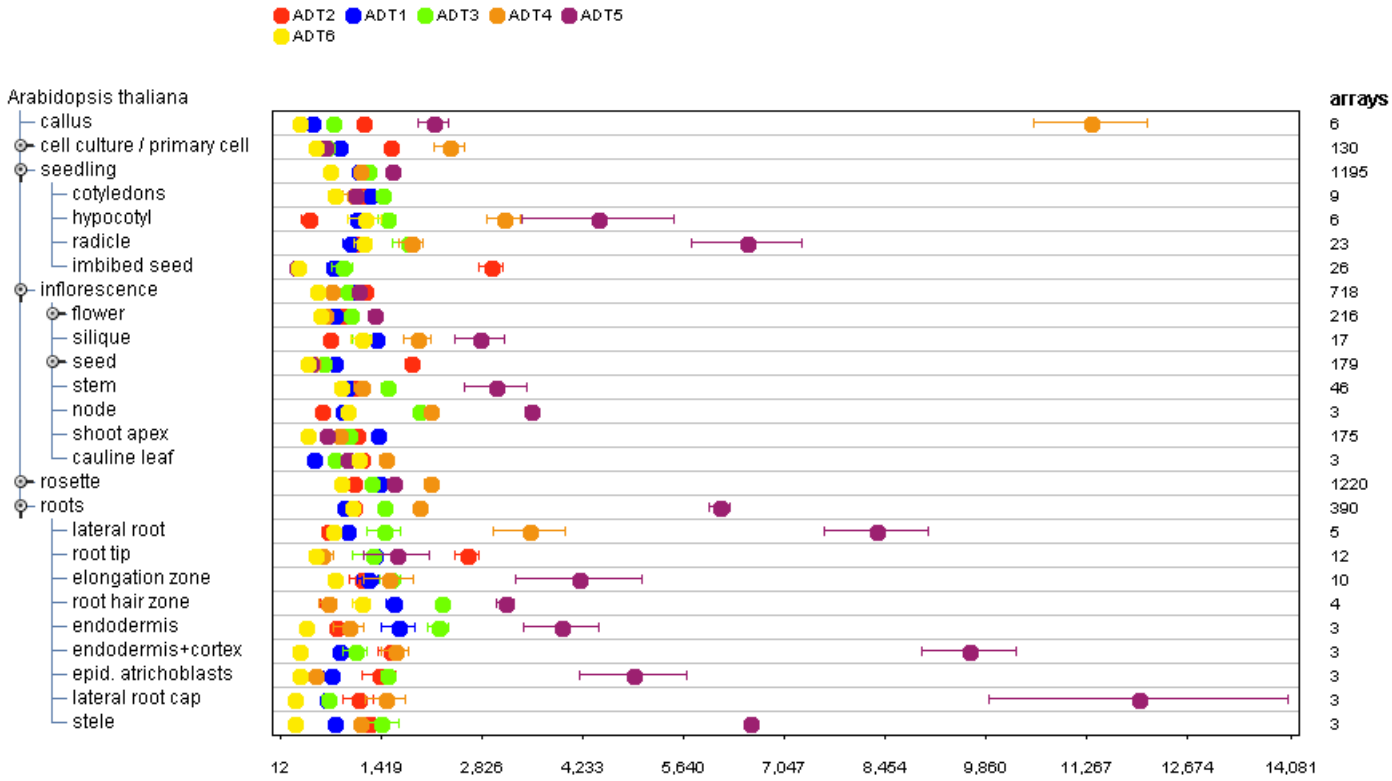
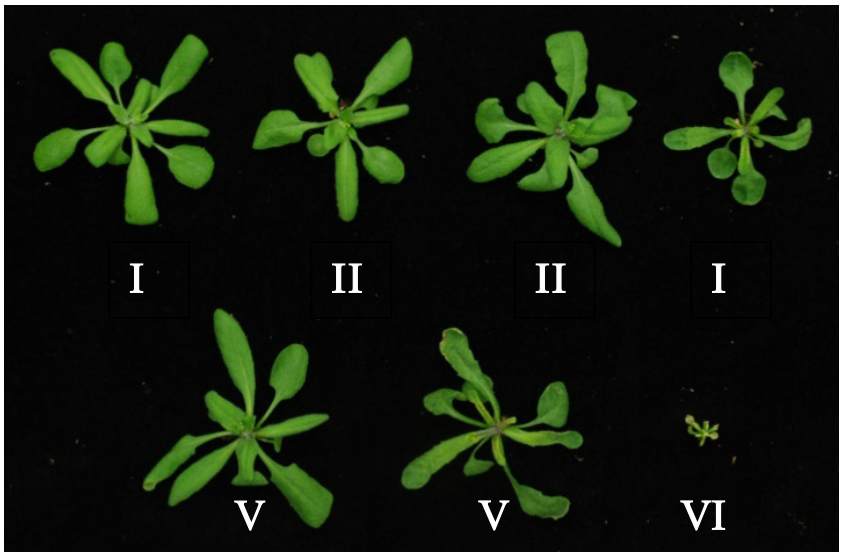


Figure 3.3. Expression analysis of the *ADT* gene family, as determined by Genevestigator. Expression levels of all six *ADT* gene family members were plotted in different tissue types. X axis indicates expression level.

a



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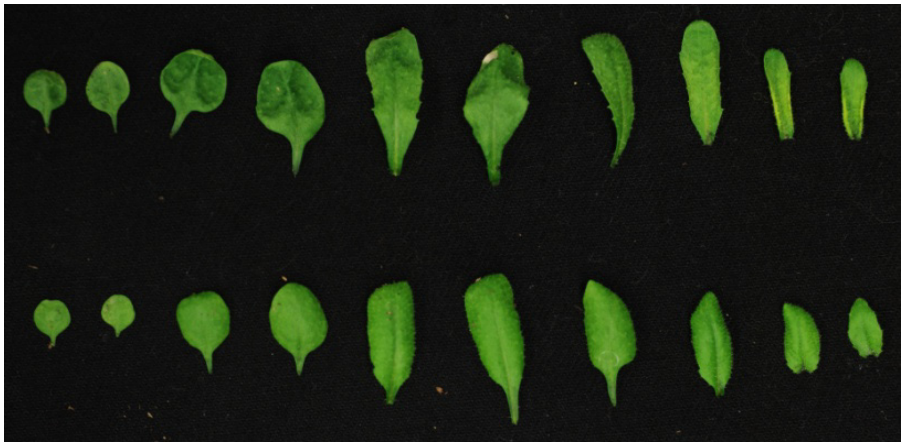


Figure 3.4. Phenotypes of mutant and transgenic lines.
(a) Rosette leaf morphology of 35 day old plants: Col (I) *adt2-1D* (II) *pADT2* (III) *padt2-1D-1* (IV), and three different plants in a segregating population of *padt2-1D-3* (V-VII). Compared to wildtype leaves, young rosette leaves of transgenic plants are smaller and narrower, with edges curved upwards. The presumably homozygous *padt2-1D-3* plant is dwarf and dies before flowering. (b) Leaf development comparison of *padt2-1D* (top panel) and Col-0 (bottom panel). Leaves were arranged according to order of emergence. Leaves of *padt2-1D* display altered surface curvature and margin serration.

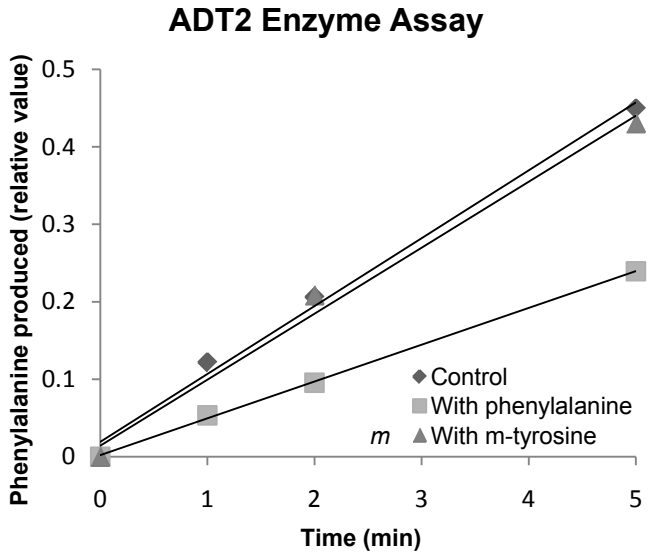
phenotypes stronger than *padt2-1D-1* and weaker than *padt2-1D-3.* Hereafter, we studied *padt2-1D-1* in more detail and use *padt2-1D* to designate this transgenic line in the text and figures, unless we are comparing it to *padt2-1D-3*.

ADT2, but not adt2-1D, is feedback inhibited by phenylalanine

Since the *adt2-1D* mutation, which confers resistance to *m*-tyrosine, is in the ADT2 regulatory domain, we hypothesized that the observed phytotoxic effects could result from ADT2 inhibition by *m*-tyrosine. To test this, the coding sequence of *adt2-1D* was cloned into the pET43.1 vector, which has been used previously for expression of wildtype ADT2 as a his-tagged fusion protein (Cho *et al.*, 2007). Both mutant and wildtype proteins were expressed in *E. coli* and purified using Ni-NTA (nickel-nitrilotriacetic acid) agarose. Measurement of the initial rate of the reaction showed that ADT2 is inhibited by 100 μM phenylalanine, whereas adt2-1D is not (Figure 3.5). *m*-Tyrosine, even at 500 μM concentration, did not inhibit ADT2 activity in this *in vitro* assay. Therefore, the *adt2-1D* mutation apparently changes an ADT2 regulatory site that mediates enzyme inhibition by phenylalanine, but not *m*-tyrosine.

The free phenylalanine content of seeds, seedlings, young rosette leaves, flowers and developing siliques was analyzed in mutant and wildtype plants. Seedlings of the *pPheA** phenylalanine-overproducing line (Tzin *et al.*, 2009) were included for comparison in the right panel and are discussed later. The results show that, in both *adt2-1D* and *padt2-1D* lines, the phenylalanine level is increased in all tissues, with leaves displaying the greatest increase (Figure 3.6a). The transgenic *padt2-1D* line also has much higher free phenylalanine than the *adt2-1D* mutant in every tissue type analyzed. Other than phenylalanine, *adt2-1D* lines do not display changes in free amino acid content relative to wildtype leaves, whereas *padt2-1D* showed reduced

a



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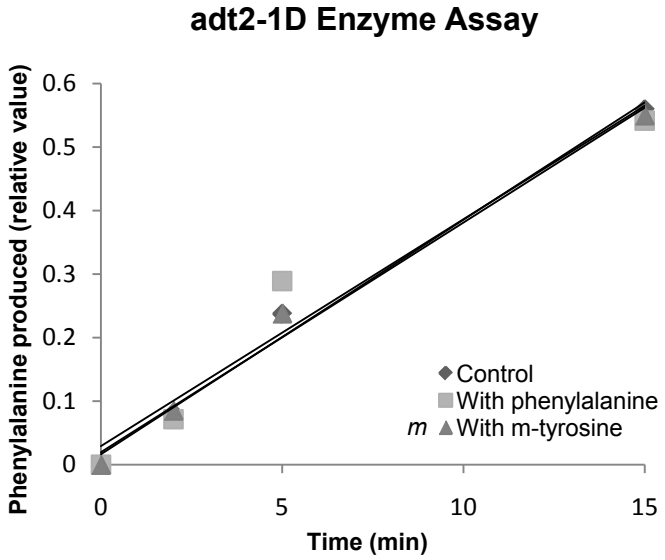

Figure 3.5. Phenylalanine inhibits the enzymatic activity of ADT2 but not adt2-1D.
(a) *In vitro* enzyme assays show that the arogenate dehydratase activity of ADT2 is significantly inhibited by 100 μM phenylalanine ($P < 0.01$). *m*-Tyrosine at 500 μM has no significant effect on enzymatic activities ($P = 0.74$). (*t*-tests based on linear regression by the ordinary least squares method) (b) Activity of adtt2-1D is not significantly inhibited by 100 μM phenylalanine ($P = 0.84$) or 500 μM *m*-tyrosine ($P = 0.90$). (*t*-tests based on linear regression by the ordinary least squares method)

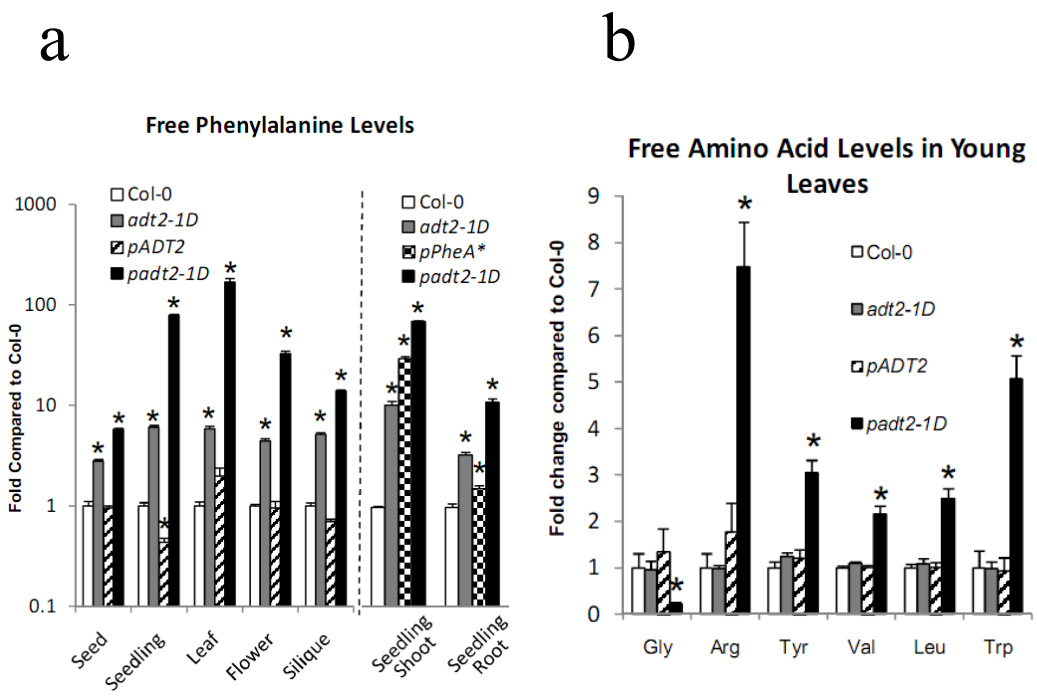

Figure 3.6. Free amino acids of *adt2-1D* mutants and transgenics.
(a) Free phenylalanine content in different tissues of wildtype Col-0, mutant, and transgenic plants. Concentration of phenylalanine in Col-0 has been set to 1 for each sample. *pPheA** lines were only measured in seedling stage and together with the controls, were further dissected to two parts: shoot and root. Note the logarithmic scale of the Y axis. Mean + s.e. of n = 5 or 6, *P < 0.05 in two tailed Student's *t*-test relative to wildtype Col-0.

(b) Amino acid changes in rosette leaves of wildtype, mutant and transgenic *A. thaliana.* Mean + s.e. of n = 5 or 6. *P < 0.05 in two tailed Student's *t*-test relative to wildtype Col-0.

levels in glycine and increased levels of some other amino acids. (Figure 3.6b for amino acids with significant changes, Table 3.1 for all 17 amino acids measured in this assay). Transgenic *padt2-1D-3* heterozygotes, which have normal rosette size but hyponastic leaves, have even higher phenylalanine levels than *padt2-1D-1*.

Since *padt2-1D* transgenics display dramatic increases in free phenylalanine compared to the EMS mutant *adt2-1D,* we performed quantitative RT-PCR to compare gene expression levels in these lines. Due to the single nucleotide difference between the *ADT2* and *adt2-1D*, the PCR primers did not differentiate between these two alleles, and all data represent combined expression levels. Homozygous plants were used for *padt2-1D-1,* whereas heterozygous plants were used for *padt2-1D-3*. Corresponding to their dramatic accumulation of free phenylalanine, transgenic lines show increased *ADT2* gene expression in the leaves (Figure 3.7). Since the genes are expressed from their native promoters, expression differences are likely due to positional effects of the transgene insertion. This suggests that phenylalanine biosynthesis can be limited by the availability of ADT2 enzymes once feedback inhibition is relaxed.

Interestingly, in *padt2-1D* plants that are just starting to flower, young rosette leaves accumulate more soluble phenylalanine than older ones. Correspondingly, the up-curled feature of young rosette leaves is also much more apparent than the older leaves. In Col-0 and *adt2-1D,* the level of free phenylalanine remains much lower than the young leaves of *padt2-1D* plants, and no up-curled leaves were found.

Metabolite analysis of adt2-1D plants

Given the central role of phenylalanine in plant metabolism, it seemed likely that the overall metabolite profile of the *padt2-1D* line would be altered relative to wildtype Col-0. To test this hypothesis, primary and secondary metabolite profiling by

Table 3.1. Free amino acids in mutant and transgenic plants. Concentrations of amino acids from rosette leaves of *adt2-1D, pADT2* and *padt2-1D* plants were compared to Col-0 by HPLC. Relative changes were shown using amino acids concentrations of Col-0 as 1. Mean ± s.e. of n = 6.

Comparison of free amino acids in young rosette leaves				
	Col	*adt2-1D*	*pADT2*	*padt2-1D*
Asp	1.00±0.05	1.05±0.03	1.18±0.09	1.22±0.04
Ser	1.00±0.10	0.93±0.09	1.26±0.21	1.42±0.14
Glu	1.00±0.04	1.09±0.05	1.15±0.08	1.12±0.03
Gly	1.00±0.30	0.96±0.18	1.34±0.50	0.22±0.03
His	1.00±0.18	1.04±0.09	1.21±0.23	1.50±0.10
Arg	1.00±0.30	0.98±0.07	1.76±0.62	7.48±0.96
Thr	1.00±0.07	1.07±0.03	1.25±0.13	0.91±0.04
Ala	1.00±0.08	0.99±0.03	1.08±0.15	1.51±0.12
Pro	1.00±0.15	0.83±0.07	1.17±0.28	0.78±0.07
Tyr	1.00±0.13	1.24±0.08	1.21±0.17	3.04±0.27
Val	1.00±0.04	1.10±0.03	1.00±0.05	2.15±0.18
Met	1.00±0.08	1.08±0.07	1.09±0.11	1.13±0.12
Lys	1.00±0.03	0.92±0.06	1.10±0.03	1.27±0.06
Ile	1.00±0.03	0.99±0.04	1.05±0.06	1.00±0.05
Leu	1.00±0.08	1.08±0.11	1.02±0.10	2.49±0.21
Phe	1.00±0.10	5.85±0.37	1.97±0.41	168.09±15.51
Trp	1.00±0.36	0.98±0.15	0.93±0.28	5.06±0.50

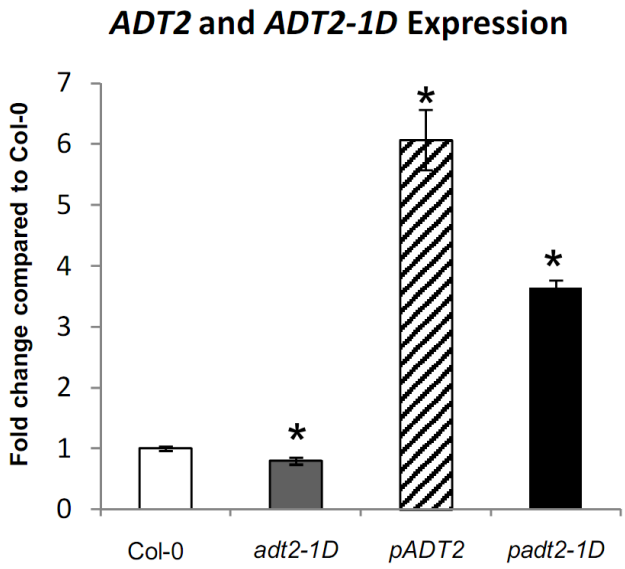

Figure 3.7. Expression of *ADT2* and *ADT2-1D* in mutants and transgenics. Expression of *ADT2* and *ADT2-1D* in rosette leaves of different genotypes. Mean ± s.e. of n = 6, *P < 0.05, two tailed Student's *t*-test relative to wildtype Col-0.

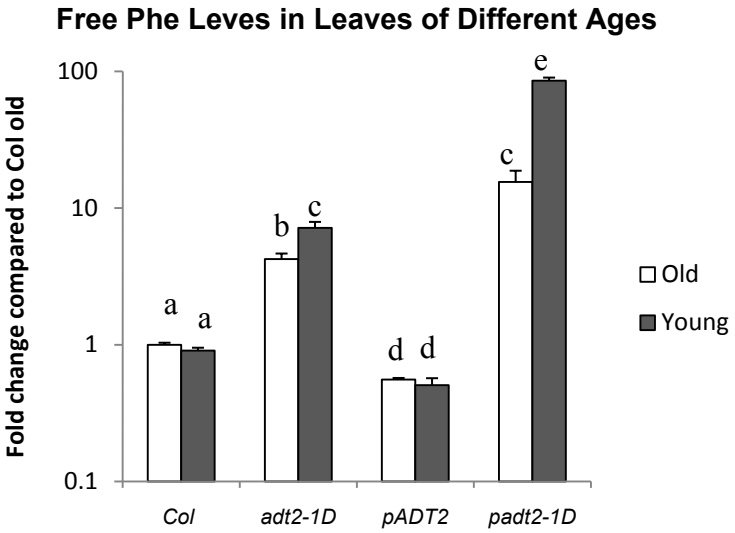

Figure 3.8. Correlation of free phenylalanine levels and leaf shapes. All data were normalized, with the concentration of phenylalanine in old Col-0 leaves set as 1. Free phenylalanine accumulation in young leaves of *padt2-1D* is much higher than in the rest of the leaves, which is correlated to their unique phenotype, as shown in Figure 3. Mean + s.e. of n=3. Note the logarithmic scale of the Y axis. Different letters on the bars indicate significant differences, P < 0.05, as determined by a two-tailed Student's *t*-test.

GC-MS and LC-MS were performed to identify changes in transgenic lines expressing *padt2-1D* and, as a control, *pADT2*. GC-MS metabolite profiling confirmed the elevated phenylalanine in *padt2-1D* (Table 3.2). Ornithine, hydroxyproline, salicylic acid (SA) and Υ-aminobutyric acid were also higher in transgenic plants, trehalose is the only significantly decreased metabolite (Table 3.2), and other tested metabolites showed no significant changes (Table 3.3).

Salicylic acid, which is derived from phenylalanine and chorismate in higher plants, is an important regulator of disease resistance (Feechan *et al.*, 2005). *PR1* (pathogenesis-related protein 1) gene expression is induced by SA and is widely used as a marker gene for plant systematic acquired resistance. In agreement with the increased level of free SA, *PR1* expression is dramatically up-regulated in *padt2-1D* (Figure 3.9), and therefore SA-dependent defense pathways might be constitutive activated in *padt2-1D*.

Table 3.2. Metabolites with significant changes in *padt2-1D* plants.

Metabolites[a]	**Fold Change**[b]	**St. Dev.**[c]
Phenylalanine	70.0	18.6**
Tyrosine	9.8	2.5**
Valine	2.0	0.3**
Tryptophan	67.3	27.3**
Hydroxyproline	2.0	0.4**
Glyceric acid	2.4	0.6**
Salicylic acid	4.7	2.3**
Gulonic acid-1,4-lactone	2.6	1.2*
4-Amino-butyric acid	2.0	0.9*
Trehalose	0.6	0.3*
Glycerol	1.2	0.1*
Putrescine	1.9	0.8*
Ornithine	4.6	3.4*
Guanidine	3.4	2.5*
Urea	1.9	0.9*

Table 3.2 (continuted)

Kaempferol-3Glc-2"Rha-7Rha	2.8	0.6*
Quercetin-3Glc-7Rha	2.9	1.0*
2-Phenylethyl glucosinolate	30.1	6.3**
Sinapoyl glucose	2.4	1.0*
Sinapoyl malate isomer	2.1	0.8*

[a] Compounds measured by GC-MS above the dashed line, by HPLC-MS below. [b]Relative changes, with metabolite concentrations of the Col-0 control as 1. [c]Standard deviation of 7 samples for GC-MS analysis and 4 samples for LC-MS analysis. *P < 0.05; **P < 0.005, one tailed Student's *t*-test comparing mutant and wildtype plants.

Table 3.3. Metabolites in *padt2-1D* plants measured by GC-MS. Concentrations of metabolites from rosette leaves of *padt2-1D* plants were compared to the *pADT2* transgenic control by GC-MS. Relative changes were shown, using metabolite concentrations in the *pADT2* control as 1. Results of two tailed Student's t-test are also shown.

Metabolite	**Mean ± s.e.**	**P-value**
Phenylalanine	69.95 ± 7.01	< 0.001
Tyrosine	9.83 ± 1.01	< 0.001
Valine	2.04 ± 0.12	< 0.001
Tryptophan	67.31 ± 11.08	< 0.001
4-Hydroxy-proline	2.03 ± 0.17	< 0.001
Glyceric acid	2.38 ± 0.25	< 0.001
Salicylic acid	4.74 ± 0.90	< 0.002
Gulonic acid-1,4-lactone	2.56 ± 0.49	0.01
4-Amino-butyric acid	2.04 ± 0.32	0.01
Trehalose	0.56 ± 0.10	0.02
Glycerol	1.24 ± 0.05	0.02
Putrescine	1.93 ± 0.32	0.02
Ornithine	4.57 ± 1.33	0.03
Guanidine	3.42 ± 0.98	0.04
Urea	1.89 ± 0.37	0.05
Alanine	2.38 ± 0.66	0.08
Fructose	1.19 ± 0.06	0.09
Isoleucine	1.20 ± 0.09	0.11
Glucose	1.28 ± 0.09	0.11

Table 3.3 (continued)

myo-Inositol	1.20 ± 0.11	0.12
1,6-Anhydro-glucose	1.62 ± 0.40	0.16
O-acetyl-serine	1.30 ± 0.17	0.17
Glucose-6-phosphate	1.60 ± 0.39	0.18
Threonine	0.75 ± 0.15	0.20
Erythritol	1.42 ± 0.31	0.21
Alanine	1.23 ± 0.16	0.22
2-Methyl-malic acid	0.81 ± 0.04	0.22
Phosphoric acid	1.30 ± 0.17	0.24
Nicotinic acid	1.17 ± 0.11	0.25
Maltose	0.82 ± 0.07	0.26
Arginine	1.42 ± 0.25	0.28
Melezitose	1.52 ± 0.33	0.28
2-Ethyl-hexanoic acid	1.29 ± 0.11	0.41
Proline	1.30 ± 0.33	0.41
Malic acid	1.24 ± 0.25	0.48
Asparagine	1.39 ± 0.52	0.50
Succinic acid	0.82 ± 0.14	0.54
Aspartic acid	1.13 ± 0.16	0.56
Lactic acid	1.10 ± 0.09	0.59
Glycine	1.54 ± 0.86	0.60
Heptadecanoic acid	0.83 ± 0.18	0.62
Tetradecanoic acid	0.88 ± 0.17	0.66
Glutamine	0.91 ± 0.16	0.67
Gluconic acid-1,5-lactone,	1.11 ± 0.14	0.71
Uracil	1.05 ± 0.07	0.74
Octadecanoic acid	0.93 ± 0.18	0.79
Glutamic acid	1.04 ± 0.12	0.79
Benzoic acid	1.04 ± 0.10	0.80
Serine	0.98 ± 0.16	0.93
Spermidine	1.01 ± 0.25	0.99

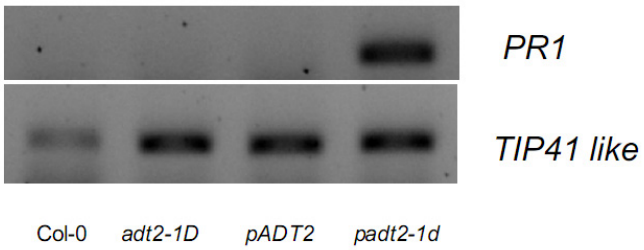

Figure 3.9. Upregulation of *PR1* expression in *padt2-1D*. Semi-quantitative RT-PCR analysis shows induced expression of the *PR1* gene in *padt2-1D* lines compared to Col-0 wildtype, *adt2-1D* and *pADT2,* using expression of the *TIP41 like* gene as an internal control.

Several categories of secondary metabolites, including flavonols, glucosinolates and phenylpropanoids were measured by LC-MS. Kaempferol-3Glc-2"Rha-7Rha, quercetin-3Glc-7Rha, sinapoyl glucose, and sinapoyl malate, were increased more than two-fold (Table 3.4). There was a trend toward increased accumulation among other phenylalanine-derived metabolites, but these effects were not significant (Table 3.4). Therefore, although the availability of free phenylalanine affects downstream metabolites, the metabolic flux of phenylalanine catabolism is still under tight control, likely mediated by the feed-back regulation of phenylalanine ammonia lyase (Blount *et al.*, 2000).

Changes in glucosinolate content affect insect resistance

A notable exception to the lack of substantial secondary metabolite changes was a compound with *m/z* $[M-H]^-$ 422, which was increased more than 30-fold in rosette leaves of *padt2-1D* lines. The *m/z* ratio suggested that this compound could be 2-phenylethylglucosinolate (2PE), which was previously reported to be increased in *A. thaliana* overproducing phenylalanine (Tzin *et al.*, 2009). Therefore, we measured the glucosinolate content of wildtype Col-0, *adt2-1D, pADT2,* and *padt2-1D* plants using an independent assay, HPLC analysis of desulphoglucosinolates. This showed a novel peak corresponding to 2PE in *padt2-1D* (Figure 3.10). 2PE, which is normally abundant only in the seeds of *A. thaliana* Col-0 (Brown *et al.*, 2003), is present at greatly elevated levels in both seedlings and mature leaves of *padt2-1D* lines. Much smaller increases are observed in the *adt2-1D* mutant (Figure 3.10). When compared to *pADT2*, *padt2-1D* transgenics also have smaller increases in 3-methylsulfinylpropyl-, 4-methylthiobutyl-, and indol-3-ylmethylglucosinolates (Table 3.5).

Table 3.4. Metabolites in *padt2-1D* plants measured by LC-MS. Concentrations of metabolites from rosette leaves of *padt2-1D* plants were compared to the *pADT2* transgenic control by LC-MS. Relative changes are shown, using metabolite concentrations of the control as 1. Results of one tailed Student's *t*-test were also shown.

Compound	MW	Mean ± s.e.	P-value
Kaempferol-3Glc-2"Rha-7Rha	756	2.78 ± 0.30	0.01
Quercetin-3Glc-2"Rha-7Rha	740	0.84 ± 0.12	0.35
Quercetin-3Glc-7Rha	610	2.85 ± 0.48	0.01
Kaempferol-3Glc-7Rha	594	1.48 ± 0.25	0.11
Kaempferol-3Rha-7Rha	578	0.95 ± 0.13	0.45
3-Indolylmethyl glucosinolate	448	1.40 ± 0.08	0.05
Glucoraphanin	437	1.24 ± 0.11	0.28
2-Phenylethyl glucosinolate	423	30.13 ± 3.15	< 0.001
Glucohirsutin	493	1.48 ± 0.15	0.21
Sinapoyl glucose	386	2.37 ± 0.49	0.04
Sinapoyl malate	340	1.30 ± 0.11	0.07
Sinapoyl malate isomer	340	2.14 ± 0.39	0.04

Table 3.5. Glucosinolate content in *pADT2* and *padt2-1D* plants measured by HPLC.

Concentrations of different glucosinolates from rosette leaves of *padt2-1D* plants were compared to the *pADT2* transgenic control by HPLC. Results of two tailed Student's *t*-tests are also shown. Glucosinolate abbreviations: 3MSP, 3-methylsulfinylpropyl; 4MSB, 4-methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; 8MTO, 8-methylthiooctyl; I3M, indol-3-ylmethyl; 4MI3M, 4-methoxyindol-3-ylmethyl; 1MI3M, 1-methoxyindol-3-ylmethyl; 2-PE 2-phenylethylglucosinolate(2PE).

Compound	MW	Mean ± s.e.	P-value
Kaempferol-3Glc-2"Rha-7Rha	756	2.78 ± 0.30	0.01
Quercetin-3Glc-2"Rha-7Rha	740	0.84 ± 0.12	0.35
Quercetin-3Glc-7Rha	610	2.85 ± 0.48	0.01
Kaempferol-3Glc-7Rha	594	1.48 ± 0.25	0.11
Kaempferol-3Rha-7Rha	578	0.95 ± 0.13	0.45
3-Indolylmethyl glucosinolate	448	1.40 ± 0.08	0.05
Glucoraphanin	437	1.24 ± 0.11	0.28
2-Phenylethyl glucosinolate	423	30.13 ± 3.15	< 0.001
Glucohirsutin	493	1.48 ± 0.15	0.21
Sinapoyl glucose	386	2.37 ± 0.49	0.04
Sinapoyl malate	340	1.30 ± 0.11	0.07
Sinapoyl malate isomer	340	2.14 ± 0.39	0.04

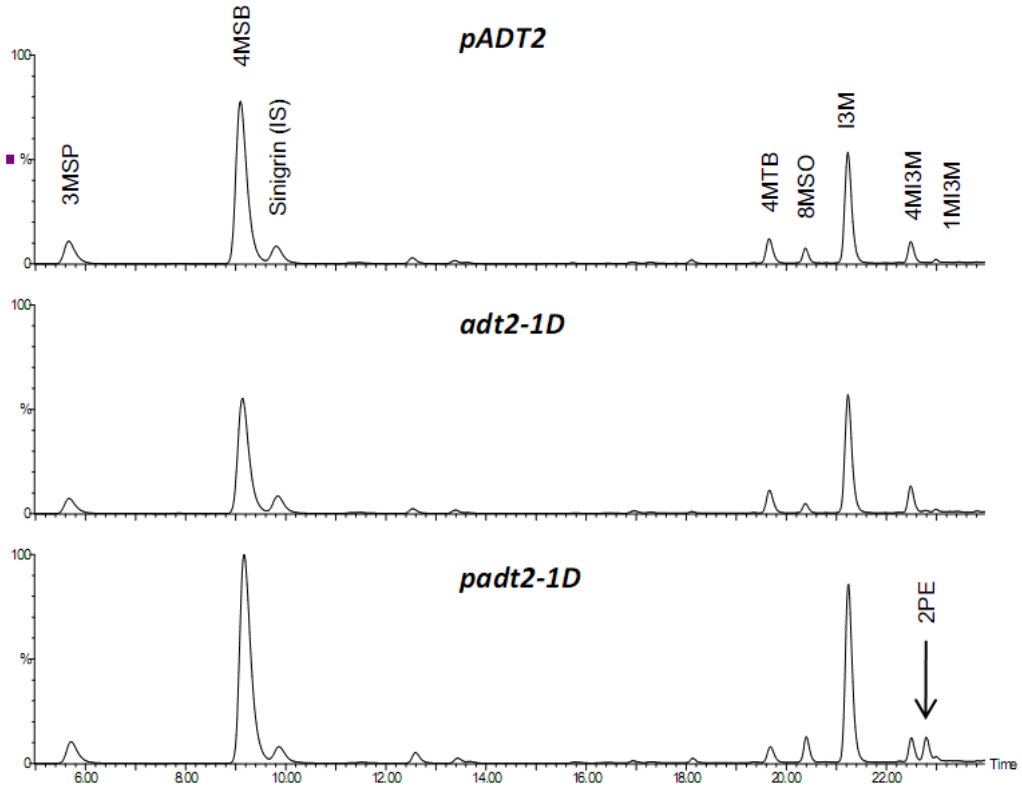

Figure 3.10. Change of glucosinolate profile in *adt2-1D* and *padt2-1D*. Representative UV absorbance (λ=229) chromatograms of desulfoglucosinolates from *pADT2* (top), *adt2-1D* (middle) and *padt2-1D* (bottom) mature rosette leaves. Glucosinolate side chain abbreviations: 3MSP, 3-methylsulfinylpropyl; 4MSB, 4-methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; 8MTO, 8-methylthiooctyl; I3M, indol-3-ylmethyl; 4MI3M, 4-methoxyindol-3-ylmethyl; 1MI3M, 1-methoxyindol-3-ylmethyl; the arrow points to the novel peak resulting from 2-phenylethylglucosinolate (2PE) in the *padt2-1D*. Sinigrin was used as an internal standard (IS).

To test the plant defense consequences of 2PE over-accumulation, we performed experiments using larvae of the cabbage looper (*Trichoplusia ni*), a generalist lepidopteran herbivore that is sensitive to alterations in glucosinolate content (Barth and Jander, 2006). When given a choice between rosette leaves of *pADT2* and *padt2-1D, T. ni* larvae showed a strong preference for feeding on *pADT2* leaves, suggesting aversion to 2PE (Figure 3.11a,). In a second run of this experiment, *T. ni* larvae hardly consumed any *adt2-1D* leaf material (Figure 3.12). In no-choice experiments, *T. ni* larvae gained significantly less weight on plants with elevated 2PE (Figure 3.11b) than on controls.

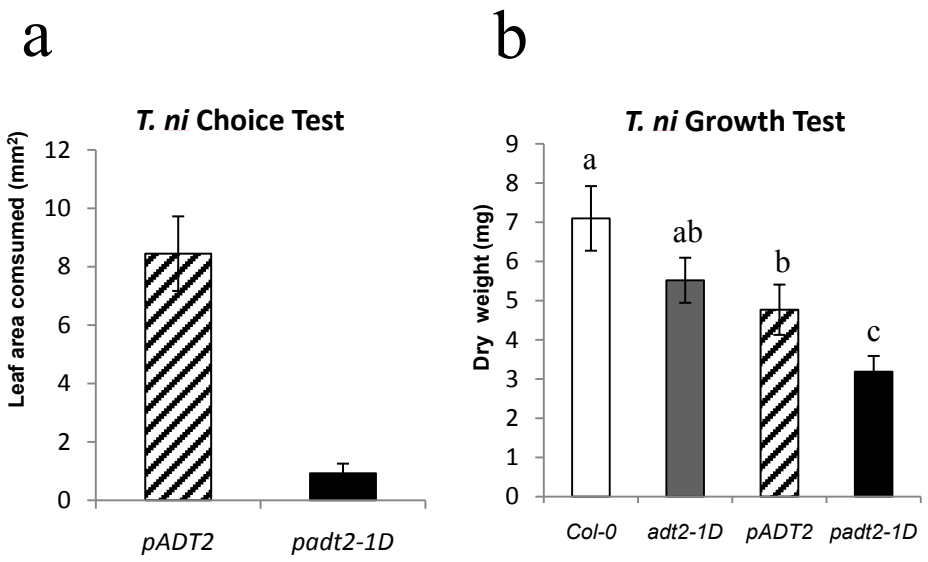

Figure 3.11. Increased insect resistance in *padt2-1D*.
(a) Leaf area consumed in *T. ni* feeding choice tests. *T. ni* consumes sigifincantly less leaves in *padt2-1D*. Mean ± s.e. of n = 16, $P < 0.0001$, Student's *t*-test.
(b) Caterpillar weight gain in a *T. ni* feeding no-choice test. Mean ± s.e. of n = 14 to19. Growth of *T. ni* is significantly inhibited in *padt2-1D*. Different letters on the bars indicate significant differences, $P < 0.05$, as determined by a two-tailed Student's *t*-test.

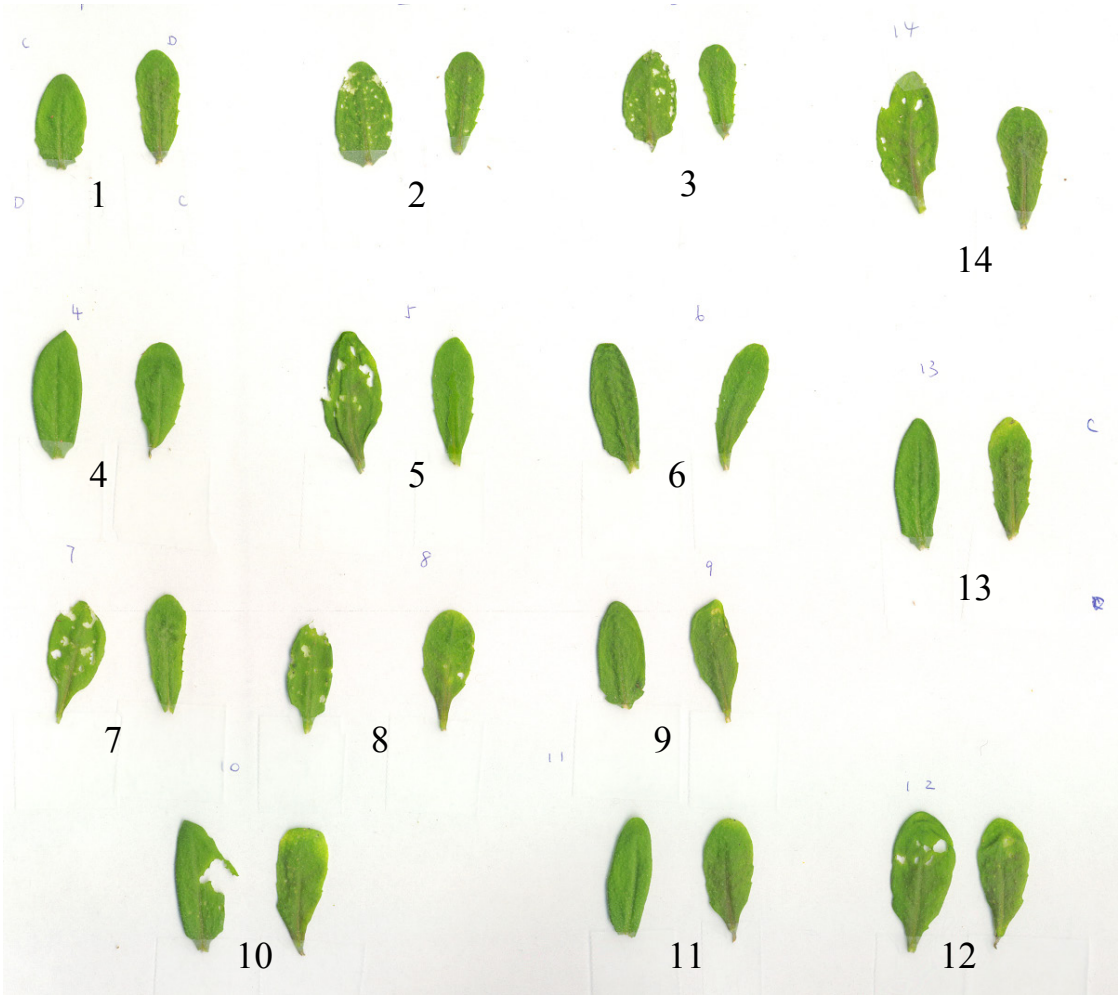

Figure 3.12. When given a choice, *T.ni* prefers to feed from *padt2-1D* rather than *pADT2* leaves. *T. ni* consumes significantly more leaf material of *pADT2* (left of each pair) than *padt2-1D* (right of each pair).

Although glucosinolate degradation products deter generalist insect feeding, some crucifer-specialist insects are resistant to these negative effects (Ratzka *et al.*, 2002; Wittstock *et al.*, 2004). Consistent with this, larval weight gain of the white cabbage butterfly (*Pieris rapae*), a crucifer-specialist herbivore, was not significantly affected when comparing *pADT2* and *padt2-1D* plants to wildtype Col-0. When given a choice between rosette leaves of *pADT2* and *padt2-1D, P. rapae* did not show a significant feeding preference. We also did not observe significant differences in diamondback moth (*Plutella xylostella*) oviposition when given a choice of *pADT2* and *padt2-1D* plants, or Col-0 and *adt2-1D* plants.

The *adt2-1D* mutation increases salt tolerance

In response to osmotic stress, *A. thaliana* and other plants accumulate elevated amounts of free amino acids, including phenylalanine (Nambara *et al.*, 1998). For instance, in dehydrated *A. thaliana* leaves, flowers, and siliques, free phenylalanine levels were increased 100-, 50-, and 30-fold, respectively (Joshi *et al.*, 2010). The osmoprotective functions of proline during osmotic stress have been studied extensively (Verbruggen and Hermans, 2008). However, for most other amino acids, it is not known whether the elevated accumulation is merely a consequence of osmotic stress or part of a protective response. Previous studies showed that increased accumulation of branched-chain amino acids in the *omr1-5* mutant of *A. thaliana* causes increased salinity tolerance during root elongation on agar plates (Joshi and Jander, 2009). Root growth of *adt2-1D* is similarly improved compared to wildtype Col-0 on agar with 100 mM NaCl (Figure 3.13). In control experiments without added NaCl, root growth of none of these three genotypes showed any significant differences between mutant and wildtype seedlings.

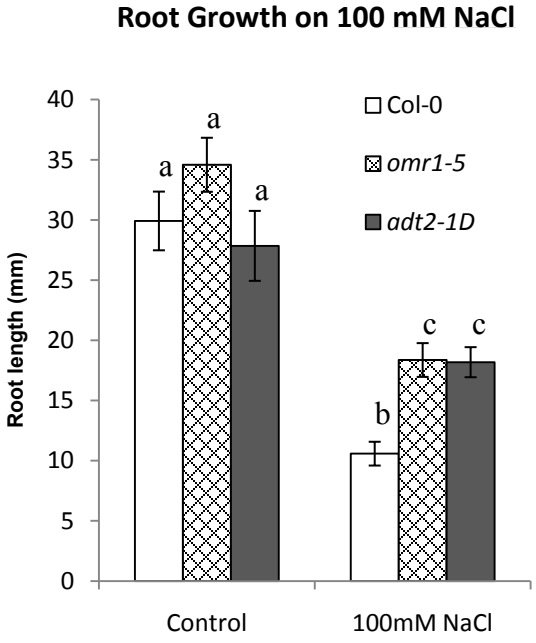

Figure 3.13. Salinity tolerance assays. Root growth of Col-0, *omr1-5,* and *adt2-1D* on MS agar with and without 100 mM NaCl. Mean ± s.e. of n = 10 to 13. Different letters on the bars indicate significant differences, $P < 0.05$, as determined by Student's *t*-test.

Resistance to 5-methyltryptophan

5-Methyltryptophan (5MT), a toxic analog of tryptophan, has been used as a tool to study tryptophan metabolism (Widholm, 1972b). However, analysis of 5MT-resistant rice identified a dominant mutation in the rice *ADT2* homolog (Yamada *et al.*, 2008). The rice mutation changes an amino acid residue in the same position as the *A. thaliana adt2-1D* mutation. It was hypothesized that the over-accumulation of free phenylalanine in rice callus and seedlings is accompanied by a dramatic increase of free tryptophan (from 4- to 20-fold), thus providing 5MT resistance. However, although the *A. thaliana adt2-1D* and *padt2-1D* lines are also resistant to 5MT (Figure

3.14a), they only accumulate only about 50% more soluble tryptophan in the seedlings (Figure 3.14b).

The *A. thaliana* transcription factor mutants *atr1D* and *atr1D atr2D* increase 5MT resistance to a level that is similar or higher than that observed with *adt2-1D* (Figure 3.14a; Bender and Fink, 1998; Smolen *et al.*, 2002). This phenotype depends on increased transcription of the *CYP79B2* and *CYP79B3* genes in the mutant lines (Celenza *et al.*, 2005). *CYP79B2* and *CYP79B3* catalyze the conversion of tryptophan to indole-3-acetaldoxime (IAOx), a precursor of indole-3-acetic acid (IAA) and indole glucosinolates (Hull *et al*., 2000). The two enzymes might also oxidize 5MT to a less toxic product by a similar reaction. However, quantitative PCR showed that in *adt2-1D* and *padt2-1D* lines, there is no significantly increased expression of *CYP79B2*, *CYP79B3,* or *ATR1(MYB34)* (Figure 3.14c).

Although *pPheA** transgenics produce a high level of free phenylalanine in *A. thaliana* seedlings (through pathway indicated in Figure 3.1; Tzin *et al.*, 2009), these plants are not resistant to 5MT or *m*-tyrosine added to agar medium. A further investigation revealed that, unlike the *adt2-1D* mutant, *pPheA** seedlings accumulate most of their phenylalanine in the shoots rather than in the roots (Figure 3.6a). Since 5MT and *m*-tyrosine in agar medium enter *A. thaliana* via the roots, the lack of phenylalanine overaccumulation in *pPheA** roots might explain why this line does not show increased resistance. Alternatively, since PheA* is a bifunctional enzyme that also encodes a feedback-insensitive chorismate mutase, the effect on tryptophan metabolism might be different from that in adt2-1D, thus producing a different 5MT resistance phenotype.

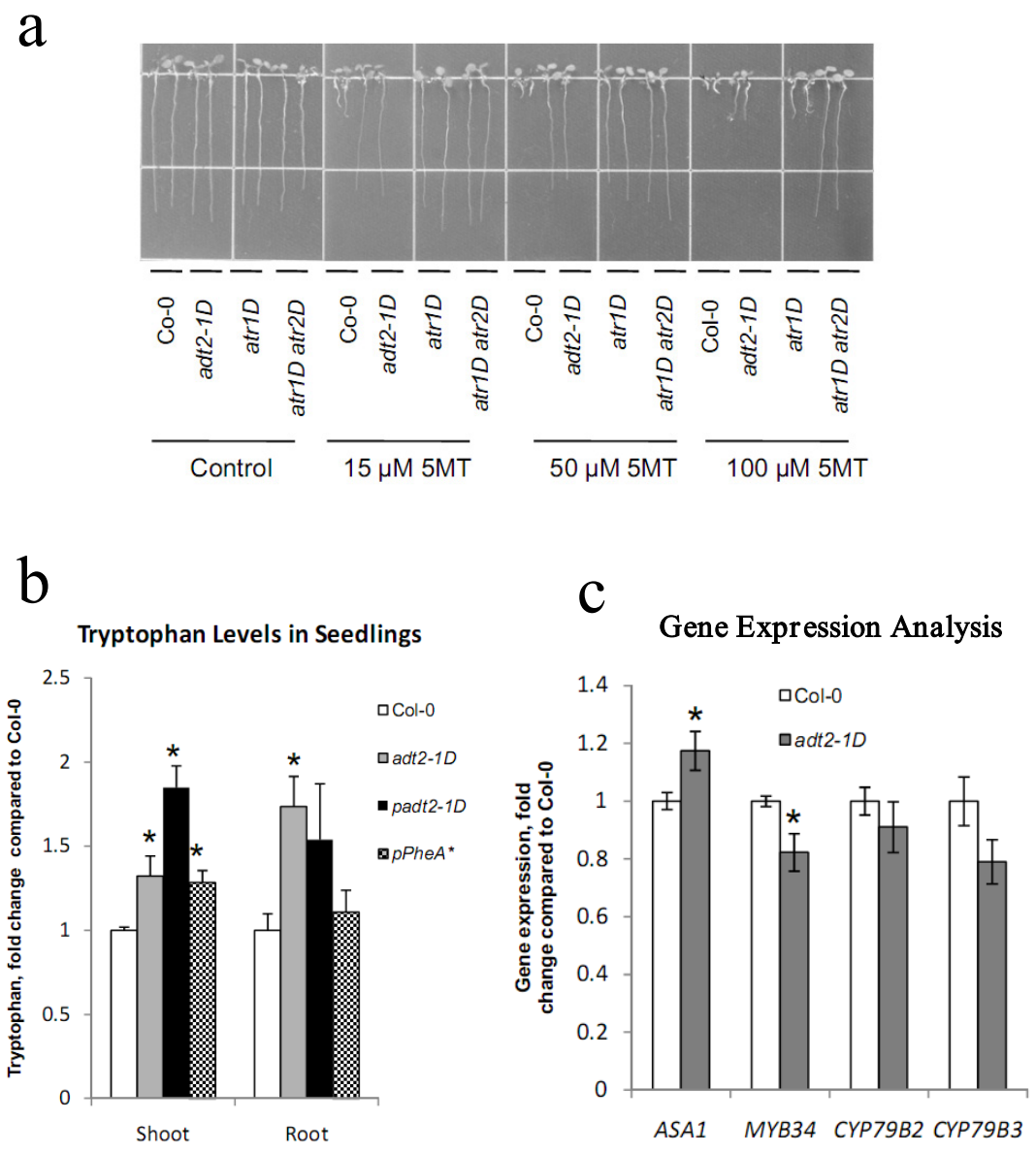

Figure 3.14. Resistance to 5-methyltryptophan.
(a) Representative seedlings of Col-0, *adt2-1D, atr1D,* and *atr1D atr2D* grown on agar with different concentrations of 5-methyltryptophan (5MT) are shown.
(b) Free tryptophan levels in seedling shoots and roots of different genotypes. Mean + s.e. of n = 6, *P < 0.05, two tailed Student's *t*-test relative to wildtype Col-0.
(c) Expression of *ASA1, MYB34, CYP79B2 and CYP79B3* in rosette leaves of Col-0 and *adt2-1D.* In *adt2-1D*, there is no significant increase of gene expression in the set of genes that are responsible for 5MT resistance phenotype in the *atr1D* mutant. Mean ± s.e. of n = 6, *P < 0.05, two tailed Student's *t*-test relative to wildtype Col-0.

Discussion

Our EMS mutant screen for *m*-tyrosine resistance identified only a single mutation in the ESRP motif of the ACT regulatory domain in ADT2. Given that a different mutant screen in rice identified a mutation in exactly the same position (Wakasa and Widholm, 1987; Yamada *et al.*, 2008), it is possible that this specific ADT2 serine residue plays a pivotal role in the regulation of plant phenylalanine biosynthesis. Although the ACT domain ESRP motif is also present in the other five *A. thaliana* ADT proteins (Cho *et al.*, 2007), no mutations were discovered in our *m*-tyrosine resistance screen. This lack of mutations could occur by chance, and it would be interesting to determine whether similar ESRP mutations in the other *A. thaliana* ADTs result in phenylalanine over-accumulation in other plant developmental stages or specific tissues.

Given that we did not see enzyme inhibition by *m*-tyrosine in our *in vitro* ADT2 activity assays (Figure 3.5), a direct effect of *m*-tyrosine on ADT2 is probably not the cause of phytotoxicity *in vivo*. Instead, *m*-tyrosine resistance is likely caused by the localized increase in phenylalanine, which reduces uptake of *m*-tyrosine and/or competes with *m*-tyrosine for access to other enzymes. It is also possible that elevated phenylalanine outcompetes *m*-tyrosine for incorporation into protein by phenylalanyl-tRNA synthetases (Gurer-Orhan *et al.*, 2006; Klipcan *et al.*, 2009). If there are multiple mechanisms of *m*-tyrosine toxicity, this would explain why no target-site resistance was found in our mutant screen. The proposed allelopathic activity of *m*-tyrosine that is released by fescue grasses may be mediated through similar effects on phenylalanine metabolism (Bertin *et al.*, 2007). In the absence of isogenic plant lines that are resistant and sensitive to *m*-tyrosine, it has not been possible to confirm this likely subtle *in vivo* allelopathic activity (Kaur *et al.*, 2009). However, the isolation of an *m*-tyrosine-resistant *A. thaliana* mutant may make it possible to perform such

ecological experiments.

Compared to the rice *mtr1* mutant, in which soluble phenylalanine and tryptophan increase more than 50-fold in callus (Yamada *et al.*, 2008), *adt2-1D* has much smaller increases (Figure 3.6a and 3.14b). This could be due either to inherent differences between *A. thaliana* and rice, or to unusual amino acid metabolism in callus, which is not a typical plant tissue. Recent studies involving suppression of *ADT1* expression by RNA interference in petunia revealed decreased free phenylalanine coupled with decreased free tryptophan and shikimate (Maeda *et al.*, 2010). Despite the relatively modest increase in soluble tryptophan (Figure 3.14b), the *adt2-1D* mutant is as resistant to 5MT as *atr1D,* the strongest 5MT-resistant mutant reported previously (Smolen and Bender, 2002) (Figure 3.14a). Although up-regulation of *CYP79B2* and *CYP79B3* was shown to be essential for 5MT resistance in the *atr1D* transcription factor mutant (Celenza *et al.*, 2005), neither of these genes is up-regulated in *adt2-1D*. Together, these experiments with rice, petunia, and *A. thaliana* demonstrate that levels of free phenylalanine and free tryptophan are positively correlated in a manner that suggest additional, as yet uninvestigated regulation of the aromatic amino acid biosynthesis pathway (Figure 3.1a). Given the observed increases in both phenylalanine and tryptophan, the overall metabolic flux through the shikimate pathway to chorismate is likely to be increased in *adt2-1D*. Feedback inhibition of chorismate mutase by elevated phenylalanine could also increase tryptophan accumulation, but reduced chorismate mutase activity would be inconsistent with the large phenylalanine increase observed in *adt2-1D.* Therefore, although phenylalanine feedback inhibits both chorismate mutatse and ADT in plastids, our study suggests that feedback inhibition of ADT, rather than chorismate mutase, plays a more major role in maintaining phenylalanine homeostasis. An attractive hypothesis to explain the increased tryptophan accumulation is that

anthranilate synthase, or some other enzyme(s) of the tryptophan branch of the pathway are activated by increased phenylalanine or a downstream metabolite. Conversely, tryptophan biosynthesis might be inhibited by arogenate, which would presumably be less abundant in *pADT2-1D*.

Compared to *adt2-1D*, in which phenylalanine and tryptophan are the only two amino acids that are significantly increased, expression of the *padt2-1D* transgene affects a much larger array of plant metabolites (Table 3.2, Table 3.3). In response to a high level of external phenylalanine, *A. thaliana* has increased accumulation of most free amino acids (Voll *et al.*, 2004). These effects allowed the selection of as yet unidentified mutations that provide resistance to toxic amounts of exogenous phenylalanine (Voll *et al.*, 2004). It is possible that similar regulatory mechanisms occur due to endogenous overproduction, reaching lethal levels in the *padt2-1D*-3 line. Notably, overexpression of *OASA1D,* a feedback insensitive anthranilate synthase gene in rice also increased the overall free amino acid levels, in addition to the expected overaccumulation of tryptophan (Wakasa *et al.*, 2006). Taken together, these data strongly suggest a co-regulated network of amino acid biosynthesis.

Increased 2PE is one of the more significant changes in the metabolite profile of *padt2-1D* leaves (Figure 3.10a). In Col-0 *A. thaliana*, substantial amounts of 2PE are only found in seeds and siliques (Brown *et al.*, 2003). Therefore, the *adt2-1D* mutant line may provide new opportunities for finding currently unknown enzymes specific to 2PE biosynthesis. In *pPheA** lines, 2PE was increased in young seedlings (Tzin *et al.*, 2009), which could represent residual glucosinolate from germinating seeds. Here we show that 2PE is abundant in mature leaves, which provided an opportunity to test its defensive role in *A. thaliana.* Choice and no-choice tests using *T. ni*, which is sensitive to glucosinolates, revealed that *padt2-1D* is more resistant than *pADT2* (Figure 3.14b, 14c). However, due to the increase of several other glucosinolates in this line, further

experiments will need to be performed to confirm that 2PE accumulation contributes to the observed resistance effect.

Another interesting finding is the increased accumulation of free SA in *padt2-1D1* plants, which results in activation of *PR1* gene expression. Since phenylalanine and chorismate are metabolic precursors of SA (Chong *et al.*, 2001; Wildermuth *et al.*, 2001; Yalpani *et al.*, 1993), increased accumulation of phenylalanine and/or chorismate in *padt2-1D1* lines could contribute to the increased level of SA. Alternatively, increased SA could be the result of a stress response caused by the unusually high level of soluble phenylalanine in the transgenic plants. Exogenous SA application caused increased 2PE accumulation in oilseed rape (*Brassica napus* L.) leaves (Kiddle *et al*., 1994), a regulatory mechanism that could also contribute to the observed 2PE increase in *padt2-1D A. thaliana*.

Previous research with *pPheA** transgenic *A. thaliana* showed very limited alteration of primary metabolites and transcription (Tzin *et al.*, 2009). Our current study showed somewhat different results. Three possible causes of this difference are: (i) Our *padt2-1D* lines produce considerably higher levels of free phenylalanine; (ii) we characterized our plant material at the rosette stage as opposed to the seedling stage, which might identify differences that are not apparent in younger plants; and (iii) although both studies revealed an increase in free phenylalanine, differences in the genes and promoters used may explain the observed phenotypic differences.

Given the role of phenylalanine as a precursor for numerous secondary metabolites, there are potential practical applications in upregulating this biosynthetic pathway. Similar effects observed in rice, petunia, and *A. thaliana* suggest that ADT activity may be a key regulated step in phenylalanine biosynthesis. Our study underscores the importance of phenylalanine regulation by ADT2 and reveals the far-reaching physiological consequences of phenylalanine overaccumulation, including

changes in leaf development, resistance to phytotoxic amino acids, decreased feeding by a generalist herbivore, and increased salt tolerance.

Experimental procedures

Plant Material and Growth Conditions

A. thaliana Col-0, L*er,* and CS21995, a T-DNA activation tagged line collection in the Col-0 genetic background, were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/). M2 EMS and fast neutron mutated Col-0 seeds were purchased from Lehle Seeds (http://www.arabidopsis.com/).

10,000 seeds representing 8600 independent T-DNA activation tagged lines, 100,000 seeds representing 12,500 independent fast neutron deletion lines, and 267,000 M2 seeds representing 33,400 independent EMS mutation lines were screened for elevated *m*-tyrosine resistance. Plants were grown in soil as described previously (Joshi *et al.*, 2006). To screen for strong *m*-tyrosine-resistant *A. thaliana* mutants, surface-sterilized seeds (Weigel and Glazebrook, 2002) were mixed in 0.5x MS medium (Sigma-Aldrich, http://www.sigmaaldrich.com/) with 1% sucrose, 0.3% Phytagar (Invitrogen, http://www.invitrogen.com/), and 40 μM *m*-Tyr (Sigma-Aldrich), and were plated in Petri dishes. Germination was scored after one week. To screen for weaker *m*-tyrosine-resistant mutants, sterilized seeds were plated in square Petri dishes with 0.5x MS medium, 1% sucrose, 1.5% Phytagar, and 3 μM *m*-tyrosine. Square Petri dishes were placed vertically in growth chambers and root length was measured after 10 days. To test 5MT resistance, 15 μM 5MT (Sigma-Aldrich) was added into 0.5x MS (Murashige and Skoog) medium, 1% sucrose and 1.5% Phytagar, and *A. thaliana* seeds were plated after sterilization. To test salt tolerance, *A. thaliana* seeds were grown on 1x MS medium, 1% sucrose, 1.5% Phytagar with 100 mM NaCl.

Positional Cloning and Transgenic Plants

The adt2-1D mutant was crossed to wildtype Ler A. thaliana, and an F2 population was used for positional cloning. The resistance phenotype was scored by measuring the root length of seedlings grown on MS agar plates with 3 μM m-tyrosine. For all plants that showed a short root phenotype and were thus sensitive to *m*-tyrosine, DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method (Weigel and Glazebrook, 2002).

DNA from 50 *m*-tyrosine-sensitive plants was extracted, pooled, and genotyped with a set of 25 insertion/deletion markers (Table 3.6). This showed genetic linkage to chromosome 3. DNA from 400 *m*-tyrosine sensitive F2 plants was extracted and tested with additional markers. The results confirmed the linkage with markers CER470676 and CER469707 on the top half of the chromosome 3. PCR primers used for genotyping are listed in Table 3.7.

Table 3.6. PCR primers used for map-based cloning.

First Pass Mapping Primers				
InDel name	**Forward primer**	**Reverse primer**	**Chr.**	**Genome location**
CER450763	CGTAAGCTGGAAGAAGAGACGTTG	TGATCGCTCAATCCTTTCGACCT	1	3243901
481865	CAGCCAGATCAAGCCACTGAACTA	GCAGCTAGTGAAGACATGAAACAGC	1	5722101
CER450309	GCCCAGTCTAGGATTGAACATAGG	CCATACTGCAAAGAATGGAAGGGC	1	10131301
CER452606	GTCATGTGACCACCACACTCACTT	CGTAAGACAATGCATGACAGCACG	1	13464201
CER449068	ATCGACTCCGGTACCACTCTAACT	AGACACATCTTCAGACACTGCCAC	1	24095201
CER464729	CCAAAGCTAAAGCTGAAGCGGAAG	TGTATTCAAGGGCTACACCAACA	1	30394601
CER458312	CTTGGCTCAGACGATTTGTCCAAC	CCGGAGTAAGCGGCCATAACTAAA	2	6148901
CER452492	AGCAGGTCTCGCGATTTATTAGGG	GTGAGTGACTCTCAGTTTCCACATC	2	9025301
CER450199	GAGTCTCTTCCATGACAAGATTTAACG	TCGTGTCCAAACTAACCGTGAAC	2	14740601
CER448839	TCACTCCTCTTGGTTTCAGTAGTGG	ATCCCTGAACTTCACATCTGCTGC	2	16473101
CER457693	TCCGTAAAGAGAAAGCCCAAGCTC	CACATCCAGAGTCAAGTAGCCCAA	3	7442001
CER464872	GGTCAACCCAATTTCGGTCTCCTA	TCACCGTGGAAGGAGAAACATCT	3	9550701
F18P9	AACAGATACCAACTCCACAGGGACAA	TCAATGGCAGCAAAGTCCATTAAATC	3	14950000
469961	CTCGCAAGCGAAACTTGACTGTTC	GAACGAGCTTTGGAAGACGAGAGT	3	18995301
T20O10	CGATCATGCGTTTACCATACATCTAA	TCATTTACGCTGTAGCAAACGTGGTA	3	23290000

Table 3.6 (continued)

CER458426	CGAGATCGACAGCCGAGATGATTA	GAATATGATCGCAAGGGAGGAAGT	4	6017401
CER466221	TCAAACGCCAACTGAATCAATCAA	TACGTACCTGGTGTTGTGTGTGGA	4	8007901
CER450255	AGGAATGGCTTCATCTATACGCGG	TTCTCCGCCTTCACCTTCTTCTTC	4	10872001
CER449044	TCGGTAAACCCTAGACACGAAAGC	TCTCAGTGGTGTTCGTCAATGGAG	4	12849001
CER452101	TTGGATGTAACGTTGGGTGTCG	GGCAAAGATACTATGCGTTCCACA	4	15294301
T16L1	GGCAATGTATTTTACACATACTTGATT	TTTCACTACTTCCAGCACCAG	4	16200000
477141	GCATGAGGGACCAATCTGGTCTAA	GAGCCAAACCAGAACTGACAACGA	5	3184101
CER457559	ACGACTCAACAAGTCACAACG	CTTTGAGACGCATAGCCATTTCGC	5	8453301
CER451893	AAAGGCGCGTGGATTTGGATAC	GCCTTTGGACATTAATCGATGGAGC	5	9445401
469672	GTTTGGTCTCTAGCGTATGTTTGGG	CGGTTTGCCCACGATTGTTTGT	5	9936001
Second Pass Mapping Primers (Chromosome 3)				
Marker	**Forward primer**	**Reverse primer**		**Location on genome**
CER455914	TTCACGAATGGCCCTTTAAT	TGGCATCACGAGGAGATACA		7585501
ATDMC1.1	GCAACTGAATTTGTTTTCGTTTG	TTGATTAGTGGATCCGCAAACAA		8100601
CER456238	GTGGACGAACTTGTAGTACACATGAC	TCGCTATTACTTTCTTCCCACCGC		8403901
CER457822	AACCTCACCGGTTGTCTCTG	CCTTTGAACCAGGTGGAAGA		8579401
CER456071	ATGTTGGTCTCGCGGATGAGTATC	GATAATCCAATGAGCGCATGCCAG		9160901
CER464872	GGTCAACCCAATTTCGGTCTCCTA	TCACCGTGGAAGGAGAAACATCT		9550701
CIW1.1	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATTC		9775545
456227	TTAGACCCGAGTTCCCAAAG	ACAAACGAACATTGCCAACA		9829901
CER464957	GGTCGGAAAATCAAAGCTGA	TTTGCTTGGGTCAAAACTTG		115201
473863	AGGACCCACTTCACACCAAC	TGGTAACACCCTCTTTCTCCA		1583000
CER464947	TGCAAATTGTTTTTGGTTGC	TCGGTGGTAAAGGTTTAGGC		3108900
CER460222	GCCGAATGCTAAAAAGCAAG	GTGTATCGTACGCCCCACTC		5599201
CER455523	GGGAGAAGAGTCAGGCAAAA	TGATCAAAAATCACATGCAAGA		6117101
469491	TTCAACGGCGGAGATAGTTC	ATTTTTAATTGGGCCGGTTC		1780101
469977	CAAGCTCTCCAACTCGTCTTC	ATCAACGCTGGAATTTGGAC		1959201
470676	CTTTTTATGGCCCAAATCCA	CACGCTGTGTCGTTTTTCTG		2299601
470456	CGGAATCCACTGCTCAGTTT	GAAGGAGGAACGAAGAGCAA		2451701
469707	CCTGCCGCCTATATGTGACT	TGCGTAGAGATATGGCTGATG		2563301
470259	CTGCCGAGTTTCCTTGTTG	TTCCCGGAAACGTAACACTC		2851101
469729	ACGAAATCCGCCTCTTCTTC	TTTTGGCTTTAATTTGCTCCA		2223301
470461	AAAAATTCCGCAAAAAGAAACA	TACTCACGACGCGCAGTAAC		2492001

Table 3.7. Other PCR primers used in this study.

gADT2 F	ACCTTTTCCATTCTAATTCCTATTCCATTA
gADT2 R	GCTTATTGCACAAGAAGCGATTATTG
ADT2 f446	CACAAATTATGGGCCTTAGGATTGG
ADT2 r1294	GAACAAAGCAAGACTAATGGCAACCA
ADT2 Q-PCR 2 F	GAAGATCTTTGAAGACTCGCCTC
ADT2 Q-PCR 2 R	TGATTCGCTGTATGCACCTC
TIP4 1-like F	GCGATTTTGGCTGAGAGTTGAT
TIP4 1-like R	GGATACCCTTTCGCAGATAGAGAC
CYP79B2 Q-PCR F	CATTAAGGAGCTTGTAATGGCGGC
CYP79B2 Q-PCR R	TCCCGACGACTCTGTCGATCT
CYP79B3 Q-PCR F	CCATTAAGGAACTTGTAATGGCGGC
CYP79B3 Q-PCR R	GCTTTGTGGAGGATCTCCGGT
MYB34 Q-PCR F	AAGGGTAACAAGTGGGCCGC
MYB34 Q-PCR R	TGCCTTTTTGCTTCAACCGCT
ASA1 Q-PCR F	TCAAAGGCCCCTGAGGATGA
ASA1 Q-PCR R	TGCCTTTTCGTAAGGAAGGCTCC
UBQ10 Q-PCR F	TTGGAGGATGGCAGAACTCTTGCT
UBQ10 Q-PCR R	AGTTTTCCCAGTCAACGTCTTAACGAAA
PR-1 Semi-Q RT PCR F	ACTGGCTATTCTCGATTTTTAATCGTC
PR-2 Semi-Q RT PCR R	CGTTCACATAATTCCCACGAGGA
TIP41-like Semi-Q RT-PCR F	GAAATTCAGGAGCAAGCCGT
TIP41-like Semi-Q RT-PCR R	GGAAGCCTCTGACTGATGGA

To transform the *adt2-1D* gene into *A. thaliana*, the entire gene, including the promoter and the 5' and 3' untranslated regions, was amplified from genomic DNA of the *adt2-1D* mutant using the primers gADT2F and gADT2R (see Table 3.7) and cloned into the pCR8/GW-TOPO vector (Invitrogen) and subsequently into pMDC123 (Curtis and Grossniklaus, 2003). The rest of the procedure was as described previously (Joshi *et al.*, 2006).

Metabolite Analysis

AccQ Tag (Waters, http://www.waters.com/) amino acid analysis followed a method described previously (Joshi *et al.*, 2006), using 10-50 mg plant tissue. For PITC (phenyl isothiocyanate) derivatization and amino acid measurement, amino acids were extracted as above and 100µL of the extract was dried using a Savant Speedvac (Thermo scientific, www.thermo.com). Ten µL of TEA (triethylamine) buffer (1:1:1 of MeOH:H_2O:TEA) was added and each sample was dried again. Twenty µL PITC buffer (7:1:1:1 of MeOH:H_2O:TEA:PITC) was added and dried. Ten µL of another TEA buffer (2:1:1 of MeOH:H_2O:TEA) was added and dried. Finally, 150 µL of a 1:1 HPLC solvent A:solvent B mixture was added to dissolve the sample. Solvent A was 140 mM sodium acetate, with 0.05% TEA and final pH adjusted to 6.33 with acetic acid. Solvent B was 60% acetonitrile. Thirty µL of sample was injected into a Waters Symmetry C18 5 µm 2.1x150mm column. The gradient used was as follows: 0 to 10 min, 99% A; 10 to 35 min, linear gradient to 65% A; 35 to 40 min, 65% A; 40 to 42 min, linear gradient to 100% B; 42 to 44 min, 100% B.

GC-MS analysis was carried out as described previously (Lisec *et al.*, 2006). Data were analyzed using TagFinder (Luedemann *et al.*, 2008) by comparison to libraries of chemical standards (Kopka *et al.*, 2005). LC-MS profiling was carried out as described (Tohge and Fernie, 2010). Glucosinolate assays were carried out as described previously (De Vos and Jander, 2009).

Heterologous Expression and Enzyme Assays

Cloning and expression of transgenes was similar to a previeous study (Cho *et al.*, 2007), with minor modifications. A construct with wildtype ADT2 in the pET43.1 vector was provided by N. Lewis (Cho *et al.*, 2007). *E. coli* cells expressing the target

protein were disrupted by sonication and the target protein was purified by NTA-agarose (Qiagen, http://www.qiagen.com/) according to the manufacturer's instructions.

Arogenate, the substrate for the enzymatic reaction, was purified from a *Neurospora crassa* mutant (ATCC 36373) as described previously (Yamada *et al.*, 2008; Zamir *et al.*, 1980), without the last step of HPLC purification. Arogenate was quantified by its conversion to phenylalanine in the presence of HCl. For enzyme assays, each reaction consisted of 50 µL KH_2PO_4-K_2HPO_4 pH 7.0 buffer, 150 µL partially purified arogenate, norleucine as internal standard, and 50 µL protein. The reaction was kept at 37^{o}C for the desired amount of time and frozen immediately. For phenylalanine feedback inhibition assays, 100 µM of phenylalanine was added before the reaction. The reaction product was analyzed by HPLC after AccQ-Tag derivatization. Data analysis was conducted using SAS software (www.sas.com).

RNA Extraction, Quantitative RT-PCR and Semi Quantitative PCR Analysis

RNA was extracted using the SV Total RNA Isolation System™ (Promega, http://www.promega.com/) and digested with Turbo DNase™ (Ambion, http://www.ambion.com/) according to the manufacturer's instructions. PCR reactions with primers ADT2 f442 and ADT2 r1294, designed to span intron sequences of *ADT2,* were used to check for genomic DNA contamination. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT20 primers, 10 mM dNTPs, and Clontech SMART™ MMLV Reverse Transcriptase (Clontech, http://www.clontech.com/) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-RT-PCR using primers that amplify the constitutively expressed genes *UBI10* or *TIP41-like* (At4g05320 and At4g34270).

Gene-specific primers were designed using Primer3 v. 0.4.0. (Rozen and Skaletsky, 2000). Q-RT-PCR analysis was done in optical 384 well clear optical reaction plates and optical adhesive covers (Applied Biosystems, http://www.appliedbiosystems.com/) with an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems), using SYBR Green to monitor double-stranded DNA synthesis. Each reaction contained 0.75 μL of cDNA solution, 0.5 μL of each of the two gene-specific primers (10 pmol μL^{-1}), and 5 μL of 2× SYBR Green PCR mix reagent (Applied Biosystems) in a final volume of 10 μL. The following PCR program was used for all PCR reactions: 95 ºC for 2 min, followed by 45 cycles of 95ºC for 15 s and 60 ºC for 60 s. When primers were used for the first time, the program was followed by a dissociation curve to determine if only one product was formed. Threshold cycle (Ct) values were calculated using Applied Biosystems Software (SDS version 2.3, for Windows XP). Subsequently, Ct values were normalized for differences in dsDNA synthesis using the *UBI10* or *TIP41-like* Ct values. Normalized transcript levels of all genes were compared with those of the mock-treated controls and the fold change in expression level was calculated based on the ΔΔCt.

Semi-quantitative PCR was conducted using a C1000 Thermal Cycler (Bio-rad, http://www.bio-rad.com/) with the following program: 95 ºC for 2min, followed by 30 ºC (for control *TIP41-like* primers) or 32 cycles (for *PR1* primers) of 95 ºC for 60 s, 51 ºC for 30 s and 72 ºC for 50 s. Cycle numbers were determined after optimization.

Sequences of all primers are provided in Table 3.7.

Insect Bioassays

T. ni and *P. xylostella* eggs were obtained from Benzon Research (http://www.benzonresearch.com/). *P. rapae* were from a colony maintained by the

Jander lab, which is descended from insects collected in the wild on the Cornell University campus in July, 2008. *P. rapae* larvae were confined on the leaves of 3-week-old plants with mesh-covered cups and allowed to feed on plants for 7 days (*P. rapae*) or 9 days (*T. ni*) before being harvested and lyophilized (Labconco, http://www.labconco.com/). Larval dry weight was determined using a precision balance (Sartorius, http://www.sartorius.com/).

For insect choice experiments, leaves were harvested from similarly-sized 2-week-old plants growing together in the same tray. Mutant and wildtype leaves were placed about 5 mm apart on moist paper towels in 55 mm diameter Petri plates. Single 2-day-old *T. ni* or 4-day-old *P. xylostella* larvae raised on artificial diet (Southland Products, www.tecinfo.com/~southland/) prior to being placed lengthwise between each pair of leaves. After 48 h, the remaining leaf material was scanned and the area eaten was calculated with ImageJ (available at http://rsb.info.nih.gov/ij).

For whole plant oviposition assays, 60 adult *P. xylostella* (~50% females) were allowed to oviposit on 32 paired mutant and wildtype plants, in complete darkness in a 30 × 60 cm nursery flat under a dome cover. After 24 h *P. xylostella* eggs were counted.

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CHAPTER FOUR:

INVESTIGATION OF *M*-TYROSINE TOXICITY TO PLANTS[*]

Introduction

Allelopathy refers to the growth interference among neighboring plants mediated by chemical compounds. In most cases, the functional chemicals, named allelochemicals, are secreted from roots or leached from decomposing leaves into the rhizosphere. In a recent study evaluating the allelopathy potential of fine leaf fescues, *m*-tyrosine, a structural isomer of the common *p*-tyrosine, was discovered to be present in a massive amount in the roots of Chewings fescue *Festuca rubra* L. ssp *commutata*, and can act as a broad spectrum phytotoxin in very low concentrations (Bertin 2005). This, together with the observation that Chewings fescue has great weed suppressive ability, indicated that Chewings fescue might have allelopathy potential (Belz and Hurle 2004). *A. thaliana*, the model plant species, is also among the plants that show great sensitivity to synthetic *m*-tyrosine in laboratory assays. The hypothesis was then proposed that, by exuding *m*-tyrosine from their roots, certain species of fine leaf fescues are able to inhibit the growth of neighboring plants. However, the physiological aspects of this allelopathic interaction are not clear.

Research on the physiological mode of action is not only an essential part of allelopathy, but can also provide new ideas for herbicide design (Macias *et al.* 2007). Hypotheses were often tested based on the structure of allelochemicals and their toxic effects at the cellular and biochemical level. Although *A. thaliana* has been the leading subject for all kinds of plant research in the past decade, it is just beginning to join the cast of allelopathy research. A recent study has taken advantage of the *A. thaliana*

[*] Part of this chapter is modified from an article with permission from *PNAS,* published in October 2007, DOI: 10.1073/pnas.0707198104

genomics resources by using microarray experiments to examine the gene expression profile upon treatment by a potential allelochemical catechin (Bais *et al.* 2003). In the present study, we investigate the mode of actions of *m*-tyrosine toxicity using *A. thaliana* as the model species.

Results and discussion

Toxicity of *m*-tyrosine to *A. thaliana* can be alleviated by supplementation of other amino acids

Given the chemical structure of *m*-tyrosine, it seemed possible that this compound would interfere with plant amino acid metabolism. Indeed, using agar plate growth test, the toxicity of 3 μM DL-*m*-tyrosine for *A. thaliana* root growth was counteracted to some extent by the addition of 14 of the 20 protein amino acids at 40 μM concentrations (Figure 4.1). Addition of charged amino acids caused little or no improvement in root growth, which may indicate that aromatic and neutral amino acids compete with *m*-tyrosine for uptake or transport within the roots. In control experiments, the protein amino acids by themselves did not significantly improve root growth at these concentrations.

This broad range of rescue by amino acids could be results of competition for root uptake via various amino acid transporters located in *A. thaliana* roots. Amino acid permease 1 (AAP1, At1g58360) is such a transporter whose substrates are similar to the pattern of amino acids that rescue the toxicity of *m*-tyrosine (Fischer *et al.* 1995; Lee *et al.* 2007). However, our results showed that two putative *A. thaliana* Columbia *aap1* mutants (SAIL_95_B01 and SAIL_508_H11) did not show elevated resistance to *m*-tyrosine, although they are resistant to 6 mM of phenylalanine, a typical phenotype of *aap1* mutants. Lysine histidine transporter 1 (LHT1, At5g40780) is another high-affinity transporter for cellular amino acid uptake in the root epidermis

(Chen and Bush 1997; Hirner *et al.* 2006; Svennerstam *et al.* 2007). We obtained two T-DNA insertion lines SALK_026389 and SALK_034566 with predicted T-DNA insertions in the genomic region of AT5g40780. Although both of these lines were resistant to D-alanine, a characteristic of loss of function of LHT1, neither of them displayed enhanced tolerance to *m*-tyrosine. The *A. thaliana* genome encodes a large number of predicted and proven amino acid transporters, and it is possible that other such proteins play redundant roles in the uptake or within-plant movement of *m*-tyrosine.

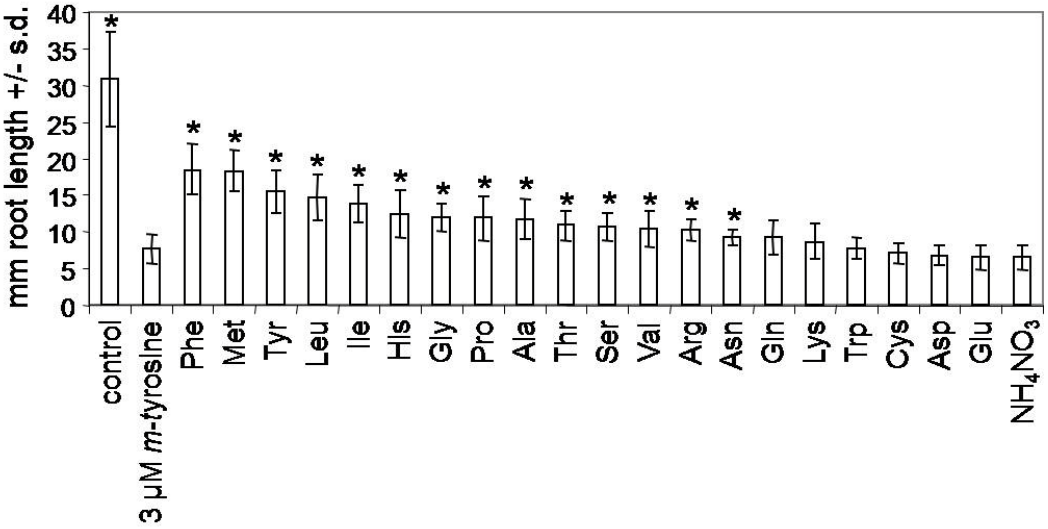

Figure 4.1. Rescue of 3 µM *m*-tyrosine toxicity by 40 uM or individual amino acids or NH_4NO_3.
Root length after one week of growth on MS agar with 3 µM m-tyrosine and 40 µM of other amino acids. Mean +/- s.d. of N = 16 to 23. *P < 0.01, t-test, relative to 3 µM *m*-tyrosine only treatment.

Auxin does not play a significant role in *m*-tyrosine toxicity

Our observations that low concentrations of *m*-tyrosine inhibit the primary root and promote lateral root elongation in *A. thaliana* and some lettuce isolates (data not shown) suggested that interference with plant growth hormones, in particular auxin (indole-3-acetic acid), could be a mechanism of *m*-tyrosine toxicity. Generally, high

concentrations of auxin inhibit root growth, whereas very low concentrations stimulate root development (Taiz and Zeiger 2002). However, when tested, expression of an auxin-responsive *DR5:GUS* fusion (Ulmasov *et al.* 1997) was unaffected by *m*-tyrosine treatment (Figure 4.2). Furthermore, six *A. thaliana* auxin-response mutants including *ilr1-1*, *aux1-7*, *tir1-1*, *axr1-3*, *axr2-1*, and *axr3-1* (Leyser 2002) did not show altered sensitivity to *m*-tyrosine. Lastly, NPA and TIBA, two auxin transport inhibitors (Estelle 2001), failed to rescue *m*-tyrosine. Taken together, these results suggest that *m*-tyrosine does not interfere directly with auxin metabolism or activity.

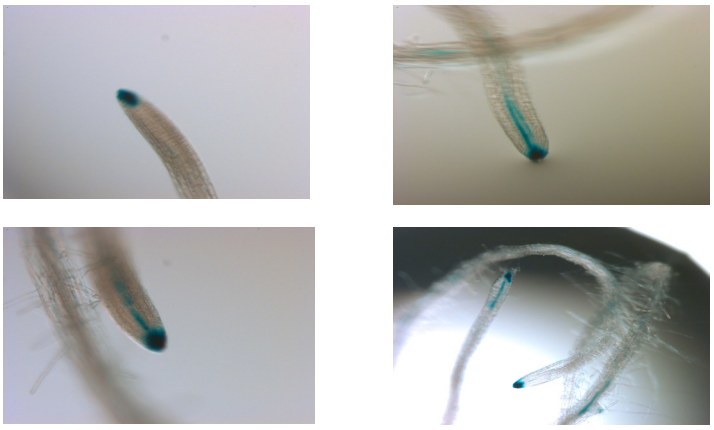

Figure 4.2. *DR5:GUS* expression is not changed by *m*-tyrosine.
Five day old seedlings of *Arabidopsis DR5:GUS* transgenic plants grown with 10 µM *m*-tyrsoine (right) or without *m*-tysoine (left) were stained to show GUS activity.

m-Tyrosine can be incorporated into proteins of *A. thaliana*

m-Tyrosine is incorporated into proteins in place of phenylalanine in bacteria (Aronson and Wermus 1965) and mammalian cells (Gurer-Orhan *et al.* 2006), where it is associated with increased protein turnover (Rodgers *et al.* 2002). *Vigna radiata* (mung bean) phenylalanine t-RNA synthase accepts *m*-tyrosine with 25% of the efficiency of phenylalanine (Smith and Fowden 1968), suggesting that *m*-tyrosine

might also be miss-incorporated into plant proteins. Two days after transfer of *A. thaliana* seedlings to agar plates with 10 µM *m*-tyrosine, the roots contained low but measurable amounts of incorporated *m*-tyrosine, representing less than 1% of the total phenylalanine in the protein fraction (Figure 4.3) (0.88% +/- 0.006%; mean +/- s.d. of N = 3). Protein samples were filtered so that no *m*-tyrosine could be detected before acid hydrolysis. This misincorporation of *m*-tyrosine into plant proteins could cause structural disruptions, or might interfere with what are normally *p*-tyrosine-specific functions such as the formation of tyrosine cross-links in cell walls (Held *et al.* 2004) or regulation of protein function by tyrosine phosphorylation (Luan 2002). However, since significant growth inhibition is observed at more than 100-fold lower concentrations in *A. thaliana* than in *Bacillus subtilis* (Aronson and Wermus 1965) or Chinese hamster ovary cells (Gurer-Orhan *et al*., 2006), plant proteins would have to be uniquely sensitive to *m*-tyrosine incorporation.

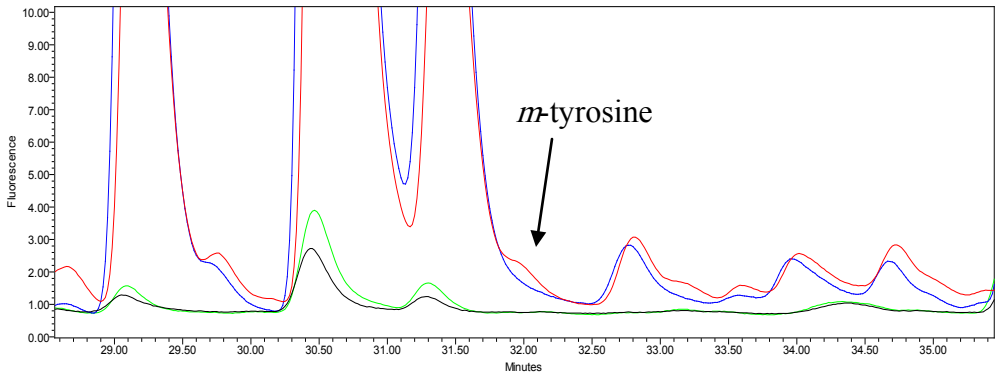

Figure 4.3. Incorporation of *m*-tyrosine into *A. thaliana* root proteins. Amino acid analysis after protein hydrolysis showed incorporation of *m*-tyrosine in treated sample (red), but not in untreated sample (blue) or sample before hydrolysis (green and black)

Test of A. thaliana ADT2 sensitivity to *m*-tyrosine in a yeast model

The identification of adt2-1D mutation in an *A. thaliana m*-tyrosine resistant mutant suggested that ADT2 enzyme might be a critical target site for *m*-tyrosine (Chapter 2, Huang et al). Although *in vitro* enzyme assay showed that *m*-tyrosine does not inhibit the enzymatic activity, *in vivo* tests would help to confirm this conclusion. In the search for a suitable *in vivo* system, yeast seems to be an ideal choice due to its small genome and resourceful collection of mutants in biochemical pathways. In yeast, the biosynthesis of phenylalanine depends on PHA2, a bifunctional enzyme of chorismate mutase / prephenate dehydratase (Ma *et al.* 2007). A yeast *PHA2* knockout mutant is a phenylalanine auxotroph. Since the chorismate mutase enzyme function is redundant in yeast (Ball *et al.* 1986), we hypothesize that if inhibition of ADT2 is the only mode of action of *m*-tyrosine toxicity, transformation of *A. thaliana ADT2* gene into yeast *PHA2* knock-out mutant would complement its function for phenylalanine biosynthesis but also make it sensitive to *m*-tyrosine, whereas complementation with *adt2-1D* gene will produce *m*-tyrosine resistant yeast strain. However, our results indicated that although *ADT2* gene indeed restored the biosynthetic pathway in yeast, it does not make the transgenic yeast strain sensitive to *m*-tyrosine (Figure 4.4).

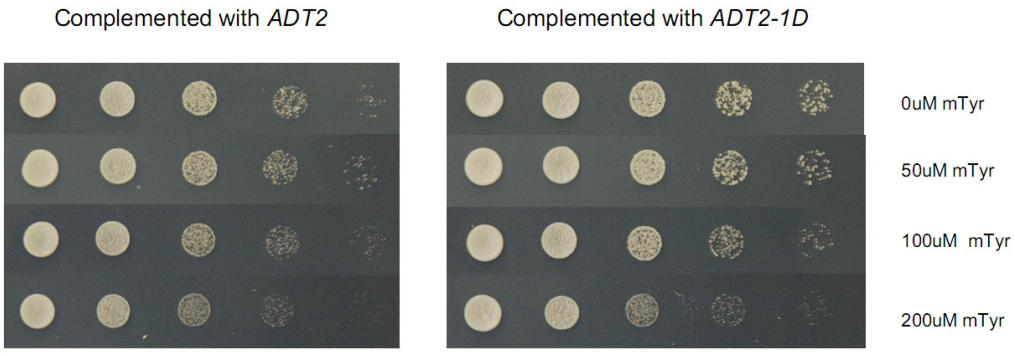

Figure 4.4. *ADT2* and *adt2-1D* rescue yeast *PHA2* phenylalanine auxotroph without changing sensitivity to *m*-tysosine.
Yeast *PHA2* mutants were transformed with *ADT2* or *ADT2-1D* and dilution series of yeast culture were plated on phenylalanine-free synthetic defined media with various concentration of *m*-tysoine.

Self-resistance to *m*-tyrosine in Fescue does not depend on the mutation found in the *A. thaniana adt2-1D* mutant

The highly toxic effect of *m*-tyrosine on plant growth raises an interesting question: how do the *m*-tyrosine producing fescues avoid self-toxicity? One possible mechanism suggested by cloning of *adt2-1D* mutation is that Chewings fescue also posses such a mutation in the same enzyme, thereby providing resistance to *m*-tyrosine by similar mechanism as in *A. thaliana*. The adt2-1D protein has an S to A mutation at the ESPR motif of the ACT domain, a regulatory domain conserved in several enzymes involved in amino acid metabolism (Chipman and Shaanan 2001). In another recent study of rice *mtr* mutant, the protein normally encoded by Os07g0694600, an arogenate dehydratase, was also found to contain an S to I mutation at the same ESRP motif of the ACT domain (Yamada *et al.* 2008). To clone the ACT domain of *ADT2* in Chewings fescue, degenerate primers were designed based on homologs of *ADT2* sequences identified from several grass species. Two rounds of PCR using Chewings fescue cDNA as template produced a single band which was sequenced. The translated peptide sequence was aligned by ClustalW and showed very high similarity to rice Os7g0694600 gene and is in the same cluster with *ADT2* when compared to all 6 genes in *A. thaliana ADT* gene family. This sequence was then designated as the partial cDNA sequence of the *A. thaliana ADT2* homolog in Chewings fescue (Figure 4.5). Interestingly, the ACT domain of fescue ADT2 share the conserved ESRP motif as wild type *A. thaliana* ADT2, indicating that the resistance to *m-tyrosine* in Chewings fescue is caused by a different mechanism than in the *adt2-1D* mutant of *A. thaliana* (Figure 4.5). For instance, Chewings fescue might have different resistant ADT enzymes or the phenylalanyl-tRNA synthetase from Chewings fescue could better discriminate *m*-tyrosine from phenylalanine. Alternatively, *m*-tyrosine could be sequestered in distinct cellular compartments so that it does not interfere with the

normal metabolism of Chewings fescue.

a

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Fescue_ADT        AGAAKPVAEQNLQDIGAVASSLAAQLYGLDILAENIQDDTDNVTRFMMLAREPIIARTDK 60
Os07g0694600      AGAAKLIAEQKLQDTGAVASSLAAQLYGLDILAENIQDDTDNVTRFMMLAREPIIPRTDK 60
Os03g0286200      AGAAKTVAEQNLQDTGAIASSLAAELYGLNVLAENIQDDKDNVTRFMMLAREPIIPRTDK 60
ADT2              AGAAKQIAFENLNDAAAVASEKAAKIYGLNIVAKDIQDDCDNVTRFLMLAREPIIPGTNR 60
ADT1              ATAAQTVSSSGKIDVGAIASVRAANIYGLDILAENIQDDVNNVTRFLILAREPMIPRTDR 60
ADT3              AGAAEFIAANNIRDTAAIASARAAEIYGLEILEDGIQDDASNVTRFVMLAREPIIPRTDR 60
ADT6              AGAAEFIASNNLRDTAAIASARAAEIYGLEILEDGIQDDVSNVTRFVMLAREPIIPRTDR 60
ADT4              AAAAEYISANDLHDTAAVASARAAELYNLQILADGIQDDPGNVTRFLMLAREPIIPRTDR 60
ADT5              AAAAEYIAANNLHDTAAVASARAAELYGLQILADGIQDDAGNVTRFLMLARDPIIPRTDR 60
                  * **: :: .   * .*:**  **::*.*::: ..**** .*****::***:*:*. *::

Fescue_ADT        PFKTSIVFSLE--EGPGQLFKALAVFALRKINLTKMESRPHKKRPLRVADDNCSTPLKHF 118
Os07g0694600      PFKTSIVFSLE--EGPGQLFKALAVFALRKINLTKMESRPHKKKPLRIADDNCSAPLKHF 118
Os03g0286200      PFKTSIVFSLE--EGPGQLFKALGVFALREINLTKIESRPHKKRPLRITDDSFSTPSKQF 118
ADT2              LFKTSIVFSLE--EGPGVLFKALAVFALRQINLTKIESRPLRKHPLRASGG-----LKYF 113
ADT1              PYKTSIVFSLE--EGPGVLFKALAVFALRSINLSKIESRPQRRRPLRVVDGSNNGSAKYF 118
ADT3              PFKTSIVFAH--EKGTCVLFKVLSAFAFRNISLTKIESRPNHNVPIRLVDEANVGTAKHF 118
ADT6              PFKTSIVFAH--EKGTSVLFKVLSAFAFRDISLTKIESRPNHNRPIRVVDDANVGTAKHF 118
ADT4              PFKTSIVFAAQEHKGTSVLFKVLSAFAFRDISLTKIESRPHHNRPLRVVGDGSFGTSKNF 120
ADT5              PFKTSIVFAAQEHKGTSVLFKVLSAFAFRNISLTKIESRPHQNCPVRVVGDENVGTSKHF 120
                   :******:    :*.  ***.*..**:*.*.*:*.**** :. *:*  .       * *

Fescue_ADT        LYLF 122
Os07g0694600      DYLF 122
Os03g0286200      DYLF 122
ADT2              DYLF 117
ADT1              DYLF 122
ADT3              EYMF 122
ADT6              EYMF 122
ADT4              EYMF 124
ADT5              EYTF 124
                   * *
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b

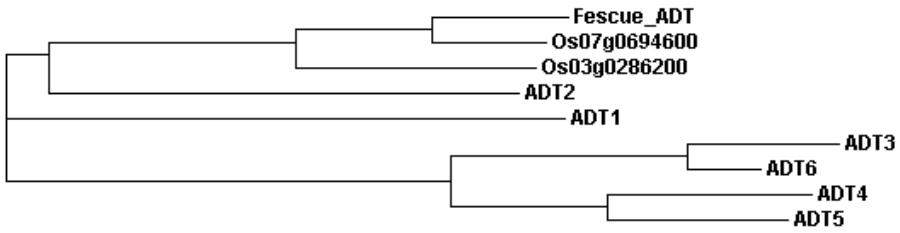

Figure 4.5. Sequence analysis of ACT domain in putative fescue ADT2 protein.
(a) Peptide sequence containing the ACT domain of a putative fescue ADD2 protein is aligned with all six genes in *Arabidopsis* ADT family and two proteins from rice that show high similarity to *Arabidopsis* ADT2. Conserved ESPR motif is boxed.
(b) Phylogenetic tree of sequenced analyzed showed the fescue ADT protein being a close homolog of *Arabidopsis* ADT2 and two genes in rice, Os07g0694600 and Os03g0286200.

Concluding remarks

In this chapter, several hypotheses were tested for the modes of action of *m*-tyrosine toxicity. Evidence of *m*-tyrosine incorporation into *A. thaliana* proteins was shown and could account for the inhibition of plant growth. Other results suggest that *m*-tyrosine shares the same transporters with other common amino acids when being absorbed by roots of *A. thaliana.* Interference with auxin, an important plant hormone, might have little or nothing to do with the *m*-tyrosine mode of action. Generation of a yeast model proved that the *A. thaliana ADT2* gene functionally supplements the prephenate dehydratase activity encoded by yeast *PHA2* gene and suggests that ADT2 might not be the direct target of *m*-tyrosine. Cloning of the ACT regulatory domain from the ADT2 homolog of Chewings fescue showed a conserved ESPR domain, the same as *A. thaliana* wildtype *ADT2* gene, indicating that Chewings fescue avoids the toxicity of *m*-tyrosine in a different manner than the *A. thaliana adt2-1D* mutant.

Experimental procedures

Plants and materials

Seeds of *A. thaliana* were purchase from ABRC (Arabidopsis Biological Resource Center, www.arabidopsis.org). The following seeds stocks were used for auxin mutant tests: CS8099 (*ilr1-1*), CS3074 (*aux1-7*), CS3798 (*tir1-1*), CS3075 (*axr1-3*), CS3077 (*axr2-1*), CS57504 (*axr3-1*). Yeast *PHA2* strain (ATCC number: 4006272) was purchased from ATCC (American Type Culture Collection, www.atcc.org). Chemicals were purchased from Sigma-Aldrich (www.sigmaaldrich.com) if not otherwise specified.

Agar plate bioassays with *A. thaliana*

To assess effects of *m*-tyrosine and other compounds on *A. thaliana* root growth,

formulated solutions of each were added to ½-strength Murashige and Skoog medium (½x MS) (31), 1% Phytagar (Invitrogen, Carlsbad, California) and 1% sucrose in Petri dishes. *A. thaliana* seeds were sterilized by shaking in 30% bleach, 0.3% TritonX-100 for 10 min, followed by three rinses with sterile distilled water. Petri dishes with seeds on agar medium were cold-stratified for 24 h at 4 °C, and were subsequently placed vertically in Conviron (Winnipeg, Canada) growth chambers at 23 °C, 180 µmols $m^{-2}s^{-1}$ photosynthetic photon flux density, and a 16:8 h light-dark cycle. After five days of growth, the root lengths of 10 seedlings per plate were measured. Experiments were repeated three times, and each replicate consisted of three agar plates. Using this assay, rescue of 3 µM *m*-tyrosine toxicity was assessed by adding the 20 protein amino acids individually at 40 µM concentration to the assay.

GUS staining

GUS staining was performed according to the protocol described by (Weigel and Glazebrook 2002).

Measurement of protein-incorporated *m*-tyrosine

A. thaliana land race Col-0 seeds were sterilized and sown on ½x MS agar plates that were placed vertically in the growth chamber. After eight days, seedlings were transferred to new plates with control agar or agar containing 10 µM *m*-tyrosine. Two days after transfer, plant roots were harvested into 1.5 mL tubes (~ 60 mg for each sample) and immediately frozen with liquid nitrogen. One 3-mm steel ball was placed into each tube, and plant tissue was crushed by shaking on a Harbil 5G-HD paint shaker (Fluid Management, Wheeling, Illinois). Five hundred µl extraction buffer (1 x phosphate buffered saline, pH 7.4, with 2 mM phenylmethanesulfonylfluoride) were

then added to the crushed sample and mixed using the same shaker. Samples were centrifuged for 10 min at 14,000 rpm at 4 °C. Supernatant was transferred to Millipore YM-10 spin columns (Millipore, Billerica, Massachusetts), centrifuged at 14000 rpm for 30 min at room temperature, washed twice with 500 µl extraction buffer, and centrifuged at 14,000 rpm. Samples were adjusted to a final volume of 100 µL using extraction buffer. Free amino acid profiles of these samples were analyzed using the AccQ tag HPLC detection system (Waters, Milford, Massachusetts) to confirm that they did not contain free *m*-tyrosine. Eighty µl of each sample were adjusted to a final volume of 400 µl with 1% phenol and a final HCl concentration of 6N. Samples were then transferred to Kontes valved NMR tubes (Kontes, Vineland, New Jersey) and the tubes were flushed with argon gas. Sealed tubes were then incubated using a 110 °C oil bath for 24 h. After hydrolysis, samples were dried by evaporation, re-dissolved in 20 mM HCl and analyzed using the AccQ tag HPLC detection system as described in (Joshi *et al.* 2006), except that a column temperature of 30° C were used to improve separation of the *m*-tyrosine derivative from the methionine derivative.

Complementation of yeast *PHA2* with A. thaliana *ADT2* and *adt2-1D* genes

Coding sequences without transit peptides of *A. thaliana ADT2* and *adt2-1D* genes were amplified using forward primer 5'-CCGGAATTCATGGAAGTGAAGAAGA TCT-3' and reverse primer 5'-CGCGGATCCTTAGAGCATTGTAGTGTC-3' and cloned into pWV3 vector using *BamHI* and *EcoRI* restriction sites. After sequence confirmation, both clones were transformed into the yeast *PHA2* mutant using the lithium acetate method (Gietz and Schiestl 1991). Transformed yeast colonies were inoculated into yeast SD (synthetic defined) media with 0.67 g/L yeast nitrogen base, 20 mg/L L-adenine hemisulfate, 20 mg/L L-methionine, 20 mg/L L-histidine HCl monohydrate, 100 mg/L L-leucine, and 20 mg/L L-uracil. After shaking at 29 °C for

two days, the OD values of the culture were measured, and all cultures were diluted to the same density. This adjusted stock solution was then again diluted to 1:10, 1:100, 1:1000 and 1:10000. Three μL of each dilution was dropped onto yeast SD agar plates with various concentrations of *m*-tyrosine and incubated at 29 °C before pictures were taken.

Cloning of the cDNA sequence encoding the ACT domain of Chewings fescue *ADT2* enzyme

cDNA of Chewings fescue was prepared as described in Chapter 2 of this thesis. For the first round PCR, forward degenerate primer AGASARCCTGTYGAYGAYAC and reverse degenerate primer CTYCGYAGWTACCGACTAGG were used. PCR was conducted at 94°C for 30 s, 47°C for 90 s, and 72°C for 60 s, for a total of 35 cycles. The PCR product was cleaned and used for nested PCR with forward degenerate primer 5'-GCDGGTGCAGCMAAGHHWRTHGC-3' and reverse primer 5'-TARAAVAGGTA RAGRAAVTDCTT-3'. The PCR program is the same, except that the annealing temperature is 52°C. The PCR product was then sequenced. Sequence alignment was performed by ClustalW program (www.ebi.ac.uk/clustalw). Rice and *A. thaliana* sequences used for comparison were downloaded from Genbank (www.ncbi.nlm.nih.gov/genbank/).

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SUMMARY AND FUTURE DIRECTIONS

The research presented in this dissertation shows a new pathway for *m*-tyrosine biosynthesis in Chewings fescue (*Festuca rubra* L. ssp *commutate*), where *m*-tyrosine is directly derived from phenylalanine. Genomic resources were also generated, providing several candidate genes for functional tests. These genes will be expressed in yeast together with *A. thaliana ATR1* (*Arabidopsis P450 reductase 1*) gene to verify their function. Identification of an *m*-tyrosine biosynthesis gene would provide information needed to manipulate the production of *m*-tyrosine in Chewings fescue. Genetically engineered Chewings fescue could then be used to verify the potential function of *m*-tyrosine, especially as an allelochemical. Understanding the function and biosynthesis of *m*-tyrosine would open the door to improving plant performance using this nonprotein amino acid.

The *A. thaliana m*-tyrosine resistant mutant *adt2-1D* and transgenic *padt2-1D* plants have many interesting phenotypes that merit further investigation. For example, the level of free phenylalanine seems to activate the metabolic flux to tryptophan and the overall shikimate pathway, which suggests a novel regulatory mechanism for aromatic amino acid biosynthesis. The detection of 2-phenylethyl glucosinolate (2PE) in mature *padt2-1D* makes this transgenic line an excellent plant material to study the enzymes involved in 2PE biosynthesis. The improved root growth of *adt2-1D* compared to wildtype Col-0 under salt stress conditions suggests involvement of free amino acids in abiotic stress tolerance. However, the exact mechanism will need further investigation.

The exact mode of action of *m*-tyrosine toxicity is still not clear. *m*-Tyrosine could have multiple target sites and likely interferes with metabolism and functions of phenylalanine, which explains why an *A. thaliana* mutant overproducing

phenylalanine is resistant to *m*-tyrosine. Misincorporation of *m*-tyrosine into proteins could be one of such reasons that disrupts normal protein functions and inhibits plant growth. This misincorporation could be further verified by *in vitro* translation assays. How Chewings fescue avoids self-toxicity is another interesting question that needs further investigation.

Finally, the identification of *m*-tyrosine resistant *A. thaliana*, as well as potentially genetically engineered Chewings fescue that does not produce *m*-tyrosine, would provide many opportunities for testing the significance of *m*-tyrosine in Chewings fescue allelopathy. This would contribute to the overall understanding of allelopathy, a research field that is still full of uncertainty.

APPENDIX

For each gene, 4 different sequences were presented. The differences may due to PCR errors produced in cloning and sequencing.

Cloned coding sequences of CYP450 candidates:

>Seq1 [organism=Festuca rubra] Chewing's fescue root CYP81A20 mRNA, CDS A
ATGGATAATTTTTACATTGCCATCCTTTCATTCGCTTTCATCTTCCTGCTCCACCACCTCCTG
GGCAGGAAGCCAAACAAGGCGCCTCTGCCGAGCCCTCCGGCCATCCCATTCGCCGGCCAT
ATCCATCTCGTGAAGAAGCCATTCCACGCCGTGCTATCCCGCCTCGCAGAGCGCCATGGGC
CCCTGTTCTCGTTGCGGCTCGGCACCTTCAATGCTGTGGTGGTGTCCTCACCGGCATTAGCG
AAGGAGTGCTTCACGGAAATGGACGTGACCTTCGCCAACCGCCCAAGGCTCCCCTCGTGG
AAATTCCTGTCCGAGGACTACTCAATGATCGCCACGAGCAGCTATAACCCGCACTGGCGA
AACCTCCGCCGCGTCGCGGCCGTGCAGCTCCTCTCGACGCACCGTGTCAGCTGCATGTCAG
GCGTCATCTCCGGTGAGGTGCACGCCATGGTGCGGCGGTTGAATCGATCGGCCACGGAGT
CCCCGCGCATCCGGCTGAAGCAGAGGCTGTTCGAGCTCTCCCTCAGCGTACTCATGGAGAC
CATCGCAAACACCAAGGCAACTCGTTCAGAAGCCACCGCCGCCGACACGGACATGTCGGT
CGAGGCCCAGGAATTCAGGACGGTGATGGACAATATCAACCCACTTGTCGGCCTGGCCAA
CCTGTGGGAGCTATTGCCAATTGTTCGCTGGTTCGACGTGTCCGGCATCAAGAACAAGCTC
CTGGCTTCGGTGACTAGAAGGGATGCGTTCCTGCAGAGGCTCATCGACGCCGAGCGGCGG
AAGGTAGAGGACGGCGGCAGTGAAGACGGCAAGAAGAGTATGATTTCCGTCATGTTCGGT
CTGCAGAAGAAAGAGCCAAATGTCTATACCGACAAAATGATCAGGGGTCTGATCACGAGT
TTATTTAGTGCTGGAACAGATACCATCTTGGCTACGACAGAATGGGCTATGTCGCTCCTAC
TAAACCATCCAACGGCCCTAAAAAAGGCACATGCACAGATCGACCAAGTTGTTGGGACCT
CCCGCCTAGTTTCCTCTGAGGACCTTTCCCGCCTCACTTACCTCCAGTGTATCATTAGTGAG
ACCCTCCGTCTTTACCCTGCTGCACCGCTTCTGCTGCCGCGTCAGACTTACGTCGACTGTAA
GATTGGTGGCCACACAATTCCGAGTGGGACCATGCTGATCTGTAATGCGTACGCCATCCAT
AGGGACCCAAACGTGTGGGAGGATCCCCTGGAGTTCAAGCCGGATCGGTTTGAGGATGGC
AAGGCGGAAGGGTTATTCATGATACCATTTGGAATGGGGAGGAGGAAGTGTCCGGGAGAG
GCAATGGCTTTACGGACAATGGGACTTGTTCTTGGGGCACTTATACAATGCTTCGAATGGG
ACCGGGTAGACGATGCGAAGGTGGACATGAAGGAATATGGAGAAGAATTTGTGGTCTTTA
GGGCCATACCATTTGAGGCTTTGTGCAAGCCACGTGCATCTATGTATGACGTGCTCGAGAG
GCTCTGA

>Seq2 [organism=Festuca rubra] Chewing's fescue root CYP81A20 mRNA, CDS B
ATGGATAATTTTTACATTGCCATCCTTTCATTCGCTTTCATCTTCCTGCTCCACCACCTCCTG
GGCAGGAAGCCAAACAAGGCGCCTCTGCCGAGCCCTCCGGCCATCCCATTCGCCGGCCAT
ATCCATCTGGTGAAGAAGCCATTCCACGCCGTGCTAGCCCGCCTCGCAGAGCGCCATGGGC
CCCTGTTCTCGTTGCGGCTCGGCACCTTCAATGCTGTGGTGGTGTCCTCACCGGCGTTAGCG
AAGGAGTGCTTCACGGAAATGGACGTGACCTTCGCCAACCGCCCAAGGCTCCCCTCGTGG
AAATTCCTGTCCGAGGACTACTCAATGATCGCCACGAGCAGCTATAACCCACACTGGCGA
AACCTCCGCCGCGTCGCGGCCGTGCAGCTCCTCTCGACGCACCGTGTCAGCTGCATGTCAG
GCGTCATCTCCGGTGAGGTGCACGCCATGGTGCGGCGGTTGAATCGATCGGCCACGGAGT
CCCCGCGCATCCGGCTGAAGCAGAGGCTGTTCGAGCTCTCCCTCAGCGTACTCATGGAGAC
CATCGCAAACACCAAGGCAACTCGTTCAGAAGCCACCGCCGCCGACACGGACATGTCGGT
CGAGGCCCAGGAATTCAGGACGGTGATGGACAATATCAACCCACTTGTCGGCCTGGCCAA
CCTGTGGGAGCTATTGCCAATTGTTCGCTGGTTCGACGTGTCCGGCATCAAGAACAAGCTC
CTGGCTTCGGTGACTAGAAGGGATGCGTTCCTGCAGAGGCTCATCGACGCCGAGCGGCGG

AAGGTAGAGGACGGCGGCAGTGAAGACGGCAAGAAGAGTATGATTTCCGTCATGTTCGGT
CTGCAGAAGAAAGAGCCAAATGTCTATACCGACAAAATGATCAGGGGTCTGATCACGAGT
TTATTTAGTGCTGGAACAGATACCATCTTGGCTACGACAGAATGGGCTATGTCGCTCCTAC
TAAACCATCCAACGGCCCTAAAAAAGGCACATGCACAGATCGACCAAGTTGTTGGGACCT
CCCGCCTAGTTTCCTCTGAGGACCTTTCCCGCCTCACTTACCTCCACTGTATCATTAGTGAG
ACCCTCCGTCTTTACCCTGCTGCACCGCTTCTGCTGCCGCATCAGACTTACGTCGACTGTAA
GATTGGTGGCCACACAATTCCGAGTGGGACCATGCTGATCTGTAATGCGTACGCCATCCAT
AGGGACCCAAACGTGTGGGAGGATCCCCTGGAGTTCAAGCCGGATCGGTTTGAGGATGGC
AAGGCGGAAGGGTTATTCATGATACCATTTGGAATGGGGAGGAGGAAGTGTCCGGGAGAG
GCAATGGCTTTACGGACAATGGGACTTGTTCTTGGGGCACTTATACAATGCTTCGAATGGG
ACCGGGTAGACGATGCGAAGGTGGACATGAAGGAATATGGAGAAGAATTTGTGGTCTTTA
GGGCCATACCATTTGAGGCTTTGTGCAAGCCACGTGCATCTATGTATGACGTGCTCGAGAG
GCTCTGA

>Seq3 [organism=Festuca rubra] Chewing's fescue root CYP81A20 mRNA, CDS C
ATGGATAATTTTTACATTGCCATCCTTTCATTCACTTTCATCTTCCTGCTCCACCACCTCCTG
GGCAGGAAGCCAAACAAGGCGCCTCTGCCGAGCCCTCCGGCCATCCCATTCGCCGGCCAT
ATCCATCTCGTGAAGAAGCCATTCCACGCCGTGCTAGCCCGCCTCACAGAGCGCCATGGGC
CCCTGTTCTCGTTGCGGCTCGGCACCTTCAATGCTGTGGTGGTGTCCTCACCGGCATTAGCG
AAGGAGTGCTTCACGGAAATGGACGTGACCTTCGCCAACCGCCCAAGGCTCCCTTCGTGG
AAATTCCTGTCCGAGGACTACTCAATGATCGCCACGAGCAGCTATAACCCGCACTGGCGA
AACCTCCGCCGCGTCGCGGCCGTGCAGCTCCTCTCGACGCACCGTGTCAGCTGCATGTCAG
GCGTCATCTCCGGTGAGGTGCACGCCATGGTGCGGCGGTTGAATCGATCGGCCACGGAGT
CCCCGCGCATCCGGCTGAAGCAGAGGCTGTTCGAGCTCTCCCTCAGCGTACTCATGGAGAC
CATCGCAAACACCAAGGCAACTCGTTCAGAAGCCACCGCCGCCGACACGGACATGTCGGT
CGAGGCCCAGGAATTCAGGACGGTGATGGACAATATCAACCCACTTGTCGGCCTGGCCAA
CCTGTGGGAGCTATTGCCAATTGTTCGCTGGTTCGACGTGTCCGGCATCAAGAACAAGCTC
CTGGCTTCGGTGACTAGAAGGGATGCGTTCCTGCAGAGGCTCATCGACGCCGAGCGGCGG
AAGGTAGAGGACGGCGGCAGTGAAGACGGCAAGAAGAGTATGATTTCCGTCATGTTCGGT
CTGCAGAAGAAAGAGCCAAATGTCTATACCGACAAAATGATCAGGGGTCTGATCACGAGT
TTATTTAGTGCTGGAACAGATACCATCTTGGCTACGACAGAATGGGCTATGTCGCTCCTAC
TAAACCATCCAACGGCCCTAAAAAAGGCACATGCACAGATCGACCAAGTTGTTGGGACCT
CCCGCCTAGTTTCCTCTGAGGACCTTTCCCGCCTCACTTACCTCCAGTGTATCATTAGTGAG
ACCCTCCGTCTTTACCCTGCTGCACCGCTTCTGCTGCCGCATCAGACTTACGTCGACTGTAA
GATTGGTGGCCACACAATTCCGAGTGGGACCATGCTGATCTGTAATGCGTACGCCATCCAT
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AAGGCGGAAGGGTTATTCATGATACCATTTGGAATGGGGAGGAGGAAGTGTCCGGGAGAG
GCAATGGCTTTACGGACAATGGGACTTGTTCTTGGGGCACTTATACAATGCTTCGAATGGG
ACCGGGTAGACGATGCGAAGGTGGACATGAAGGAATATGGAGAAGAATTTGTGGTCTTTA
GGGCCATACCATTTGAGGCTTTGTGCAAGCCACGTGCATCTATGTATGACGTGCTCGAGAG
GCTCTGA

>Seq4 [organism=Festuca rubra] Chewing's fescue root CYP81A20 mRNA, CDS D
ATGGATAATTTTTACATTGCCATCCTTTCATTCGCTTTCATCTTCCTGCTCCACCACCTCCTG
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ATCCATCTCGTGAAGAAGCCATTCCACGCCGTGCTAGCCCGCCTCGCAGAGCGCCATGGGC
CCCTGTTCTCGTTGCGGCTCGGCACCTTCAATGCTGTGGTGGTGTCCTCACCGGCGTTAGCG
AAGGAGTGCTTCACGGAAATGGACGTGACCTTCGCCAACCGCCCAAGGCTCCCCTCGTGG
AAATTCCTGTCCGAGGACTACTCAATGATCGCCACGAGCAGCTATAACCCACACTGGCGA
AACCTCCGCCGCGTCGCGGCCGTGCAGCTCCTCTCGACGCACCGTGTCAGCTGCATGTCAG
GCGTCATCTCCGGTGAGGTGCACGCCATGGTGCGGCGGTTGAATCGATCGGCCACGGAGT
CCCCGCGCATCCGGCTGAAGCAGAGGCTGTTCGAGCTCTCCCTCAGCGTACTCATGGAGAC
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CCTGTGGGAGCTATTGCCAATTGTTCGCTGGTTCGACGTGTCCGGCATCAAGAACAAGCTC

CTGGCTTCGGTGACTAGAAGGGATGCGTTCCTGCAGAGGCTCATCGACGCCGAGCGGCGG
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CTGCAGAAGAAAGAGCCAAATGTCTATACCGACAAAATGATCAGGGGTCTGATCACGAGT
TTATTTAGTGCTGGAACAGATACCATCTTGGCTACGACAGAATGGGCTATGTCGCTCCTAC
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CCCGCCTAGTTTCCTCTGAGGACCTTTCCCGCCTCACTTACCTCCACTGTATCATTAGTGAG
ACCCTCCGTCTTTACCCTGCTGCACCGCTTCTGCTGCCGCATCAGACTTACGTCGACTGTAA
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GCAATGGCTTTACGGACAATGGGACTTGTTCTTGGGGCACTTATACAATGCTTCGAATGGG
ACCGGGTAGACGATGCGAAGGTGGACATGAAGGAATATGGAGAAGAATTTGTGGTCTTTA
GGGCCATACCATTTGAGGCTTTGTGCAAGCCACGTGCATCTATGTATGACGTGCTCGAGAG
GCTCTGA

>Seq5 [organism=Festuca rubra] Chewing's fescue root CYP75A47 mRNA, CDS A
ATGTCCCTCCTCACCGGCACCGGGCTGTTCAACATCAGCGACTTCGTGCCGGCGCTGGCGT
GGATGGACCTGCAGGGCGTGCAGGCCAGGCTGCGCGGTGTTCACCGGCAGTTCGACGGCC
TCATCACCAAGCTTCTGGCGGAGCACGCCGCTACGGCCGAGGACCGCGCCCGGGAGGGCC
GCCTGGACTTCGTCGACAAGCTCCGCGCCAGCAAGGACGACGATGACGGCGAGACCATTA
CCGAGATCAACATCAAGGGGCTCATCTTCGACATGTTCACGGCAGGCACGGACACGTCGT
CGGTGATCGTGGAGTGGGCGATGGCGGAGATGATGGCGAACCCGTCCATCATGGCGCGCG
CGCAGGAGGAGATTGACCGCGTCGTCGGCCGCGACCGGCGGCTGGAGGAGTCCGACATCG
CCGACCTCCCCTACCTGCAGGCCGTCTGCAAGGAGGCCATGCGCCTCCACCCCTCCACGCC
GCTCAGCCTCCCGCACTTCTCCTTCCAGGAGACCCAGGTGGACGGCTACCACGTCCCCGCC
AACACACAGCTCCTCGTCAACATCTGGGCCATCGGCCGCGACCCGGACGCCTGGGAGGAT
CCCCTCCAGTTCTGCCCCGAGCGCTTCCTCTCCGACGGGCCGGCGGCCAAGGTCGACCCCA
TGGGCAACTACTTCGAGCTCATACCGTTCGGTGCCGGCAGGAGGATCTGTGCTGGGAAGCT
GGCCGGCATGGTGTTCGTGCAGTACTTCCTGGGCACGCTCGTGCACGCGTTCGAGTGGCGC
CTGCCGGATGGCGAGGAGATGGTCGACATGGCCGAGACCTCCGGCCTGGCGCTGCCCAAG
GCTGTGCCGCTCAGGGCCCTCGTCACGCCGCGTCTTGCGCCGGTCGCCTACGCCTGA

>Seq6 [organism=Festuca rubra] Chewing's fescue root CYP75A47 mRNA, CDS B
ATGTCCCTCCTCACCGGAACCGGGCTGTTCAACATCAGCGACTTCGTGCCGGCGCTGTCGT
GGATGGACCTGCAGGGCGTGCAGGCCAGGCTGCGCCGGGTTCACAAGCAGTTCGACGGCC
TCATCACTAAGCTTCTGGCGGAGCACGCCGCCACGGCCGAGGACCGCGCCCGGGAGGGCC
GCCTGGACTTCGTCGACAAGCTCCGCGCCAGCAAGGACGATGAGGACGGCGAGACCATCA
CCGAGATCAACATCAAGGGGCTCATCTTCGACATGTTCACGGCAGGCACGGACACGTCGT
CGGTGATCGTGGAGTGGGCGATGGCGGAGATGATGGCGAACCCGTCCATCATGGCGCGCA
CGCAGGAGGAGCTTGACCGCGTCGTCGGCCGCGACCGTCGGCTGGAGGAGTCCGACATCG
CCGACCTCCCCTACCTGCAGGCCGTCTGCAAGGAGGCCATGCGCCTCCACCCCTCCACGCC
GCTCAGCCTCCCGCACTTCTCCTTCCAGGAGACCCAGGTGGACGGCTACCACGTCCCCGCC
AACACACAGCTCCTCGTCAACATCTGGGCCATCGGCCGCGACCCGGACGCCTGGGAGGAT
CCCCTCCAGTTCTGCCCCGAGCGCTTCCTCTCCGACGGGCCGGCGGCCAAGGTCGACCCCA
TGGGCAACTACTTCGAGCTCATACCGTTCGGTGCCGGCAGGAGGATCTGTGCTGGGAAGCT
GGCCGGCATGGTGTTCGTGCAGTACTTCCTGGGCACGCTCGTGCACGCGTTCGAGTGGCGC
CTGCCGAATGGCGAGGAGATGGTCGACATGGCCGAGACCTCCGGCCTGGCGCTGCCCAAG
GCTGTGCCGCTCAGGGCCCTCGTCACGCCGCGTCTTGCGCCGGTCGCCTACGCCTGA

>Seq7 [organism=Festuca rubra] Chewing's fescue root CYP75A47 mRNA, CDS C
ATGTCCCTCCTCACCGGAACCGGGCTGTTCAACATCAGCGACTTCGTGCCGGCGCTGTCGT
GGATGGACCTGCAGGGCGTGCAGGCCAGGCTGCGCCGGGTTCACAAGCAGTTCGACGGCC
TCATCACTAAGCTTCTGGCGGAGCACGCCGCCACGGCCGAGGACCGCGCCCGGGAGGGCC
GCCTGGACTTCGTCGACAAGCTCCGCGCCAGCAAGGACGATGAGGACGGCGAGACCATCA
CCGAGATCAACATCAAGGGGCTCATCTTCGACATGTTCACGGCAGGCACGGACACGTCGT

CGGTGATCGTGGAGTGGGCGATGGCGGAGATGATGGCGAACCCGTCCATCATGGCGCGCA
CGCAGGAGGAGCTTGACCGCGTCGTCGGCCGCGACCGTCGGCTGGAGGAGTCCGACATCG
CCGACCTCCCCTACCTGCAGGCCGTCTGCAAGGAGGCCATGCGCCTCCACCCCTCCACGCC
GCTCAGCCTCCCGCACTTCTCCTTCCAGGAGACCCAGGTGGACGGCTACCACGTCCCCGCC
AACACACAGCTCCTCGTCAACATCTGGGCCATCGGCCGCGACCCGGACGCCTGGGAGGAT
CCCCTCCAGTTCTGCCCCGAGCGCTTCCTCTCCGACGGGCCGGCGGCCAAGGTCGACCCCA
TGGGCAACTACTTCGAGCTCATACCGTTCGGTGCCGGCAGGAGGATCTGTGCTGGGAAGCT
GGCCGGCATGGTGTTCGTGCAGTACTTCCTGGGCACGCTCGTGCACGCGTTCGAGTGGCGC
CTGCCGAATGGCGAGGAGATGGTCGACATGGCCGAGACCTCCGGCCTGGCGCTGCCCAAG
GCTGTGCCGCTCAGGGCCCTCGTCACGCCGCGTCTTGCGCCGGTCGCCTACGCCTGA

>Seq8 [organism=Festuca rubra] Chewing's fescue root CYP75A47 mRNA, CDS D
ATGTCCCTCCTCACCGGCACTGGGCTGTTCAACATCAGCGACTTCGTGCCGGCCCTGTCGT
GGATGGACCTGCAGGGCGTGCAGGCCAGGCTGCGCCGGGTCCACCGCCAGTTCGACGACC
TCATCACCAAGCTTCTGGCGGAGCACGCCGCCACGGCCGACGACCGCGCCCGGGAGGGAC
GCCTGGACTTCGTCGACAAGCTCCGCGCCAGCAAGGACGACGAGGACGGCGAGACCATCA
CCGAGATCAACATCAAGGGGCTCATCTTCGACATGTTCACGGCTGGCACGGACACGTCGTC
GGTGATCGTGGAGTGGGCGATGGCGGAGATGATGGCGAACCCGTCCATCATGGCGCGCAC
GCAGGAGGAGCTTGACCGCGTCGTCGGCCGCGACCGTCGGCTGGAGGAGTCCGACATCGC
CGACCTCCCCTACCTGCAGGCCGTCTGCAAGGAGGCCATGCGCCTCCACCCCTCCACGCCG
CTCAGCCTCCCGCACTTCTCCTTCCAGGAGACCCAGGTGGACGGCTACCACGTCCCCGCCA
ACACACAGCTCCTCGTCAACATCTGGGCCATCGGCCGCGACCCGGACGCCTGGGAGGATC
CCCTCCAGTTCTGCCCCGAGCGCTTCCTCTCCGACGGGCCGGCGGCCAAGGTCGACCCCAT
GGGCAACTACTTCGAGCTCATACCGTTCGGTGCCGGCAGGAGGATCTGTGCTGGGAAGCT
GGCCGGCATGGTGTTCGTGCAGTACTTCCTGGGCACGCTCGTGCACGCGTTCGAGTGGCGC
CTGCCGGATGGCGAGGAGATGGTCGACATGGCCGAGACCTCCGGCCTGGCGCTGCCCAAG
GCTGTGCCGCTCAGGGCCCTCGTCACGCCGCGTCTTGCGCCGGTCGCCTACGCCTGA

>Seq9 [organism=Festuca rubra] Chewing's fescue root CYP92A44 mRNA, CDS A
ATGGAGTTTCCTCAGTGGGCGTCCTTCCTGGTCGTCGTGCTCGCCACGGTGCTCTTTCTCAA
GGCCGTCCTCCGGCGCCGAAGCAGCCGCAAGTACAACCTCCCGCCGGGTCCCAAGGCGTG
GCCGATCATCGGCAACCTGAACCTCATCGGCACGCTCCCACATCGCTCCATCCACGCGCTC
TCCAAGCAGTACGGCCCGCTCTTGCAGCTCCAGTTTGGCTCCTTTCCCTGCGTCGTCGGCTC
CTCCGTCGAGATGGCTAAGTTCTTCCTCAAGACCCACGACGTGATGTTCACCGACCGGCCC
AAGTTCGCCGCCGGCAAGCACACCACCTACAACTACAGCGATATCACCTGGTCCCCCTACG
GCGCCTACTGGAGGCAGGCCCGCAAGATGTGCCTCACGGAGCTCTTCAGCGCCAGGCGGC
TCCAGTCGTACGAGTACATCCGCAGCGAGGAGGTACTCGCCCTCCTCCGCGACCTGCATCG
CGGCGCCACCGTCGGCGCCGGCCGCGCCCTGGTGCTCAAGGACTACCTGTCCACGGTGAG
CCTGAACGTGATCACGCGCATGGTGATGGGCAAGAAATACCTGGAGAAGGAGGTGAGGG
ACGGGAGCGGCGCGGTGATCACGACGCCGGACGAGTTCAAGTGGATGATCGACGAGCTGT
TCCTTCTCAACGGCGTGCTGAACATCGGTGACTCCATCCCGTGGCTCGACTGGATGGACCT
GCAGGGTTACATTAAGAGGATGAAGAAGCTCAGCAAGATGTTTGACCGGTTCCTGGAGCA
CGTCGTCGACGAGCACAGCGAGCGACGCCGCCACGAGGCGGAGAGCTTCGTTGCCAAGGA
CATGGTTGACGTGCTTCTGCAGTTCGCGAGCAACCCCGATCTCGAGGTCAAGCTCAACAGG
GAGGGCGTCAAGGCTTTCACTCAGGATCTTATCGCTGGCGGCACAGAAAGCTCGGCGGTG
ACGGTGGAGTGGGCACTCTCGGAGCTCCTGAAGAAGCCCGAGGTGTTCGCCAGGGCGACC
GAGGAGCTGGACCGCGTCGTCGGAAGAGGCCGTTGGGTCACCGAGAAGGACATGCCGAGC
CTCCCCTACGTGGACGCCATCGTGAAGGAGACGATGCGGCTGCACCCAGTGGCGCCGATG
CTGGTGCCCCGCCTCTCCCGCGAGGTCACGACCATCGGCGGCTATGACATCCCCGCCGGCA
CCCGGGTGCTCGTCAGTGTGTGGACCATCGGCCGGGACCCAGAGCTATGGGACGCGCCTG
AAGAGTTCATGCCAGAGAGGTTCCTCGGCAGCAGGCTCGACGTCAAGGGGCAGGACTACG
AGCTACTGCCGTTCGGATCGGGACGCAGGATGTGCCCTGGGTACAGTCCTGGACTAAAAG
TCATCCAGGTGAGCCTGGCCAACCTACTGCACGGCTTCGAGTGGAAGCTCCCCGACGGCGT
GGAGCTGAGCATGGAGGAGATCTTCGGCCTCTCCACGCCGCGCAAGTTCCCGCTGGAGGC
CGTCGTGGAGCCCAAGCTCCCGGCTCATCTCTACGAATGA

>Seq10 [organism=Festuca rubra] Chewing's fescue root CYP92A44 mRNA, CDS B
ATGGAGTTTCCTCAGTGGGCGTCCTTCCTGGGTGTCGTGCTCGCCACGGTGCTCTTTCTCAA
GGCCGTCCTCCGGCGCCGAAGCGGCCGAAAGTACAACCTCCCGCCGGGTCCCAAGGCGTG
GCCGATCATCGGCAACCTGAACCTCATGGGCACGCTCCCACATCGCTCCATCCACGCGCTC
TCCAAGCAGTACGGCCCGCTCTTGCAGCTCCAGTTCGGCTCCTTCCCCTGCGTCGTCGGCTC
CTCCGTCGAGATGGCCAAGTTCTTCCTTAAGACCCACGACGTGTCGTTCACCGACCGGCCC
AAGTTCGCCTCCGGCAAGCACACCACCTACAACTACAGCGATATCACCTGGTCCCCCTACG
GCGCCTACTGGCGGCAGGCCCGCAAGATGTGCCTCACCGAGCTCTTCAGCGCCAGGCGGC
TCCGGTCGTACGAGTACATCCGCAGCGAGGAGGTGCTGGCCCTCGTCCGCGACCTGCATCG
CGGCGCCACCGCCGGCGCCGGTCGCGCCCTGGTGCTCAAGGACTACCTGTCCACAGTGAG
CCTGAACGTGATCACGCGCATGGTGATGGGTAAGAAGTACCTGGAGAAGGAGGTGAGGGA
CGAGAGCGGCGCGGTGATCACGACGCCGGACGAGTTCAAGTGGATGATCGACGAGCTGTT
CCTTCTCAACGGCGTCCTCAACATCGGCGACTCCATCCCGTGTCTCGACTGGATGGACCTG
CAGGGGTACATTAAGAGGATGAAGAAACTCAGCAAGATGTTCGACCGCTTCCTGGAGCAC
GTCGTGGAAGAGCACAGCGAGCGGCGCCGCCGTGACGGGGAAAGCTTCGTGGCGAAGGA
CATGGTCGACGTGCTGCTGCAGTTCGCCAGCAACCCCGATCTCGAGGTCAAGCTCAACAGG
GAGGGCGTCAAGGCTTTCACTCAGGATCTTATCGCTGGCGGCACAGAAAGCTCGGCGGTG
ACAGTGGAGTGGGCTCTCTCGGAGCTCCTGAAGAAGCCCGAGGTGTTCGCCAGGGCGACG
GAGGAGCTGGACCGCGTCGTCGGCAGAGGCCGCTGGGTCACCGAGAAGGACATGCCGAGC
CTCCCCTACGTGGACGCCATCGTGAAGGAGACGATGCGGCTGCACCCGGTGGCGCCGATG
CTGGTGCCCCGCCTCTCCCGCGAGGACACGTCCATCGGCGGCTACGACATCCCCGCCGGCA
CGCGGGTGCTCGTCAGCGTGTGGTCCATCGGCCGCGACCCGGAGCTATGGGAAGCGCCGG
AGGAGTTCATGCCAGAGCGGTTCATCGGCAGCAGGCTCGATGTTAAGGGGCAGGACTACG
AGCTGCTGCCGTTCGGGTCGGGGCGCAGGATGTGCCCCGGGTACAGTCTGGGGCTGAAGG
TGATCCAGGTGAGCCTGGCGAACCTACTGCACGGGTTCGAGTGGAAGCTCCCCCATGGCGT
GGAGCTGAGCATGGAGGAGATCTTTGGGCTGTCCACGCCGCGCAAGTTCCCACTGGAGGC
CGTCTTGGAGCCCAAGCTCCCGGCTCATCTCTACGAATGA

>Seq11 [organism=Festuca rubra] Chewing's fescue root CYP92A44 mRNA, CDS C
ATGGAGTTTCCTCAGTGGGCGTCCTTCCTGGGAGTCGTGCTCGCCACGGTGCTCTTTCTCAT
GGCCGTCCTCCAACGCCGAAGCAGCCGCAAGTACAACCTCCCGCCGGGTCCCAAGGCGTG
GCCGATCATCGGCAACCTGAACCTCATCGGCACGCTCCCGCATCGCTCCATCCACGCGCTC
TCCAAACAGTACGGCCCGCTCTTGCAGCTCCAGTTTGGCTCCTTTCCCTGCGTCGTCGGCTC
CTCCGTCGAGATGGCTAAGTTCTTCCTCAAGACCCACGACGTGATGTTCACCGACCGGCCC
AAGTTCGCCGCCGGCAAGCACACCACCTACAACTACAGCGATATCACCTGGTCCCCCTACG
GCGCCTACTGGAGGCAGGCCCGCAAGATGTGCCTCACGGAGCTCTTCAGCGCCAGGCGGC
TCCAGTCGTACGAGTACATCCGCAGCGAGGAGGTACTCGCCCTCCTCCGCGACCTGCATCG
CGGCGCCACCGTCGGCGCCGGCCGCGCCCTGGTGCTCAAGGACTACCTGTCCACGGTGAG
CCTGAACGTGATCACGCGCATGGTGATGGGCAAGAAATACCTGGAGAAGGAGGTGAGGG
ACGGGAGCGGCGCGGTGATCACGACGCCGGACGAGTTCAAGTGGATGATCGACGAGCTGT
TCCTTCTCAACGGCGTGCTGAACATCGGTGACTCCATCCCGTGGCTCGACTGGATGGACCT
GCAGGGTTACATTAAGAGGATGAAGAAGCTCAGCAAGATGTTTGACCGGTTCCTGGAGCA
CGTCGTCGACGAGCACAGCGAGCGACGCCGCCACGAGGCGGAGAGCTTCGTTGCCAAGGA
CATGGTTGACGTGCTTCTGCAGTTCGCGAGCAACCCCGATCTCGAGGTCAAGCTCAACAGG
GAGGGCGTCAAGGCTTTCACTCAGGATCTTATCGCTGGCGGCACAGAAAGCTCGGCGGTG
ACGGTGGAGTGGGCACTCTCGGAGCTCCTGAAGAAGCCCGAGGTGTTCGCCAGGGCGACC
GAGGAGCTGGACCGCGTCGTCGGAAGAGGCCGTTGGGTCACCGAGAAGGACATGCCGAGC
CTCCCCTACGTGGACGCCATCGTGAAGGAGACGATGCGGCTGCACCCAGTGGCGCCGATG
CTGGTGCCCCGCCTCTCCCGCGAGGTCACGACCATCGGCGGCTATGACATCCCCGCCGGCA
CCCGGGTGCTCGTCAGTGTGTGGACCATCGGCCGGGACCCAGAGCTATGGGACGCGCCTG
AAGAGTTCATGCCAGAGAGGTTCCTCGGCAGCAGGCTCGACGTCAAGGGGCAGGACTACG
AGCTACTGCCGTTCGGATCGGGACGCAGGATGTGCCCTGGGTACAGTCTTGGACTAAAAGT
CATCCAGGTGAGCCTGGCCAACCTACTGCACGGCTTCGAGTGGAAGCTCCCCGACGGCGT
GGAGCTGAGCATGGAGGAGATCTTCGGCCTCTCCACGCCGCGCAAGTTCCCGCTGGAGGC

CGTCGTGGAGCCCAAGCTCCCGGCTCATCTCTACGAATGA

>Seq12 [organism=Festuca rubra] Chewing's fescue root CYP92A44 mRNA, CDS D
ATGGAGTTTCCTCAGTGGGCGTCCTTCCTGGGTGTCGTGCTCGCCACGGTGCTCTTTCTCAA
GGCCGTCCTCCGGCGCCGAAGCGGCCGAAAGTACAACCTCCCGCCGGGTCCCAAGGCGTG
GCCGATCATCGGCAACCTGAACCTCATGGGCACGCTCCCACATCGCTCCATCCACGCGCTC
TCCAAGCAGTACGGCCCGCTCTTGCAGCTCCAGTTCGGCTCCTTCCCCTGCGTCGTCGGCTC
CTCCGTCGAGATGGCCAAGTTCTTCCTTAAGACCCACGACGTGTCGTTCACCGACCGGCCC
AAGTTCGCCTCCGGCAAGCACACCACCTACAACTACAGCGATATCACCTGGTCCCCCTACG
GCGCCTACTGGCGGCAGGCCCGCAAGATGTGCCTCACCGAGCTCTTCAGCGCCAGGCGGC
TCCGGTCGTACGAGTACATCCGCAGCGAGGAGGTGCTGGCCCTCGTCCGCGACCTGCATCG
CGGCGCCACCGCCGGCGCCGGTCGCGCCCTGGTGCTCAAGGACTACCTGTCCACGGTCAGC
CTGAACGTGATCACGCGCATGGTGATGGGCAAGAAGTACCTGGAGAAGGAGGTGAGGGA
CGGGAGCGGCGCGGTGATCACGACGCCGGATGAGTTCAAGTGGATGATCGACGAGCTATT
CCTTCTCAACGGCGTGCTCAACATCGGCGACTCCATCCCGTGGCTCGACTGGATGGACCTG
CAGGGGTACATTAAGAGGATGAAGAAGCTGAGCAAGATGTTCGACCGGTTCCTGGAGCAC
GTCGTGGACGAGCACAGCGAGCGGCGCCGCCGCGATGGGGAGAGCTTCGTGGCCAAGGAC
ATGGTCGACGTGCTGCTGCAGTTCGCCAGCGACCCCAACCTCGAGGTCAAGCTCAACAGG
GATGGCGTCAAGGCTTTCACTCAGGATCTCATTGCTGGGGGCACAGAAAGCTCGGCAGTG
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GAAGAGCTGGACCGCGTCGTTGGCCGAGGCCGTTGGATCACCGAGAAGGACATGCCGAGC
CTCCCCTACGTGGACGCCATCGTGAAGGAGACGATGCGGCTGCACCCGGTGGCGCCGATG
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CCCGTGTGCTCGTCAGCGTGTGGTCCATCGGCCGCGACCCGGAGCTATGGGACGTGCCGGA
GGAGTTCATGCCAGAGCGGTTCATCGGCAGCAAGCTCGATGTTAAGGGGCAGGACTACGA
GCTGCTGCCGTTCGGGTCAGGGCGCAGGATGTGCCCCGGGTATAGCCTGGGGCTGAAGGT
GATCCAGGTGAGCTTGGCGAACCTACTGCACGGGTTCGAGTGGAAGCTCCCCGACGGCGT
GGAGCTGAACATGGAAGAGATCTTCGGCCTGTCTACGCCGCGCAAGTTCCCGCTGGAGGC
CGTCGTAGAGCCCAAGCTCCCGGCTCATCTCTACGAATGA

>Seq13 [organism=Festuca rubra] Chewing's fescue root CYP73A91 mRNA, CDS A
ATGGACGTCAACCTCCTGGAGAAAGCCCTACTGGGCCTCTTTGCGGCAGCGGTGCTCGCCA
TCGCGGTCGCGAAGCTCACCGGTAAGAAGTTCAAGCTTCCTCCAGGCCCGTCCGGTTACCC
CATCGTGGGCAACTGGCTCCAGGTCGGCGATGACCTGAACCACCGCAACCTGATGGGCAT
GGCCAAGCGCTTCGGCGAGGTGTTCCACCTCCGCATGGGCGTCCGCAACCTGGTGGTCGTC
TCCAGCCCCGAGCTCGCCAAGGAGGTCCTCCACACCCAGGGCGTGGAGTTCGGTTCCCGCA
CCCGCAACGCCGTCTTCGACATCTTCACCGGCAAGGGGCAGGACATGGTGTTCACCGTGTA
CGGCGACCACTGGCGCAAGATGCGCCGCATCATGACCGTGCCCTTCTTCACCAGCAAGGTG
GTGGCGCAGAACCGCCTCGGGTGGGAGGAGGAGGCAAGGCTGGTGGTGGAGGACGTGAA
GGCCGACCCGGCGGCGGCCACCACCGGCGTGGTCATCCGCCGCAGGCTGCAGCTCATGAT
GTACAACGACATGTTCCGCATCATGTTCGCCCGCCGCTTCGAGTCCCTCGCCGACCCGCTC
TTCAACAAGCTCAGGGCGCTCAACGCCGAGCGCAGCATCCTCTCCCAGAGCTTCGACTACA
ACTACGCCGACTTCATCCCCTTCCTCCGCCCCTTCCTCCGCGGATACCTCAACCGCTGCACC
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GCATTGGAGCAGAGTGGTGAGATCAAGTGTGCCATGGACCACATCCTCGAGGCCGAAAGG
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GTCCAGTCAAAGCTGCGCAACGAGATTGCCGCCGTATTGGGCCCCAACGTGGCGGTGACG
GAGCCGGACCTGGAGCGCCTCCCCTACCTGCAGTCTGTCGTGAAGGAGACCCTCCGCCTCC
GCATGGCCATCCCGCTGCTGGTGCCACACATGAACCTCCAGGAGGCCAAGCTGTCCGGCTA
CGACATCCCCCGCCGAGTCCAAGATCCTCGTGAACGCCTGGTTCCTTGCCAACGACCCCAAG
CGTTGGCTGCGGGCCGACGAATTCAGGCCGGATCGCTTCCTCGAGGAGGAGAAGGCCGTC
CAAGCTCATGGCAATGACTTCCGCTTCGTGCCCTTCGGTGTCGGCCGCCGAAACTGCCCGG
GGATCATCCTAGCGCTGCCCATCATCGGCATTACGCTCGGCAACATGGTGCAGAACTTCCA
GCTGCTGCCGCCGCCGGGGCAGGACAAGATCGACACCACCGAGAAGCCCGGACAGTTCAG

CAACCAGATCCGCACCCACGCCAACGTCGTCTGCAAGCCCCTCAAGGCTTAGAA

>Seq14 [organism=Festuca rubra] Chewing's fescue root CYP73A91 mRNA, CDS B
ATGGACGTCAACCTCCTGGAGAAGGCCCTACTGGGCCTCTTCGCGGCGGCGGTGCTCGCCA
TCGCAGTCGCGAAGCTCACCGGTAAGCGCTTCAAGCTTCCTCCAGGCCCGTCCGGTTACCC
CATCGTGGGCAACTGGCTCCAGGTCGGCGATGACCTGAACCACCGCAACCTGATGGGCAT
GGCCAAGCGCTTCGGCGAGGTGTTCCACCTCCGCATGGGCGTCCGCAACCTGGTGGTCGTC
TCCAGCCCCGAGCTGGCCAAGGAGGTCCTCCACACCCAGGGCGTGGAGTTCGGCTCCCGC
ACCCGCAACGCCGTCTTCGACATCTTCACCGGCAAGGGGCAGGACATGGTGTTCACCGTCT
ACGGCGACCACTGGCGCAAGATGCGCCGGATCATGACCGTGCCCTTCTTCACCAGTAAGGT
GGTGGCGCAGAACCGCGTCGGCTGGGAGGAGGAGGCTAGGCTGGTGGTCGAGGACGTGA
AGGCCGACCCGGCGTCGGCGACGACCGGCGTGGTCATCCGCCGCAGGCTGCAGCTGATGA
TGTACAACGACATGTTCCGCATCATGTTCGACCGCCGCTTCGAGTCCCTCGCCGACCCGCT
CTTCAACAAGCTCAGGGCGCTCAACGCCGAGCGCAGCATCCTCTCCCAGAGCTTCGACTAC
AACTACGGCGACTTCATCCCCTTCCTCCGCCCATTTCTCCGCGGATACCTCAATCGCTGCAC
CAATCTCAAGACCAAGAGGATGAAAGTCTTCGAGGACGACTTCGTCACACCACGCAAGAA
GGCGTTGGAGCAGAGTGGTGAAATCAAGTGTGCCATGGACCACATCCTCGAGGCCGAAAG
GAAGGGTGAGATCAACCACGACAACGTCCTCTACATCGTCGAGAACATCAATGTCGCAGC
CATCGAGACGACACTGTGGTCAATCGAGTGGGGCATTGCTGAGCTGGTGAACCACCCAGA
AGTCCAGTCAAAGCTGCGCAACGAGATTGCCGCCGTACTCGGCCCCAATGCGGCGGTGAC
GGAGCCGGACCTGGAGCGCCTCCCCTACCTGCAGTCCGTCGTGAAGGAGACTCTCCGCCTC
CGCATGGCCATCCCGCTGCTCGTGCCACACATGAACCTCAACCAGGCGAAGCTCTCCGGCT
ACGACATCCCCGCCGAGTCCAAGATCCTCGTGAACGCCTGGTTCCTCGCCAACGACCCCAA
GCGTTGGGTGCGGGCCGATGAGTTCAGGCCGGAGCGATTCCTCGAGGAGGAGAAGGCCGT
CCAAGCTCATGGCAATGACTTCCGCTTCGTGCCCTTCGGCGTCGGCCGGCGAAACTGCCCG
GGGATCATCCTAGCACTGCCCATCATCGGCATTACGCTCGGCAACATGGTGCAGAACTTCA
ATCTGCTGCCGCCGCCGGGTCTGGACAAGATCGACACCACCGAGAAGCCGGGACAGTTCA
GCAACCAGATCCGCACCCACGCCAACGTCGTCTGCAAGCCCCTCAAGGCTTAGAA

>Seq15 [organism=Festuca rubra] Chewing's fescue root CYP73A91 mRNA, CDS C
ATGGACGTCAACCTCCTGGAGAAGGCCCTACTGGGCCTCTTCGCGGCGGCGGTGCTCGCCA
TTGCAGTCGCGAAGCTCACCGGTAAGCGCTTCAAGCTTCCTCCAGGCCCGTCCGGTTACCC
CATCGTGGGCAACTGGCTCCAGGTCGGCGATGACTTGAACCACCGCAACCTGATGGGCAT
GGCCAAGCGCTTCGGCGAGGTGTTCCACCTCCGCATGGGCGTCCGCAACCTGGTGGTCGTG
TCCAGCCCCGAGCTGGCCAAGGAGGTCCTCCACACCCAGGGCGTGGAGTTCGGCTCCCGC
ACCCGCAACGCCGTCTTCGACATCTTCACCGGCAAGGGGCAGGACATGGTGTTCACCGTCT
ACGGCGACCACTGGCGCAAGATGCGGCGGATCATGACCGTGCCCTTCTTCACCAGCAAGG
TGGTGGCGCAGAACCGCGTCGGGTGGGAGGAGGAGGCGAGGCTGGTGGTCGAGGACGTG
AAGGCCGACCCGGCGTCGGCGACGACCGGCGTGGTCATCCGCCGCAGGCTGCAGCTGATG
ATGTACAACGACATGTTCCGCATCATGTTCGACCGCCGCTTCGAGTCCCTCGCCGACCCGC
TCTTCAACAAGCTCAGGGCGCTCAACGCCGAGCGCAGCATCCTCTCCCAGAGCTTTGACTA
CAACTACGGCGACTTCATCCCCTTCCTCCGCCCATTCCTCCGCGGATACCTCAACCGCTGCA
CCAATCTCAAGACCAAGAGGATGAAAGTCTTCGAGGACGACTTCGTCACACCACGCAAGA
AGGCGTTGGAGCAGAGTGGTGAGATCAAGTGTGCCATGGACCACATCCTCGAGGCCGAAA
GGAAAGGCGAGATCAACCACGACAACGTCCTCTACATCGTCGAGAACATCAATGTTGCAG
CCATCGAGACGACGCTGTGGTCAATCGAGTGGGGCATTGCTGAGCTGGTGAACCACCCTG
AGGTCCAGTTGAAGCTGCGCAACGAGATTGCTGCTGTGCTTGGCCCCAACGTGGCGGTGAC
GGAGCCGGACCTGGAGCGTCTCCCCTACCTGCAGTCTGTGGTGAAGGAGACCCTCCGCCTC
CGCATGGCCATCCCGCTGCTCGTGCCACACATGAACCTCAACCAGGCGAAGCTGGCTGGCT
ACGACATCCCCGCAGAGTCCAAGATCCTCGTCAACGCCTGGTTCCTCGCCAACGACCCCAA
GCGCTGGGTGCGGGCCGACGAGTTCAGGCCGGAGCGATTCCTCGAGGAGGAGAAGGCCGT
CCAAGCTCATGGCAATGACTTCCGCTTCGTGCCCTTCGGTGTCGGCCGCCGAAACTGCCCG
GGGATCATCCTAGCACTGCCCATCATCGGCATTACGCTCGGCAACATGGTGCAGAACTTCA
ATCTGCTGCCGCCGCCGGGTCAGGACAAGATCGACACCACCGAGAAGCCCGGACAGTTCA
GCAACCAGATCCGCACCCACGCCAACGTCGTCTGCAAGCCCCTCAAGGCTTAGAA

>Seq16 [organism=Festuca rubra] Chewing's fescue root CYP73A91 mRNA, CDS D
ATGGACGTCAACCTCCTGGAGAAGACCCTACTGGGCCTCTTCGCGGCGGCGGTGCTCGCCA
TCGCAGTCGCGAAGCTCACCGGTAAGCGCTTCAAGCTTCCTCCAGGCCCGTCCGGTTACCC
CATCGTGGGCAACTGGCTCCAGGTCGGCGATGACCTGAACCACCGCAACCTGATGAGCAT
GGCCAAGCGCTTCGGCGAGGTGTTCCACCTCCGCATGGGCGTCCGCAACCTGGTGGTCGTC
TCCAGCCCCGAGCTGGCCAAGGAGGTCCTCCACACCCAGGGCGTGGAGTTCGGCTCCCGC
ACCCGCAACGCCGTCTTCGACATCTTCACCGGCAAGGGGCAGGACATGGTGTTCACCGTTT
ACGGCGACCTCTGGCGCAAGATGCGGCGGATCATGACCGTGCCCTTCTTCACCAGCAAGGT
GGTGGCGCAGAACCGCGTCGGGTGGGAGGAGGAGGCGAGGCTGGTGGTCGAGGACGTGA
AGGCCGACCCGGCGTCGGCGACGACCGGCGTGGTCATCCGCCGCAGGCTGCAGCTGATGA
TGTACAACGACATGTTCCGCATCATGTTCGACCGCCGCTTCGAGTCCCTCGCCGACCCGCT
CTTCAACAAGCTCAGGGCGCTCAACGCCGAGCGCAGCATCCTCTCCCAGAGCTTCGACTAC
AACTACGGCGACTTCATCCCCTTCCTCCGCCCATTTCTCCGCGGATACCTCAATCGCTGCAC
CAATCTCAAGACCAAGAGGATGAAAGTCTTCGAGGACGACTTCGTCACACCACGCAAGAA
GGCGTTGGAGCAGAGTGGTGAAATCAAGTGTGCCATGGACCACATCCTCGAGGCCGAAAG
GAAAGGCGAGATCAACCACGACAACGTCCTCTACATCGTCGAGAACATCAATGTTGCAGC
CATCGAGACGACGCTGTGGTCAATCGAGTGGGGCATTGCTGAGCTGGTGAACCACCCTGA
GGTCCAGTTGAAGCTGCGCAACGAGATTGCTGCTGTGCTTGGCCCCAACGTGGCGGTGACG
GAGCCGGACCTGGAGCGTCTCCCCTACCTGCAGTCTGTGGTGAAGGAGACCCTCCGCCTCC
GCATGGCCATCCCGCTGCTCGTGCCACACATGAACCTCAACCAGGCGAAGCTGGCTGGCTA
CGACATCCCCCGCAGAGTCCAAGATCCTCGTCAACGCCTGGTTCCTCGCCAACGACCCCAAG
CGCTGGGTGCGGGCCGACGAGTTCAGGCCGGAGCGATTCCTCGAGGAGGAGAAGGCCGTC
CAAGCTCATGGCAATGACTTCCGCTTCGTGCCCTTCGGTGTCGGCCGCCGAAACTGCCCGG
GGATCATCCTAGCACTGCCCATCATCGGCATTACGCTCGGCAACATGGTGCAGAACTTCAA
TCTGCTGCCGCCGCCGGGTCAGGACAAGATCGACACCACCGAGAAGCCCGGACAGTTCAG
CAACCAGATCCGCACCCACGCCAACGTCGTCTGCAAGCCCCTCAAGGCTTAGAA