The Roles of Threonine Deaminase in *Nicotiana attenuata*

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller Universität Jena

von Master of Science in Agriculture

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geboren am 10. März 1969 in Korea

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Tag der Doktorprüfung: 22/03/2006

Tag der öffentlichen Verteidigung: 29/03/2006

Table of Contents

Manuscript I

The role of threonine deaminase in seedling growth and flower development in *Nicotiana attenuata*

This manuscript demonstrates that threonine deaminase (TD), which catalyzes the first committed step in the biosynthesis of isoleucine (Ile), plays a role in plant development. The involvement of TD in development was determined by characterizing antisense TD (asTD) transgenic plants that had short roots; delayed opening of cotyledons, and leaf and flower senescence; and retarded growth. I demonstrated that the phenotypes of asTD transgenic plants could be partially recovered by adding α-keto butyrate, which is the first product of TD activity, Ile, or jasmonic acid-Ile conjugate. Using TD promoter:GUS-transformed plants, I confirmed the tissue specific expression of TD in cotyledons during seedling growth and in anthers, stigmas, and trichomes during flower development as well as the induction of TD in leaves upon wounding and MeJA treatment.

I isolated and characterized the TD genomic sequence, and generated transformation vectors bearing different lengths of TD promoter and GUS fusions for plant transformation. Ian T. Baldwin and I designed all the experiments to characterize the asTD and TD promoter:GUS transgenic plants. I performed all the experiments including northern and southern blots; TD activity measurement; secondary metabolite measurement; Ile, JA, and JA-Ile measurement; GUS histochemical and fluorometric assay. Plant transformation was done by T. Kruegel and M. Lim.

Manuscript II

Isolation and characterization of threonine deaminase promoter in *Nicotiana attenuata*

This manuscript reports the isolation and characterization of threonine deaminase (TD) genomic DNA. I demonstrated that in the TD promoter: β-glucuronidase (GUS) reporter gene-fused transgenic plants, GUS is constitutively expressed in seedlings and flowers, and elicited in leaves by wounding or by MeJA. I demonstrated which regions of TD promoter are important for directing minimal expression in cotyledons and anthers during development and for basal elicitation in leaves using wounding and MeJA treatment.

I generated transformation vectors bearing different lengths of TD promoter:GUS fusions for plant transformation. Ian T. Baldwin and I designed all the experiments to characterize the series of TD promoter:GUS transgenic plants. I performed all the experiments including the GUS histochemical and fluorometric assays. Plant transformation was done by T. Kruegel and M. Lim.

Manuscript III

Silencing threonine deaminase and *JAR1* homologue in *Nicotiana attenuata* impairs JA-isoleucine-mediated defense against the specialist herbivore, *Manduca sexta*

This manuscript demonstrates that a gene responsible for the biosynthesis of an essential amino acid plays an exciting role in jasmonate (JA)-mediated herbivore resistance. I demonstrated that threonine deaminase (TD), which catalyzes the first committed step in the biosynthesis of isoleucine (Ile), is involved in JA signaling, by producing the Ile pool at the wound site, which is subsequently conjugated with JA to form JA-Ile, and in turn elicits two potent direct defenses, nicotine and trypsin protease inhibitors (TPI). I demonstrated this by producing transgenic plants expressing TD in an antisense orientation and selecting transformants with intermediate levels of TD silencing; the transformants are characterized by normal growth but impaired herbivore resistance. When leaves were wounded and treated with *M. sexta* oral secretions or JA, transgenic plants had lower levels of elicited JA-Ile, which resulted in lower levels of direct defenses (e.g., nicotine, TPI) and increased susceptibility to *M. sexta* larvae attack compared to wild type (WT) plants. All of these phenotypes of the transgenetic plants could be restored to WT levels by adding Ile to the wound site or by treating plants with JA-Ile. Silencing TD and *JAR4*, the *Arabidopsis JAR1* homologue that is a JA-Ile conjugating enzyme, by virus-induced gene silencing (VIGS) further confirmed that TD and JAR4 play important roles in herbivore resistance.

Ian T. Baldwin and I designed all the experiments to characterize the antisense transformed plants and VIGS plants. I performed all the experiments including northern blot, southern blot, TD activity measurement, secondary metabolite measurement, and herbivore performance. The JA and JA-Ile measurements were done with the help of Bernd Krock. Plant transformation was done by T. Kuegel and M. Lim. The VIGS experiment was done with the help of Lei Wang.

1. Introduction

In addition to their obvious role in protein synthesis, amino acids perform essential functions in both primary and secondary plant metabolism. Some amino acids serve to transport nitrogen from sources to sinks; others serve as precursors to secondary products such as hormones and compounds involved in plant defense (Coruzzi and Last, 2000). For example, glutamate, glutamine, aspartate, and asparagines are used to transfer nitrogen from source organisms to sink tissues and to build up reserves during periods of nitrogen availability for subsequent use in growth, defense, and reproductive processes. Phenylalanine, tyrosine, and tryptophan are precursors for the plant defense compounds chlorogenic acid, dhurrin, and indole glucosinolates, respectively. In addition, phenylalanine and tryptophan are precursors for the essential phytohormones salicylic acid and indole-3-acetic acid, respectively (Celenza, 2001). Methionine serves as a component of methionyl tRNA, which is required for the initiation of protein synthesis, and is a direct precursor of *S*-adenosyl-methionine (SAM), the main biological methyl donor in many transmethylation reactions. In plant tissues, Methionine is also metabolized into the phytohormone ethylene via SAM (Matthews, 1999). Thus, the synthesis of amino acids directly or indirectly controls various aspects of plant growth and development. Recent investigations of genes involved in amino acid biosynthesis reveal that this dynamic process is controlled by metabolic, environmental, and developmental factors (Coruzzi and Last, 2000).

In the collection of manuscripts presented in this study, I investigate the roles of threonine deaminase (TD), which is the first enzyme in the isoleucine biosynthetic pathway, present in plant development and defense. For this purpose, I isolate genomic TD DNA; manipulate transgenic plants bearing TD promoter:β-glucuronidase reporter gene fusions; characterize antisense TD transgenic plants; and characterize TD and *JAR1* homologue using the virus-induced gene silencing (VIGS) method.

The aspartic acid metabolic pathway and threonine deaminase

Aspartate is the common precursor of the synthesis of the essential amino acids threonine, lysine, methionine, and isoleucine (Fig. 1). The first enzymatic

reaction involves the phosphorylation of aspartate-producing β-aspartyl phosphate, which is catalyzed by the enzyme aspartate kinase. The β-aspartyl phosphate is

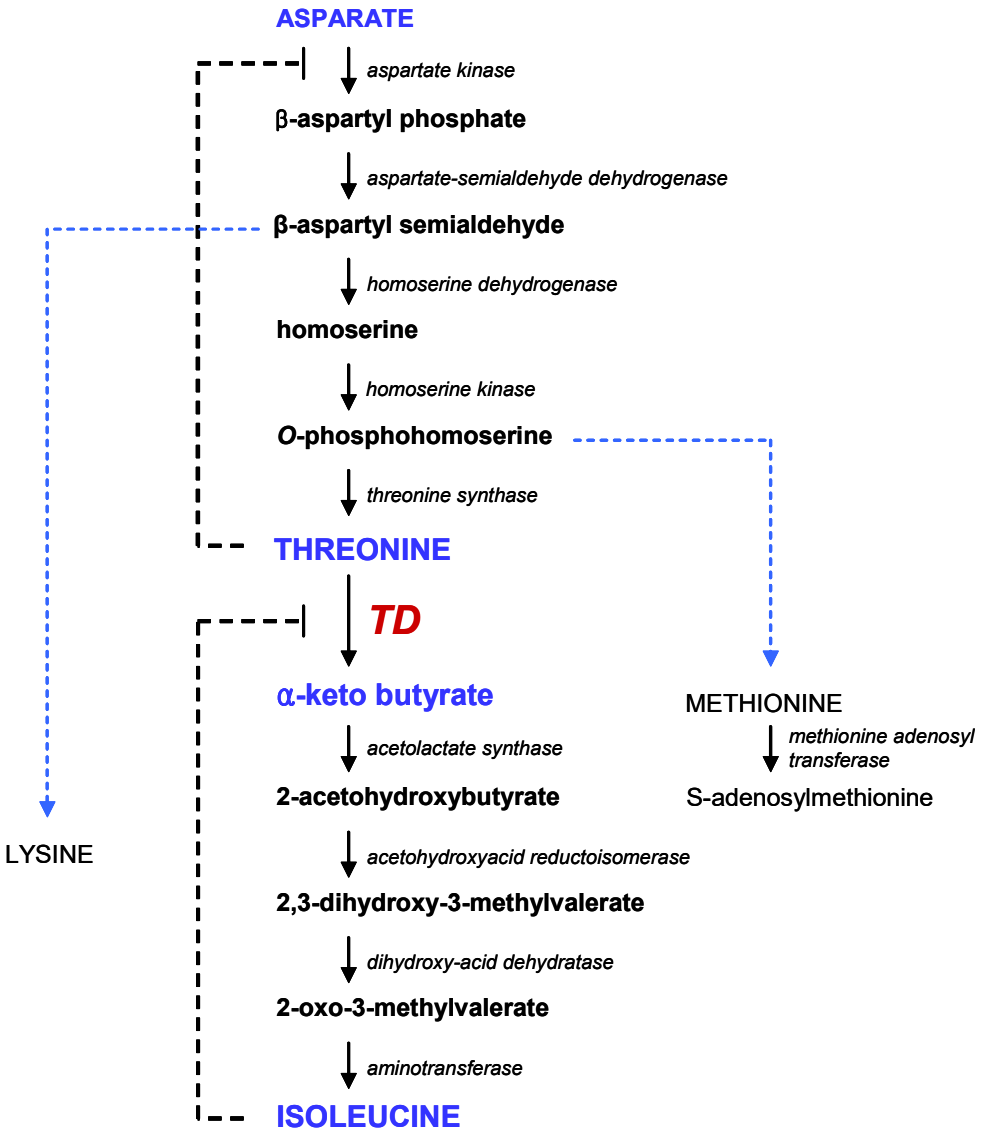

Figure 1. The aspartate metabolic pathway of higher plants. Several enzymatic steps are indicated by dotted arrows.

converted to β-aspartyl semialdehyde in a reaction catalyzed by the enzyme aspartate semialdehyde dehydrogenase. From this point, the pathway divides in two branches: one leads to lysine biosynthesis, the other branch divides into two sub-branches with one leading to the biosynthesis of threonine and isoleucine and the other to the biosynthesis of methionine. Lysine is produced from β-aspartyl semialdehyde in a

series of seven enzymatic reactions initiated by the enzyme dihydrodipicolinate synthase. In the other branch, β-aspartyl semialdehyde is reduced to homoserine in a reaction catalyzed by the enzyme homoserine dehydrogenase. Homoserine is phosphorylated to *O*-phosphohomoserine by the action of the enzyme homoserine kinase, which is then converted to threonine by the enzyme threonine synthase. Isoleucine is produced from threonine after five enzymatic reactions. The synthesis of the amino acid methionine follows a separate branch starting from *O*phosphohomoserine via three enzymatic reactions involving the enzymes cystathionine *γ*-synthase, cystathionine β-lyase, and methionine synthase. S-adenosylmethionine (SAM), a major methyl donor in plants, is synthesized from methionine in a reaction catalyzed by the enzyme *S*-adenosylmethionine synthetase (Azevedo et al., 1997; Singh, 1999; Coruzzi and Last, 2000; Azevedo, 2002).

The first committed step of isoleucine synthesis is the dehydration and deamination of threonine to yield α-keto butyrate and ammonia, catalyzed by threonine deaminase (TD). In microorganisms, two forms of the enzymes are present. One form is inhibited by threonine and is considered to be "biosynthetic." A second form is not subject to feedback inhibition and has been termed the "biodegradative" form (Umbarger, 1978). The enzyme is subject to feedback inhibition by isoleucine (Sharma and Mazumder, 1970), and is localized in the chloroplast (Kagan et al., 1969). The absolute requirement of TD for isoleucine biosynthesis was first demonstrated by the isolation of the isoleucine auxotrophic mutant in *Nicotiana plumbaginifollia*, which has no detectable TD activity (Sidorov et al., 1981). When this mutant was transformed with the *Saccharomyces cerevisiae* ILV gene that encodes TD, the transformed lines could be grown on a medium without Ile (Colau et al., 1987).

The gene encoding the biosynthetic TD has been isolated from tomato, potato, and wild tobacco *Nicotine attenuata* (Samach et al., 1991; Hildmann et al., 1992; Hermsmeier et al., 2001). The open reading frame of TD in *N. attenuata* encodes a polypeptide of 601 amino acids with a calculated molecular mass of 65.6 kD (Hermsmeier et al., 2001). Alignments of the amino acid sequence of *N. attenuata* with other sequences available in databases revealed similarities of 73% to tomato TD (Samach et al., 1991), 73% to *Arabidopsis* TD (Lin et al., 1999), 59% to yeast TD (Kiellandbrandt et al., 1984), 58% to *Escherichia coli* TD (Cox et al., 1987), and 46% to human Ser deaminase (Ogawa et al., 1989).

Tissue-specific expression of TD was investigated in undamaged plants during vegetative growth, bolting, and flowering. Surprisingly, the expression of TD was 50- to 500-fold higher in tomato floral organs than in roots and leaves (Samach et al., 1991). In *N. attenuata,* TD transcripts were readily detected in apical buds of the developing axis, barely detectable in the stems of the vegetative stage and the leaves of bolting plants, and not detectable in roots (Hermsmeier et al., 2001). The reason for this high expression of TD in floral organs is unclear. The effects of abiotic and biotic stimuli on TD mRNA accumulation were also investigated. Accumulation of TD-specific transcripts increased dramatically upon herbivory (Hermsmeier et al., 2001), application of methyl jasmonic acid (Hildmann et al., 1992; Samach et al., 1995; Hermsmeier et al., 2001), and in response to wounding (Schittko et al., 2001). Although the function of TD is not understood, the similar responses might reflect the need for precursors of flower development and stress-inducible secondary metabolites derived from the isoleucine pathway.

Plant signaling cascades

Wound- and herbivore-induced resistance are largely mediated by products of the "octadecanoid" (C18-fatty acids) pathway. The production of various defense-related compounds, e.g., toxins, antinutritive and antidigestive enzymes, requires signaling by octadecaniods, such as 12-oxophytodienoic acid (OPDA), jasmonic acid (JA), and methyl jasmonic acid (MeJA), all derived from linolenic acid (Creelman and Mullet, 1997; Kessler and Baldwin, 2002). The octadecanoid pathway also regulates developmental processes. The involvement of the JA pathway in regulating TD was demonstrated by analyzing antisense lipoxygense (aslox) transgenic plants. When attacked by *M. sexta* larvae, the aslox plants showed reduced levels of JA and TD expression compared to wild-type plants (Halitschke and Baldwin, 2003). The role of JA signaling was also shown when a sterile mutant of tomato (*jasmonic acid-insensitive1* [*jai1*]) that is defective in JA signaling was characterized. *jai1* plants exhibited several defense-related phenotypes, including the inability to express JA-responsive genes (e.g., proteinase inhibitors, cathepsin D inhibitor, and TD), severely compromised resistance to two-spotted spider mites, and abnormal development of glandular trichomes (Li et al., 2004).

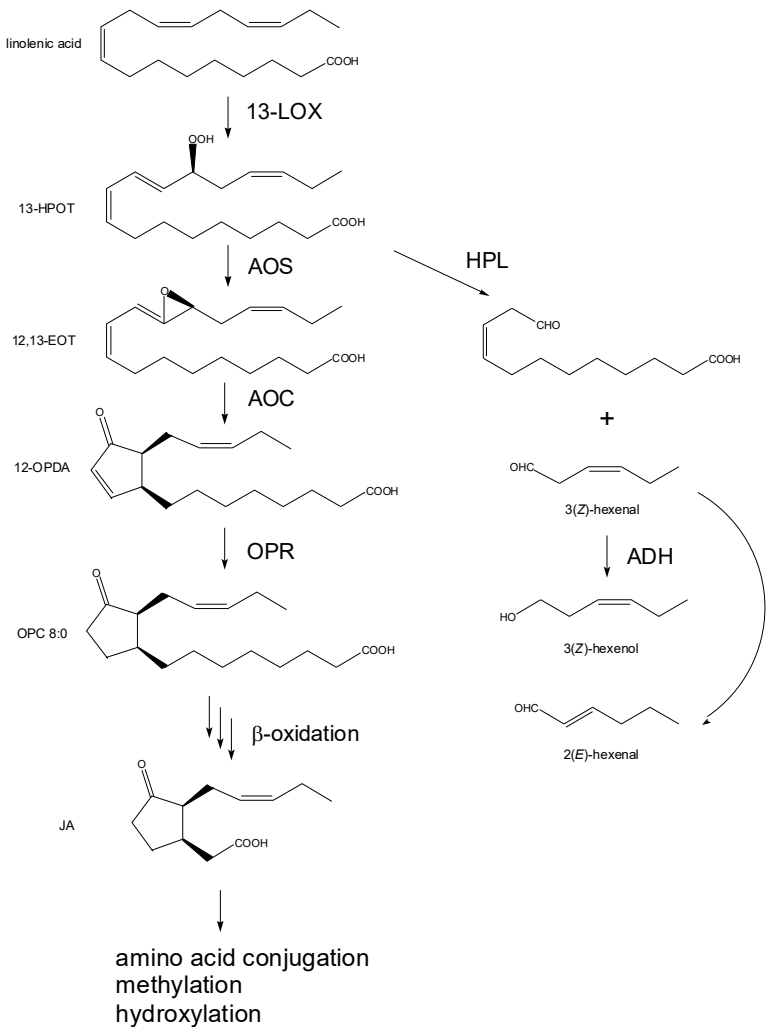

Figure 2. Biosynthesis of jasmonates and green leaf volatiles.

Abbreviations: 13-LOX, 13-lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, 12-oxo-phytodienoic acid reductase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase, 13-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadeca-dienoic acid; 12,13-EOT, 12,13(*S*)-epoxyoctadecatrienoic acid; 12-OPDA, 9(S)/13(S)-12-oxo-phytodienoic acid; OPC 8:0, 3-oxo-2(2'pentenyl)-cyclopentane-1-octanoic acid; JA, 3(R)/7(S)-jasmonic acid.

The octadecanoid pathway (Vick and Zimmerman, 1984) involves the regio- and stereospecific dioxygenation of linolenic acid (LA) by a 13-lipoxygenase (13-LOX); formation of an epoxide by allene oxide synthase (AOS); ring formation by allene oxide cyclase (AOC); reduction by OPDA reductase (OPR), and side-chain

shortening by three consecutive β-oxidation steps. A second class of oxylipins, C_6 aldehydes, alcohols, and their esters, is produced by the hydroperoxide lyase pathway (Fig. 2). Some of the naturally occurring JA derivatives are formed during JA biosynthesis, for example, 9, 10-hydro JA (Gundlach and Zenk, 1998), whereas most of them are metabolic products originating from JA. Among them are methyl, glucosyl, and gentobiosyl esters, *O*-glucosylates of 11- and 12-OH-JA, *O*-glucolytes of cucurbic acid, and conjugates of amino acids (Sembdner et al., 1994). In many plants, amino acid conjugates of JA are permanent constituents in addition to JA (Kramell et al., 1995). Predominant among them is the isoleucine conjugate, which accumulates in response to stress (Kramell et al., 1995; Kramell et al., 2000). In leaves, its level can reach up to 10% of the amount of JA, whereas in flowers, its level can far exceed that of JA (Knoefel and Sembdner, 1995; Hause et al., 2000). The biosynthesis of JA appears to involve three different compartments: the conversion of LA to OPDA is localized in the chloroplasts (Vick and Zimmerman, 1987; Song et al., 1993), while the reduction of racemic OPDA to OPC-8 : 0 occurs in the cytoplasm (Schaller and Weiler, 1997a, b); finally, the postulated steps of β-oxidation, i.e. conversion of OPC-8 : 0 to JA, are believed to occur in peroxisomes (Gerhardt, 1983; Vick and Zimmerman, 1984).

Nicotiana attenuata as a model system

The wild tobacco plant *Nicotiana attenuata* Torr. ex Watson (synonymous with *Nicotiana torreyana* Nelson & Macbr.) is an annual plant growing in disturbed desert habitats in southwestern USA. *N. attenuata* colonizes and dominates the vegetation of burned areas for the initial years after a fire (Fig. 3). Its occurrence on nitrogen-rich soils with low interspecific competition is regulated by the synchronization of seed germination by smoke-derived positive cues (Baldwin et al., 1994) and inhibition by allelochemicals in the litter of other plant species (Preston et al., 2002). The initially high population densities of this ephemeral pioneer plant decline with the immigration of stronger competitors. Potential herbivores have to recolonize burned areas and establish new populations with every new generation of plants. Hence, this native tobacco encounters highly variable herbivore and pathogen challenges. *N. attenuata* (2n=24) is largely self-compatible but has maintained features for outcrossing. Selfing and generation times of 2-3 months make this plant useful for laboratory studies in general and genetic engineering in particular.

N. attenuata is a particularly useful system in which to study herbivore-resistance responses. Not only is it well established that JA signaling mediates herbivore resistance in the field (Baldwin, 1998; Kessler and Baldwin, 2001; Kessler et al., 2004), but also, the direct and indirect defense traits with which JA signaling

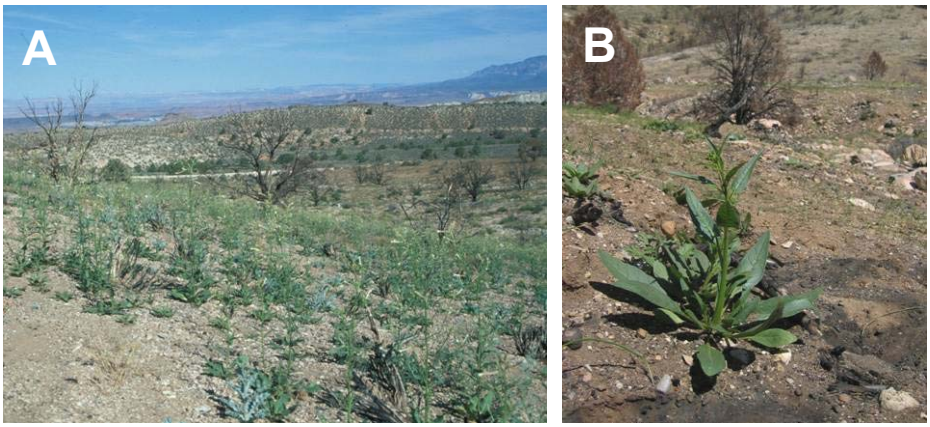

Figure 3. The wild tobacco plant *Nicotiana attenuata* in its natural habitat, the Great Basin desert in southwest Utah, USA. *N. attenuata*: population (A) and individual (B). Sources: A, Andre Kessler; B, Danny Kessler.

influences herbivore resistance are known (Halitschke et al., 2004; Steppuhn et al., 2004; Zavala et al., 2004b). *N. attenuata* is attacked by herbivores from more than 20 different taxa, including mammalian browsers that consume entire plants as well as intracellular feeding insects, and functions as a host likewise to polyphagous and oligophagous organisms. The larvae of leaf-chewing insect herbivores *Manduca sexta* (Linnaeus) and *Manduca quinquemaculata* (Haworth) are major defoliators of wild *N. attenuata* (Fig. 4). Larvae of other leaf-chewing insect herbivores, *Heliothis virescens* (Fabricius) and *Spodoptera exigua* (Huebner) (Lepidoptera, Noctuidae), feed similarly, but are both polyphagous and serious pests on many crops and only occasionally observed on native *N. attenuata* plants. The cell-content feeding herbivore *Tupiocoris notatus* (Distant; Heteroptera, Miridae, Dicyphina) is also found on *N. attenuata* and native plant populations and Solanaceaous crops (*N. tabacum, L. esculentum*).

The responses of *N. attenuata* to the Solanaceous specialist *M. sexta* are particularly well understood. The attacked plant reorganizes its wound response when 8 fatty acid amino acid (FACs) conjugates, present in the herbivore's oral secretions (OS) and regurgitants are introduced into plant wounds during feeding. The reorganization begins with a dramatic JA burst in the attacked leaves (Schittko et al., 2000), which changes the expression of numerous genes and the accumulation and release of secondary metabolites, such as nicotine, flavonoids, phenolics, diterpene sugar esters, and proteinase inhibitors (Halitschke et al., 2000; Kahl et al., 2000; van Dam et al., 2001; Halitschke et al., 2003; Roda et al., 2004; Zavala et al., 2004b). Moreover, since *N. attenuata* is a native plant that has not undergone artificial selection, functional associations between defense traits are likely to result from natural selection.

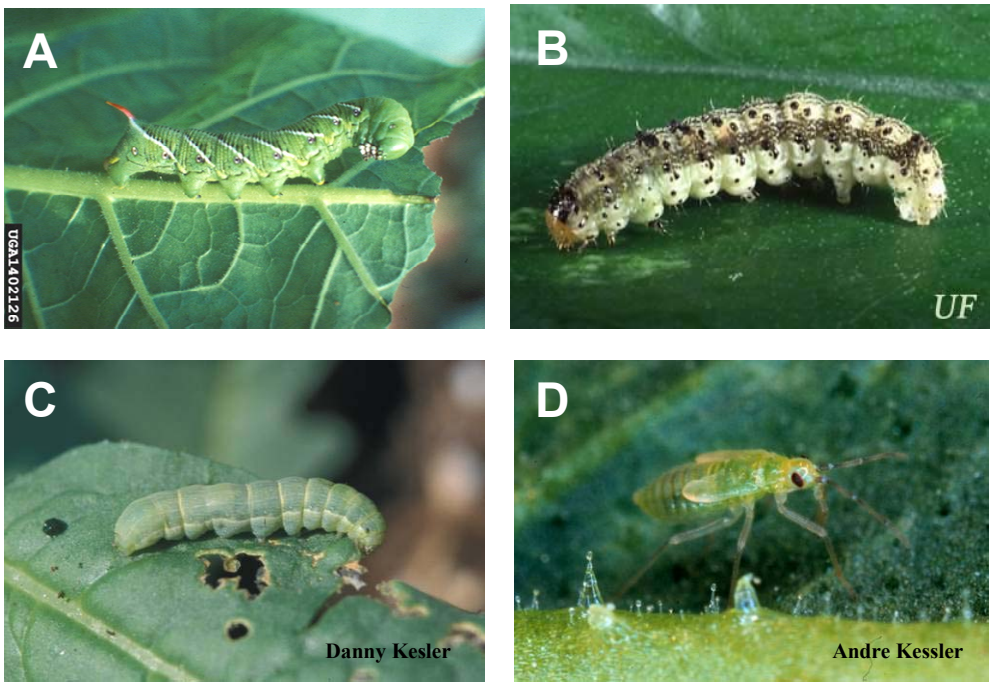

Figure 4. Herbivores of *Nicotiana attenuata*. **A,** *Manduca sexta* (tobacco hornworm); **B,** *Heliothis virescens* (tobacco budworm); **C,** *Spodoptera exigua* (beet armyworm); **D,** *Tupiocoris notatus* (suckfly). Sources: A, B, http://www.ipmimages.org; C, Danny Kessler; D, Andre Kessler.

To study plant-herbivore interaction, a subset of *N. attenuata*'s transcriptome, which had been isolated from plants attacked by *M. sexta* larvae and *Tupiocoris notatus* bugs, was analyzed by differential screening procedures such as differential display, subtractive libraries, or cDNA-AFLP (Hermsmeier et al., 2001; Halitschke et al., 2003; Hui et al., 2003; Voelckel and Baldwin, 2003). Further comparative transcriptional analyses were conducted with customized microarrays to examine which genes are regulated by herbivory, pathogen infection, or abiotic stress (Voelckel and Baldwin, 2004a, b). Among the genes isolated and tested, TD was one of the genes highly and specifically induced by herbivory.

The objective of this thesis is to elucidate why TD is elicited by herbivory and why TD is developmentally regulated during seedling growth and flower development by characterizing antisense TD transgenic plants, TD-VIGS plants, and TD promoters.

Manuscript I describes the involvement of TD in plant development. The characterization of asTD transgenic plants revealed that TD is important for root growth and cotyledon opening during seedling growth; and for stigma and anther development during flower development. Analysis of TD promoter-GUS fusion transgenic plants also showed that TD was specifically expressed in cotyledons, anthers, and stigmas, supporting the role of TD in seedling growth and flower development.

Manuscript II describes TD promoter analysis in detail. Promoter deletion analysis defined which regions of TD promoter are important for tissue-specific expression in cotyledons, stigmas, and anthers as well as which regions of TD promoter are responsible for TD induction in leaves by wounding and MeJA treatment.

Manuscript III details the involvement of TD in plant defense and shows that JA-Ile conjugate is the signal molecule for plant herbivore defenses. In JA-Ile synthesis TD produces the isoleucine pool at the wound site, which is subsequently conjugated with JA to form JA-Ile, and in turn elicits two potent direct defenses, nicotine and trypsin protease inhibitors.

Manuscript I

Reviewed: Plant Cell and Environment (2006)

Running Title: TD suppression influences plant development and JA-Ile signaling

The role of threonine deaminase in seedling growth and flower development in *Nicotiana attenuata*

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ABSTRACT

Threonine deaminase (TD), the first enzyme to convert threonine to α-keto butyrate (α-KB) in the isoleucine (Ile) biosynthetic pathway, is up-regulated after herbivore attack and methyl jasmonate (MeJA) elicitation, and constitutively expressed in floral buds in *Nicotiana attenuata*. To understand these unusual patterns of expression, we silenced TD by virus-induced gene silencing (VIGS) as well as by *Agrobacterium*-mediated transformation. Plants whose TD had been silenced by VIGS had shortened pistils and anthers that reverted to petals. Antisense-oriented TD transgenic plants (asTDS) were stunted in their growth and delayed in their cotyledon opening and leaf senescence, and produced flowers with shortened pistils and anthers that reverted to petals; that is, all tissues in which TD promotor:GUS transformants revealed high TD expression. The seedling phenotypes of asTDS plants were partially recovered by supplementing the germination media with α-KB, Ile, or jasmonic acid-isoleucine (JA-Ile). The shortened pistils, poor pollen production, petaloid anthers, and reduced trichome numbers are defects shared with other JA-signaling mutants. Floral buds had higher-than-usual jasmonic acid (JA) levels but unaltered salicylic acid levels, and reduced α-KB, Ile, JA-Ile, and trypsin proteinase inhibitor levels. Clearly, TD plays various roles in plant development, some of which are likely attributable to TD's function in supplying Ile for JA-Ile signaling.

Key-words: threonine deaminase, jasmonic acid, jasmonic acid-isoleucine conjugate, trypsin proteinase inhibitor, promoter, GUS.

INTRODUCTION

Threonine deaminase (TD) catalyzes the formation of α-keto butyrate (α-KB) from threonine (Thr), the first step in the biosynthesis of isoleucine (Ile). In plants, the enzyme is active in the biosynthetic pathway of the aspartate-derived amino acids lysine, methione, Thr, and Ile. TD is subject to feedback inhibition by Ile (Umbarger 1978) and is localized in the chloroplast. When a tobacco mutant that lacked TD activity and required Ile for growth (Sidorov, Menczel & Maliga 1981) was transformed with the *Saccharomyces cerevisiae* ILV gene that encodes TD, the transformed plant could be grown on a medium without Ile (Colau *et al.* 1987). Inhibiting TD by 2-(1-cyclohexen-3(R)-yl)-S-glycine proved lethal to *Arabidopsis* and was reversed by supplements of α-KB or Ile (Szamosi, Shaner & Singh 1994), thereby demonstrating the essential role of TD in Ile synthesis. While these experiments demonstrate that plants can't live without TD, they don't address its unusual expression pattern in solanaceous plants, a pattern that suggests it plays additional roles, namely, in development and defense.

TD was first cloned as a result of its unusual up-regulation in tomato flowers. The first 80 amino acids have a putative chloroplast transit peptide and its mRNA expression is 500-fold higher in flowers than in leaves or roots (Samach *et al.* 1995; Samach *et al.* 1991). In potato, TD expression was found to be elicited by wounding and methyl jasmonic acid (MeJA) treatment (Hildmann *et al.* 1992). TD was isolated from the diploid tobacco *Nicotiana attenuata* by mRNA differential display after plants were attacked by larvae of the solanaceous specialist herbivore *Manduca sexta*. TD was also found to be strongly elicited by MeJA treatment and constitutively expressed at high levels in floral buds, but not in leaves and roots (Hermsmeier, Schittko & Baldwin 2001). Additionally, TD has been cloned from developing tomato fruits (Tieman & Handa 1996) and immature seeds of chickpea (*Cicer arietinum* L.) (John, Srivastava & Guhamukherjee 1995). Collectively, these patterns of expression suggest similarities between TD activity and jasmonic acid (JA) accumulation.

Though JA is known to mediate various responses to biotic and abiotic stresses, such as wounding (Lee & Howe 2003; Li, Li & Howe 2001; Park *et al.* 2002), herbivory (Baldwin 1998; Halitschke & Baldwin 2003; Kessler, Halitschke & Baldwin 2004; Walling 2000), and pathogen attack (Seo *et al.* 2001; Vijayan *et al.* 1998; Xie *et al.* 1998), it has also been implicated in several developmental processes in plants, including tuber formation (Kolomiets *et al.* 2001), root growth (Staswick & Tiryaki 2004), anther and pollen development (Ishiguro *et al.* 2001; Li *et al.* 2004; Stintzi & Browse 2000; Xie *et al.* 1998), and leaf senescence (He *et al.* 2002). Flowers contain many jasmonates, some of which include JA and its volatile methyl esters and amino acid conjugates. JA and MeJA have been isolated from the pollen and anthers of *Camellia* species (Yamane, Abe & Takahashi 1982). The JA conjugates, JA-Ile and JA-tyramine, have been isolated from the pollen of *Pinus mugo* (Knöfel & Sembdner 1995) and *Petunia hybrida* (Miersch *et al.* 1998), respectively. Particular oxylipins, namely, JA, cis(+) 12-oxophytodienoic acid, JA-Ile, and their methyl esters, show different patterns of accumulation during tomato flower development. Unique oxylipin signatures in different organs (flower stalks, sepals, stamens, pistils) suggest that these compounds influence flower development (Hause *et al.* 2000; Miersch *et al.* 2004). Thus JA seems to play a dual role, in both defense and development.

To examine the effect of TD, we expressed 1.4 kb of the *Na*TD in an antisense orientation in the native tobacco *N. attenuata*. Transformed lines were easily characterized as having one of two growth phenotypes: 1) plants with mildly reduced TD expression and activity, but otherwise displaying wild type (WT) growth and development patterns (asTDM plants) and 2) plants with severely reduced TD expression and activity, displaying retarded growth (asTDS plants). In a separate paper (Kang and Baldwin, in review), we demonstrate that asTDM plants are more susceptible to *M. sexta* attack than are WT plants. This can be attributed to reductions in subtle aspects of JA signaling, specifically, the production of JA-Ile, and in the levels of direct defenses (nicotine, trypsin proteinase inhibitors [TPI]) that correlate

with reduced levels of JA-Ile. That these defense phenotypes can be restored by applying JA-Ile to asTDM plants implies that TD is involved in herbivore defense by regulating Ile formation at the wound site and that Ile is involved in the conversion of JA to JA-Ile.

Here, we report that the flowers of plants whose TD has been silenced by virus-induced gene silencing (VIGS) are morphologically different than the flowers of plants inoculated with *Agrobacterium*-containing empty vector. We demonstrate in detail that the young seedlings and flowers of plants with strongly silenced TD (asTDS) plants have morphological differences in compared to those of WT plants; moreover, their floral buds have impaired direct defenses, a state that is associated with both reduced levels of α-KB and an imbalance of JA and JA-Ile. TD promoter analysis in plants transformed with TD promoter: GUS constructs demonstrated that TD is specifically expressed in seedlings and floral organs, as well as strongly elicited by MeJA treatment in leaves. Together these observations suggest that it plays an important role in development as well as in defense.

MATERIALS AND METHODS

Plant materials and growth conditions

An inbred genotype of *Nicotiana attenuata* Torr. Ex Wats. (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae), originally collected from southwestern Utah in 1988, was transformed and used for all experiments. Seeds were sterilized and sown on germination media [Gamborg's B5 medium with minimal organics: (Sigma) and 0.6% (w/v) phytagel (Sigma)], and maintained in a growth chamber (Percival, Perry Iowa, USA) at 26ºC/16 h 155 µm/s/m² light, 24ºC/8 h dark as described previously (Krügel *et al.* 2002). Ten-day-old seedlings were planted in soil in Teku pots (Waalwijk, The Netherlands) and, once established, transferred to 1 L pots in soil and grown in the glasshouse at 26-28ºC, under 16 h light supplemented by Philips Sun-T Agro 400 or 600W Na lights.

Chemical treatments

In order to examine the effects of amino acids and jasmonates on seedling growth, seeds were germinated and grown on germination media supplemented with filter-sterilized stock solutions of Thr, α-KB, Ile, JA, JA-Ile, or MeJA. To prepare the stock solution, 50 mM of Thr, α-KB, and Ile were dissolved in water and 5 mM of JA, JA-Ile, and MeJA were dissolved in 10% ethanol and subsequently filter-sterilized. Hypocotyl and root length were measured 15 days after germination. Cotyledon opening was measured as the separation of a seed coat from cotyledons and subsequent splitting of cotyledons.

The leaf undergoing the source-sink transition was designated as growing at node 0. For MeJA-treated plants, the leaf growing at node +1, which is older by one leaf position than the source-sink transition leaf, was treated with 150 µg (0.625 µmole) of MeJA in 20 µl of lanolin paste as described previously (Halitschke *et al.* 2000).

Generation and characterization of VIGS plants

PCR was used to generate TD fragments from *N. attenuata* in antisense orientations with the following primer pairs: TD forward primer; 5'- GCGGCGGGATCCGCAC CAAATGGCTCAACTCC-3', TD reverse primer; 5'- GCGGCGGTCGACGTCAT GCCTGTTACCACACC-3'. The obtained TD (335 bp) PCR fragment were digested with *Bam*HI and *Sal*I. The resulting fragments were cloned into the pTV00 vector digested with the same enzymes. The pTV00 vector is a 5.5 kb plasmid with an origin of replication for *E. coli* and *A. tumefaciens* and a gene for kanamycin resistance (Ratcliff, Martin-Hernandez & Baulcombe 2001). *A. tumefaciens* (strain GV3101)-mediated transformation procedure was previously described (Saedler & Baldwin 2004). Phytoene desaturase (PDS) gene was used to monitor the progress of VIGS. Plant growth and inoculation conditions were as previously described (Saedler & Baldwin 2004).

Generation and characterization of asTDS transgenic lines

For the plant transformation vector, a 1349 bp portion of the *N. attenuata* TD cDNA resident on plasmid pTD13 (Hermsmeier, Schittko & Baldwin 2001) was PCR amplified using primers 5'-GCGGCGCCATGGCATAGGTCCCACAAGTTCGC-3' and 5'-GCGGCGGGTCACCTGGAAGTTCTTTGTCAAGCC-3'. The obtained 1.4 kb PCR fragment was cut with *Bst*EII and partially cut with *Nco*I. The resulting 1.4 kb fragment was cloned in pNATGUS3 (Krügel *et al.* 2002) and digested with the same enzymes, resulting in plant transformation vector pNATTD1 (10.1 kb), which contained in its T-DNA a 1.4 kb fragment of TD in an antisense orientation under the control of the 35S promoter of the cauliflower mosaic virus. The *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure and the transformation vector have been previously described (Krügel *et al.* 2002). Progeny of homozygous plants were selected by NTC resistance screening and screened for the desired phenotype, namely, reduced MeJA-induced α-KB accumulation. For all

experiments, T_2 homozygous lines, each harboring a single insertion that was confirmed by Southern blot analysis (Fig. S1), or WT plants were used.

Nucleic acid blot analysis

Extraction of total RNA and Northern blot analysis was performed as described previously (Winz & Baldwin 2001). Genomic DNA was extracted from leaves as described previously (Richard 1997) and 10 µg of DNA digested with *Eco*RI and blotted onto nylon membranes. To prepare the probe, plasmid pTD13 (GenBank accession no. AF229927) containing full-length cDNA of TD was cut with *Pst*I and gel-eluted using Geneclean Kit (BIO 101, Vista, CA, USA), labeled with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little Chalfont, UK), and purified on G50 columns (Amersham-Pharmacia). After overnight hybridization, blots were washed three times with 2xSSPE at 42°C and one time with 2xSSPE / 2% SDS at 42°C for 30 min, and analyzed on a phosphoimager (model FLA-3000; Fuji Photo Film Co., Tokyo, Japan).

TD activity measurement

Floral buds (5 - 10 mm) were homogenized in 2 volumes of extraction buffer (100 mM Tris buffer [pH 9], 100 mM KCl, and 10 mM β-mercaptoethanol) and centrifuged at 15,000 g for 15 min at 4°C. TD activity was assayed by incubating the enzyme with substrate and determining the quantity of α-KB formed. The α-KB was estimated by modifying the method as described previously (Sharma & Mazumder 1970). Protein extract (100 µL) was added to the same volume of reaction buffer (40 mM L-threonine, 100 mM Tris buffer [pH 9], and 100 mM KCl). After incubation at 37°C for 30 min, 160 µL of 7.5% trichloracetic acid was added to stop the reaction and the protein precipitate was removed by centrifugation at 10,000 g for 2 min. The α-KB was determined by adding 400 µL of 0.05% dinitrophenylhydrazine to the sample solution. After incubation at room temperature for 10 min, 400 µL of 4N sodium hydroxide was added to the sample solution and mixed well. After incubation

at room temperature for 20 min, the absorbance of the sample solution was read at 505 nm in spectrophotometer (model Ultraspec®3000; Pharmacia Biotech, Cambridge, England).

JA and SA measurement

Floral buds (5 - 10 mm) were harvested and immediately frozen in liquid nitrogen. Samples were homogenized in 3 volumes of extraction buffer (acetone: 50 mM citric acid, 7:3 [v/v]) with the FastPrep extraction system FP120 (Savant Instruments, Holbrook, NY, USA). Samples were homogenized by being shaken at 6.0 m/sec for 90 sec in extraction tubes containing 900 mg of lysing matrix (BIO 101, Vista, CA, USA). Samples were analyzed by GC-MS with labeled internal standards ([1,2-^{13}C] JA and D_4-SA) as previously described (Heidel & Baldwin 2004).

Ile and JA-Ile measurement

Floral buds (5 – 10 mm) were harvested and immediately frozen in liquid nitrogen. Samples were homogenized in 3 volumes of extraction buffer (acetone: 50 mM citric acid, 7:3 [v/v]). Samples were centrifuged at 13,000 rpm for 15 min at 4 ^{o}C and supernatants were transferred to a new tube. The pellet was re-extracted with extraction buffer and supernatants were added to first supernatants. The combined supernatants were evaporated to dryness in a heating block and the remaining aqueous phase was extracted 3 times with 1 mL of ether. The ether was evaporated completely and dissolved with acetonitrile. The samples were separated by an Agilent LC1100 HPLC system (Agilent, Waldbronn, Germany) with degasser, binary pump, autoinjector, and column thermostat, and detected by a DAD coupled with a LCQ DECA XP mass spectrometer (Thermo-Finnigan, Egelsbach, Germany). Mobile phase A consisted of 0.5% acetic acid in water and mobile phase B of 0.5% acetic acid in acetonitrile. The mobile phase gradient was increased linearly from 20% B (initial value) to 50% B at 16 min, then held constant at 50% for 25 min, then increased linearly to 100% B at 30 min. The mobile phase flow was 0.7 mL/min and

the injection volume 30 µL. Stationary phase was a Luna 5µ C18 column (250 x 4.60 mm, 5 µm particle size, Phenomenex, Aschaffenburg, Germany). The MS conditions were as follows: APCI Ion source: 500°C vaporizer temperature; 275°C capillary temperature; 10 µA discharge current; sheath gas: nitrogen, 50 (arbitrary units); auxiliary gas; nitrogen, 30 (arbitrary units). Three MS/MS ion acquisition segments were programmed as follows: 1) 1 – 10 min: m/z 132 @ 23 positive polarity for Ile; 2) 10 – 17.5 min: m/z 155 @ 28 negative polarity for 2-chloro benzoic acid (CBA, internal standard); 3) 21.5 – 30 min: m/z 324 @ 30 positive polarity for JA-Ile. Standard curves were constructed with known quantities of Ile and JA-Ile and used for quantification of those chemicals in samples.

Analysis of TPI activity

Trypsin protease inhibitor (TPI) activity was analyzed by radial diffusion activity assay as previously described (Van Dam *et al.* 2001).

Construction of the TD promoter:GUS reporter gene fusion construct, plant transformation, and measurement of GUS activity

The TD promoter region (1.9 kb) was isolated by PCR from the *N. attenuata* genomic clone (GenBank accession no. AY928105) using the primers 5'-CGGAATTCGAA ACCAAAGCTACAGGGTTCGATC-3' and 5'-CATGCCATGGATCGTACTATATT TGATAGAGG-3', which contain an *Eco*RI and an *Nco*I site, respectively. The resulting PCR product was cloned into the pUC19 vector and sequenced. Next, the TD promoter region was cut from the pUC19 vector by *Eco*RI and *Nco*I digestion and inserted into the binary vector pCAMBIA1301, replacing the 35S promoter cauliflower mosaic virus so that the TD promoter directed the expression of the uidA (β-glucuronidase [GUS]) gene. This vector was called pTD1836. For negative control, pCAMBIA1300, which does not contain GUS gene, was used. The constructs were introduced into *A. tumefaciens* strain LBA4404, which

was used to transform *N. attenuata* as previously described (Krügel *et al.* 2002). Lines transformed with the construct were selected using hygromycin.

Enzymatic assays with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide were performed to localize the GUS enzyme (Jefferson, Kavanagh & Bevan 1987). Tissue samples were incubated at 37°C in a modified GUS-staining buffer (80 mM sodium phosphate buffer [pH 7.0], 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 8 mM EDTA, 0.05% Triton X-100, 0.8 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, and 20% methanol). After overnight incubation, chlorophyll was extracted with 70% ethanol. Four fully-expanded, size-matched leaves from greenhouse-grown transgenic plants in the rosette stage were used for fluorometric GUS assays. Leaves were ground into a fine powder with liquid nitrogen and then vortexed with GUS extraction buffer (50 mM $NaPO_4$ [pH 7.0], 10 mM β-mercaptoethanol, 10 mM Na_2EDTA [pH 8.0], 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100). The extracts were centrifuged for 10 min in a microcentrifuge at 4°C, and the supernatants were used for a GUS assay using 4-methyllumbelliferyl- β-D-glucuronide (MUG) as a substrate, according to Jefferson (1987). MUG activity was expressed as fluorescence units pmole MU/mg protein/min.

RESULTS

TD suppression influences development in asTDS lines

To examine the function of TD in *Nicotiana attenuata* plants with normal patterns of development, we used a virus-induced gene silencing (VIGS) system optimized for *N. attenuata* (Saedler & Baldwin 2004) to silence *Na*TD mRNA in WT plants. To monitor the progress of VIGS, we silenced phytoene desaturase (PDS), a gene that oxidizes and cyclizes phytoene to α-and β-carotene; these are subsequently converted into xanthophylls of the antenna pigments of the photosystems of plants, which when silenced cause visible bleaching of the green tissues (Fig. 1a. left). TD-silenced (TD-VIGS) plants were similar to plants inoculated with an empty vector (EV-VIGS) during the vegetative-growth stage (Fig. 1a). However, TD-VIGS flowers have a different morphology compared to EV-VIGS flowers during the reproductive stage. EV-VIGS flowers developed normal pistils and stamens (Fig. 1b). In TD-VIGS flowers, some stamens developed into petals and their pistils were shorter than the stamens (12 out of 112 flowers, 11%, Fig. 1c). Although the VIGS procedure provides a rapid means of strongly silencing the expression of a target gene after late rosette-stage growth, it is not useful for gene silencing from seed to young seedling stages because gene silencing by VIGS with TRV-based vectors cannot be transmitted to seeds of the next generation.

To confirm and examine the function of TD in detail, we produced transgenic plants that express TD in an antisense orientation. T_2 homozygous plants were analyzed from independently transformed lines, each harboring a single copy of the transgene, as verified by segregation analysis for antibiotic resistance and Southern blot analysis (Fig. S1). Transformed lines were easily characterized as having one of two growth phenotypes: 1) plants with mildly reduced TD expression and activity, but otherwise wild type (WT) growth and development patterns (asTDM plants; Kang and Baldwin in review), and 2) plants with severely reduced TD expression and

activity, and retarded growth (asTDS plants). The two independently transformed lines (asTDS1 and asTDS2) showed similar developmental patterns (e.g., delayed

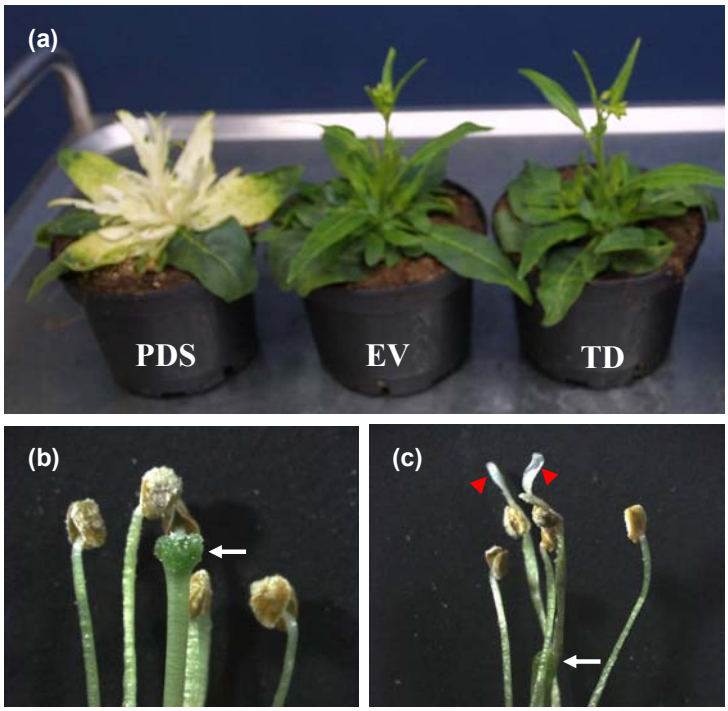


Figure 1. Phenotypic comparison between EV- and TD-VIGS plants. (a) PDS- (left), EV- (middle), and TD-suppressed plants (right) by VIGS 6 weeks after seed germination (2 weeks after inoculation with VIGS constructs). Plants were inoculated with *Agrobacterium*-harboring TRV constructs containing a 160 bp PDS fragment (PDS-VIGS), an empty vector (EV-VIGS), or a 335 bp *N. attenuata* TD fragment (TD-VIGS). (b and c) filaments and stamens of EV-VIGS (b) and TD-VIGS flowers (c). An arrow indicates the stigma. Note that the filament is shorter than the stamens in TD-VIGS flowers. An arrowhead indicates the petaloid stamens, which are only found in TD-VIGS flowers.

cotyledon opening and shortened pistils, Fig. S2) and reduced direct defenses (nicotine, trypsin proteinase inhibitor [TPI], Fig. S3) compared to WT plants. Here we characterize asTDS1 in detail to identify the role of TD in developmental processes.

One of the dramatic differences between WT and asTDS plants was the timing of cotyledon opening during seedling growth on germination media. Cotyledon

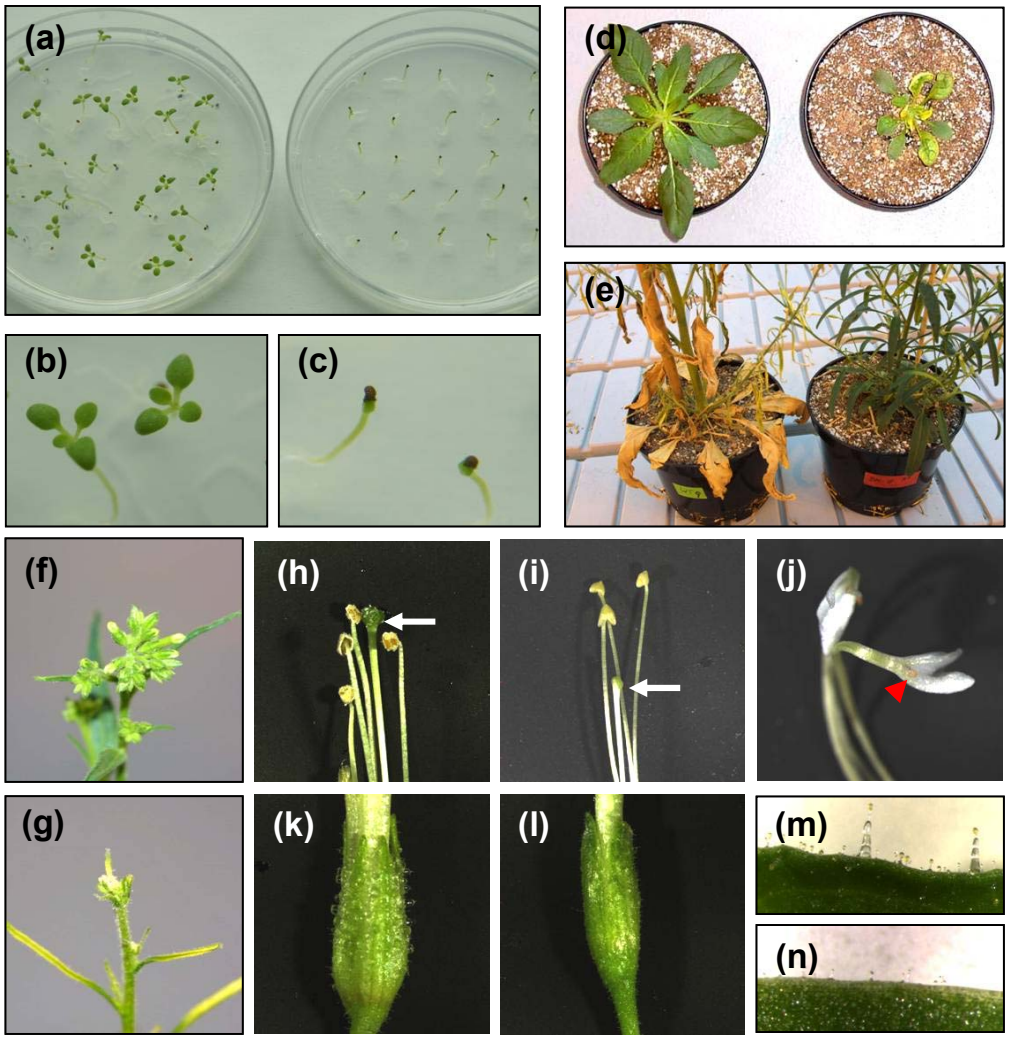


Figure 2. Phenotypic comparison between WT and asTDS1 plants. (a) 10-day-old seedlings of WT (left) and asTDS1 (right) plants. (b and c) Same magnification photographs of WT (a) and asTDS1 seedlings (b) as in (a), showing opened and unopened cotyledons in WT and asTDS1 seedlings, respectively. (d) Rosette stage of WT (left) and asTDS1 (right) plants 42 days after germination. Note the small size and yellowish green leaves in asTDS1 plants. (e) 94-day-old WT (left) and asTDS1 plants (right) after germination. Note the difference in senescence between WT and asTDS1 plants. (f and g) Young floral buds in WT (f) and asTDS1 plants (g). (h and i) Filaments and stamens of WT (h) and asTDS1 flowers (i). An arrow indicates the

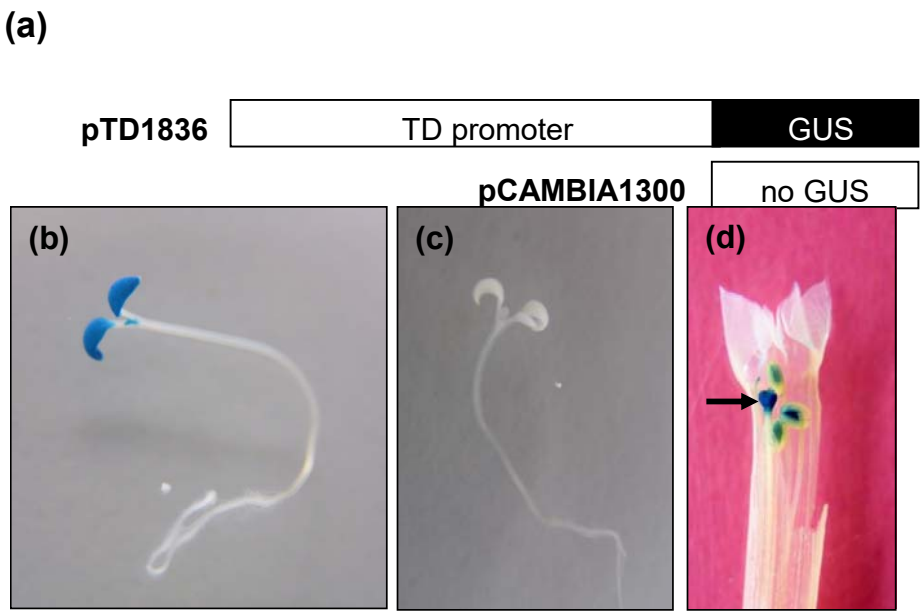
stigma. Note that the filament is shorter than the stamens in asTDS1 plants. (j) Petaloid stamens in asTDS1 flowers. An arrowhead indicates the rudimentary stamen. (k and l) Sepals of WT (k) and asTDS1 plants (l). Note trichome exudates in WT plants. (m and n) Same magnification photograph of WT (m) as of asTDS1 sepal (n). Note that only WT plants have multicellular glandular trichomes.

opening was highly delayed in asTDS1 seedlings (Figs. 2a-c). WT seeds began opening cotyledons 5 days after germination (DAG) and had fully opened cotyledons at 6 DAG, whereas asTDS1 seeds did not open their cotyledons until 10 DAG. Leaf color and size in rosette-stage plants also differed. Leaves of WT plants were larger and green, while those of asTDS1 plants were smaller and yellowish green (Fig. 2d). WT plants had completely senesced at 94 DAG, while asTDS1 plants remained green (Fig. 2e). As shown in TD-VIGS flowers, the morphology of asTDS1 flowers also differed: asTDS1 plants produced smaller and fewer floral buds compared to WT plants (Figs. 2f, g). The pistils and stamens of WT flowers are typically of similar length (Fig. 2h). However, in asTDS1 flowers, the pistils were substantially shorter than the stamens (Fig. 2i). In addition, some stamens developed into petals, though these petaloid stamens retained a structure reminiscent of normal stamens (Fig. 2j). The abnormalities in asTDS1 plants resulted in poor seed capsule production, and the seeds that matured from these capsules were lighter than those of WT plants. WT and asTDS1 plants differed in the average number of seed capsules produced per plant (44.3 ± 2.4 and 26.5 ± 2.6, respectively; mean ± SE; n = 8/genotype; unpaired t-test, P = 0.0002) and in the average seed weight (135.6 ± 2.3 and 119.8 ± 2.0, respectively; mean µg ± SE/ seed; n = 4 genotypes [100 seeds]/genotype; unpaired t-test, P = 0.002). The glandular trichomes of WT sepals produced copious quantities of exudates (Fig. 2k) but those of asTDS1 did not (Fig. 2l). WT plants produced two morphologically distinct glandular trichome types on their sepals: those with a unicellular stalk and those with an elongated multicellular stalk (Fig. 2m). Only a few

unicellular trichomes were produced by asTDS1 plants (Fig. 2n). The differences in trichome density (number/mm^2) were highly significant: unicellular trichomes (11.6 ± 0.6 [WT] and 5.2 ± 0.4 [asTDS1]; mean ± SE; n = 5/genotype; unpaired t-test, P < 0.0001) and multicellular trichomes (2.4 ± 0.2 [WT] and 0 ± 0 [asTDS1]; mean ± SE; n = 5/genotype; unpaired t-test, P < 0.0001).

Constitutive expression of the TD gene during seedling and flower development

To test whether TD was expressed in the same tissues that displayed the various developmental phenotypes observed in asTDS plants, the 1.8 kb TD promoter (Fig. S4) was fused to the β-glucuronidase (GUS) reporter gene (Fig. 3a: pTD1836). Homozygous T_2 transgenic plants containing this construct were obtained. Histochemical analysis revealed that GUS was strongly expressed in cotyledons and young primary leaves but not in hypocotyls and roots (Fig. 3b). Analysis of transgenic flowers found GUS expressed in stigma, stamens, and sepals, but not in styles, filaments, and petals (Fig. 3d, e). In addition, GUS was highly expressed in the trichomes of sepals (Fig. 3f). GUS was not expressed in the seedlings (Fig. 3c) and flowers (Fig. 3g) of homozygous T_2 transgenic plants containing pCAMBIA1300,



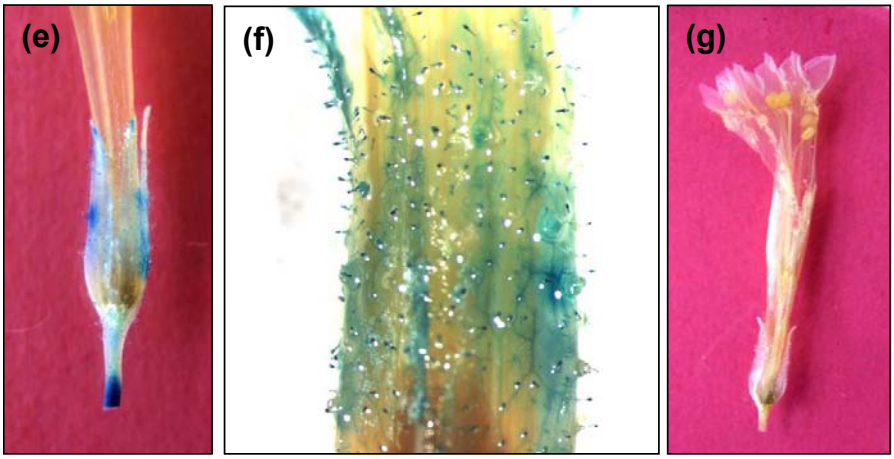


Figure 3. GUS expression of TD promoter-GUS fusion in transgenic seedlings and flowers. (a) Diagrammatic representation of the constructs used for transformation in *N. attenuata*. The 5' end of the TD promoter region was deleted to the end points indicated, with a 3' end at ATG translational start codon. These deleted promoters were inserted into the binary vector pCAMBIA1301, replacing the 35S promoter cauliflower mosaic virus, and used to transform *N. attenuata* plants (pTD1836). pCAMBIA1300, which does not contain the GUS gene, was used as a negative control. (b and c) Histochemical GUS staining of seedlings from a plant expressing TD-GUS fusion (pTD1836, b) and a control plant without GUS (pCAMBIA1300, c). (d and e) Histochemical GUS staining of flowers from a plant expressing TD-GUS fusion (pTD1836). An arrow indicates the stigma. (f) Enlargement of sepal region from a plant expressing TD-GUS fusion (pTD1836). Note the strong GUS expression in trichomes. (g) Histochemical GUS staining of flowers from a control plant without GUS (pCAMBIA1300). T_2 seedlings and flowers were incubated overnight with a GUS-staining buffer and washed with ethanol to remove chlorophyll.

which does not contain the GUS reporter gene. The organ-specific pattern of TD-GUS expression in cotyledons, stigma, stamens, and trichomes (Fig. 3) is consistent with

the growth phenotypes observed in asTDS plants, namely, delayed cotyledon opening, shortened pistils, and conversion of stamens into petals (Fig. 2).

In addition to its constitutive expression in floral buds, TD mRNA was also elicited by mechanical wounding, herbivore attacks, and MeJA treatment (Hermsmeier, Schittko & Baldwin 2001; Schittko, Hermsmeier & Baldwin 2001). When leaves of TD:GUS transgenic plants were treated with MeJA in a lanolin paste 24 h prior to analysis, GUS activity was significantly higher than in untreated controls (2420 ± 285 and 1452 ± 167, respectively; mean [pmole MU/mg protein/min] ± SE; n = 4/treatment and genotype; unpaired t-test, $P = 0.026$).

Effects of amino acids and jasmonates on asTDS seedling growth

Seedling growth differed dramatically between asTDS1 and WT lines (Fig. 2b, c). The average hypocotyl length of asTDS1 was 88% that of WT seedlings (Fig. S5a; unpaired t-test, $P = 0.0125$) and the average asTDS1 root length was 27% that of WT seedlings (Fig. S5b; unpaired t-test, $P < 0.0001$) at 15 DAG. In order to examine whether the reduced hypocotyl and root length and the delayed cotyledon opening resulted from TD suppression, seeds were germinated on medium supplemented with 0 – 400 µM Thr, α-KB, or Ile. In WT seedlings, all supplements reduced hypocotyl length by 19 – 30% (Fig. S5a; unpaired t-test, $P < 0.0001$) and root length by 41 – 57% compared to untreated controls (Fig. S5b; unpaired t-test, $P < 0.0001$). In asTDS1 seedlings, the overall reductions in hypocotyl length were less than those in WT plants (8 – 16% compared to the untreated controls [Fig. S5a; unpaired t-test, $P \leq 0.0207$]) but the patterns were similar. The responses in root growth of asTDS1 seedlings to supplemented Thr, α-KB, or Ile differed from those of WT seedlings. Thr had no significant effect, 200 µM α-KB increased root length by 20%, and both 200 and 400 µM Ile significantly increased root length by 93 - 118% (Fig. S5b; unpaired t-test, $P < 0.0001$) compared to untreated asTDS1 seedlings. Cotyledon opening in WT seedlings was slightly delayed by supplementing Thr, α-KB, and Ile. Cotyledon

opening in asTDS1 seedlings was slightly delayed by Thr but accelerated by α-KB, and Ile supplementation (Fig. 4, Fig. S6). These results demonstrate that in WT

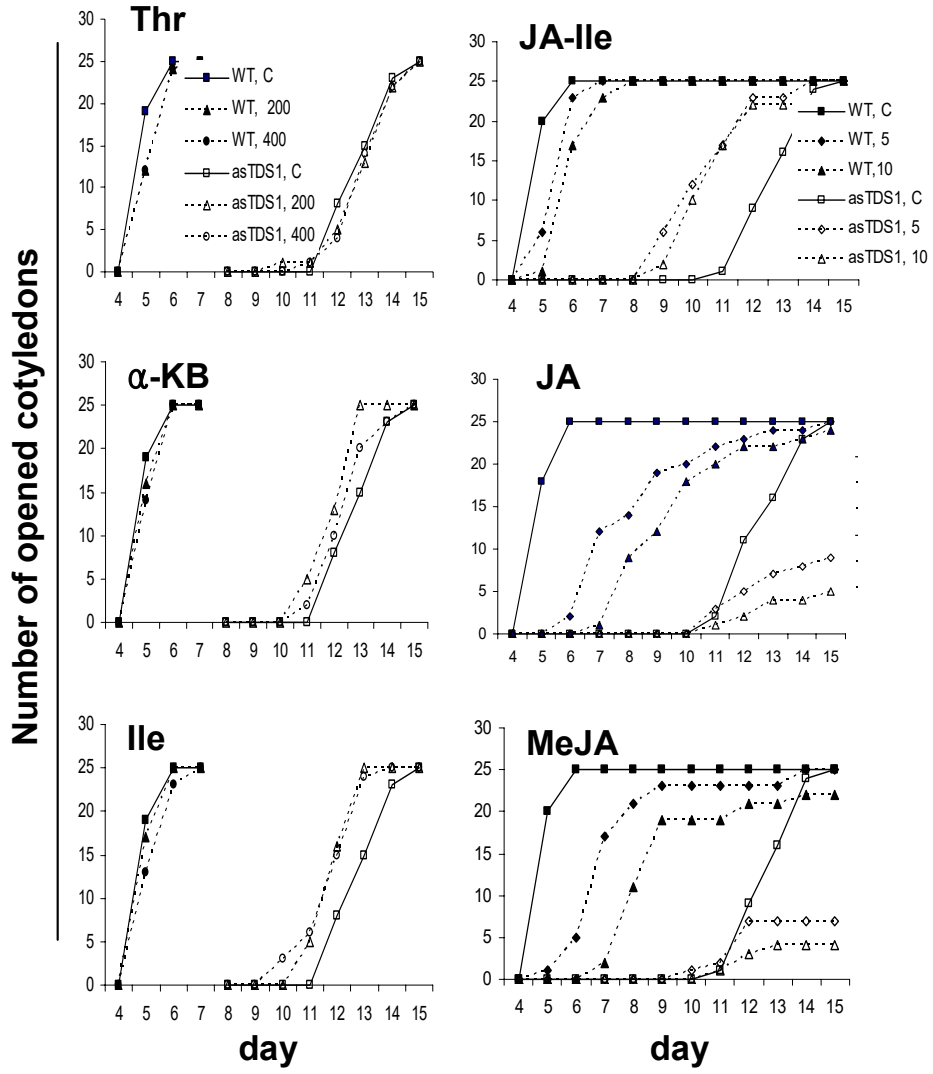


Figure 4. Effect of Thr, α-KB, Ile, JA, JA-Ile, and MeJA on cotyledon opening in WT and asTDS1 seedlings. Cotyledon opening was measured as the separation of a seed coat from cotyledons and subsequent splitting of cotyledons. The number of opened cotyledons was recorded 4 – 15 days after germination. WT and asTDS1 seeds (in replicates of 25) were grown on germination media (C), germination media containing 200 or 400 µM of Thr, α-KB, or Ile, and germination media containing 5 µM or 10 µM of JA, JA-Ile, or MeJA. Data values represent one of two independent

experiments that produced similar results. The data from the second replicate are presented in Fig. S6.

seedlings, supplementing Thr, α-KB, and Ile inhibits hypocotyl and root growth and slows cotyledon opening. In asTDS1 seedlings, Thr was also inhibitory, but α-KB and Ile had positive effects, likely reflecting the deficiencies in α-KB and Ile in asTDS1 seedlings.

In order to determine whether seedling growth is affected by JA and its conjugates, we germinated seeds on medium supplemented with 0 – 10 μM JA, JA-Ile, or MeJA., Both hypocotyl (50 – 62%) and root growth (64 – 83%) in WT seedlings were significantly reduced at 15 DAG in all treatments compared to the untreated controls (Fig. S7a, b; unpaired t-tests, *P*'s < 0.0001). At 15 DAG in all treatments, the hypocotyl length was reduced by 45 – 62% (Fig. S7a; unpaired t-test, $P < 0.0001$) and root growth was reduced by 51 – 69% compared to the untreated controls (Fig. S7b; unpaired t-test, $P < 0.0001$). When WT seeds were supplemented with JA or MeJA, cotyledon opening was delayed by 5 days compared to untreated seedlings. When WT seedlings were treated with JA-Ile, cotyledon opening was delayed by 2 days. Supplementing JA and MeJA also strongly inhibited cotyledon opening in asTDS1 seedlings, but supplementing JA-Ile accelerated cotyledon opening by 2 days compared to untreated asTDS1 seeds (Fig. 4, Fig. S6). Cotyledon opening responses were similar between asTDS2 and asTDS1 seedlings when the seeds were supplemented with amino acids and jasmonates (Fig. S8). Collectively these results point to Ile and JA-Ile deficiencies in the seedling phenotypes of asTDS plants.

Metabolites in floral buds of asTDS plants

Northern blot analysis of TD mRNA expression demonstrated that TD was highly expressed in WT floral buds. In comparison, levels of TD mRNA in asTDS1 floral

buds were not detectable. As expected, antisense-oriented TD mRNA was found only in the asTDS1 floral buds (Fig. 5a). The suppression of TD mRNA in asTDS1 plants decreased TD activity and Ile pools. The amount of α-KB and Ile in asTDS1 floral buds was reduced by 85% and 79%, respectively, compared to WT floral buds (Fig. 5b; unpaired t-test, $P = 0.0008$ and $P = 0.03$, respectively). The reduced TD activity

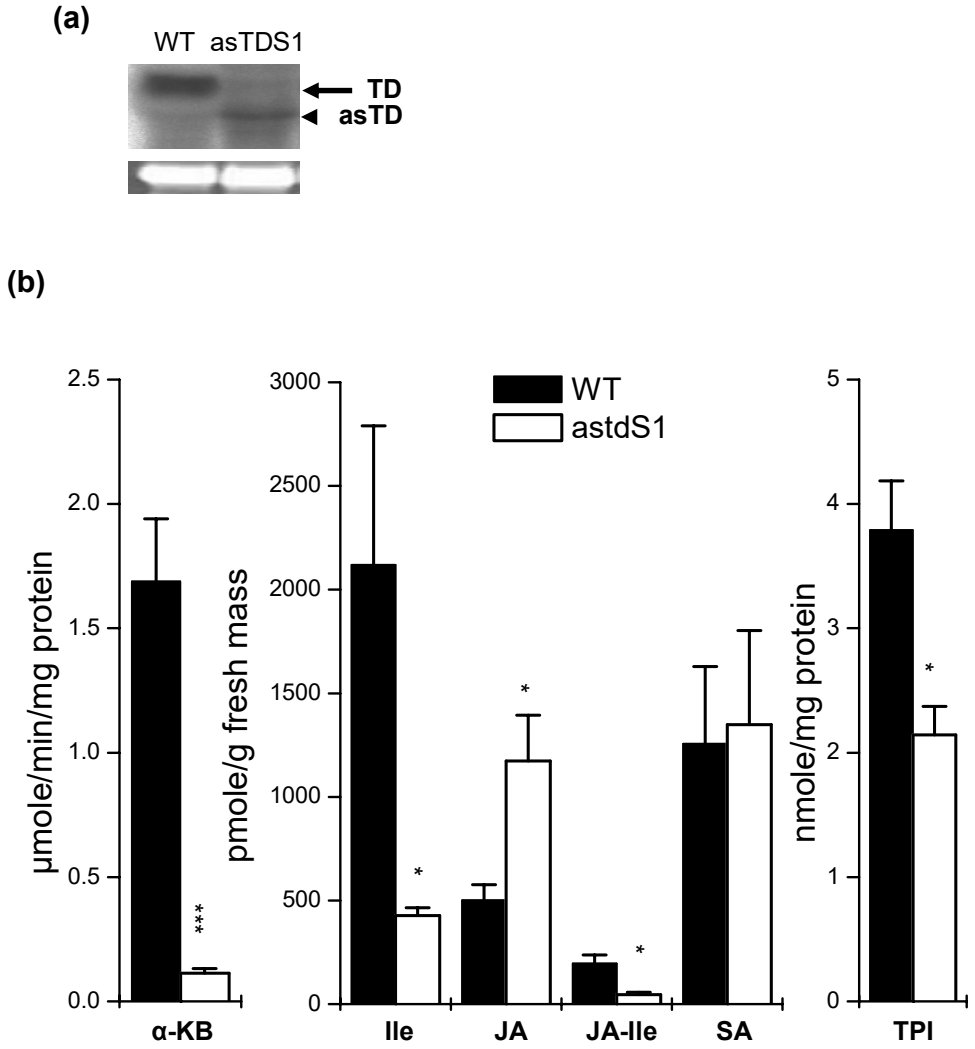


Figure 5. Suppression of TD in asTDS1 plants reduces the levels of α-KB, Ile, and JA-Ile in floral buds. (a) Accumulation of TD transcripts in floral buds of WT and asTDS1 plants. Fifteen micrograms of total RNA extracted from 5-10 mm floral buds were loaded and hybridized with TD probe (1706 bp). Arrow indicates endogenous TD RNA (TD) and arrowhead indicates antisense TD RNA (asTD). Ethidium bromide-stained 18S rRNA is shown as a loading control. (b) Mean (± SE) α-KB, Ile,

JA, JA-Ile, SA, and TPI concentration of 4 replicate of floral buds from WT and asTDS1 plants. Stars represent significant differences between WT and asTDS1 plants (unpaired t-test: *, $P < 0.05$; ***, $P < 0.001$).

was also associated with a 134% increase in constitutive JA levels (Fig. 5b; unpaired t-test, $P = 0.0281$) and a 77% reduction in constitutive JA-Ile levels (Fig. 5b; unpaired t-test, $P = 0.0127$) compared to those of WT floral buds: the levels of salicylic acid, in contrast, stayed the same (Fig. 5b; unpaired t-test, $P = 0.8806$). The reduced TD activity in asTDS1 floral buds was also associated with a 43% reduction in TPI activity compared to WT floral buds (Fig. 5b; unpaired t-test, $P = 0.0114$).

DISCUSSION

We characterized the traits of transgenic plants with strongly silenced TD mRNA and highly reduced levels of TD activity (asTDS plants). These plants are profoundly influenced in their growth and development (Fig. 2). To establish associations between an observed phenotype and TD activity, we transformed WT *N. attenuata* plants with a TD promoter:GUS fusion to localize TD expression and attempted to complement the phenotype by growing asTDS plants on media supplemented with various TD substrates and products. We did not expect to fully complement the WT phenotype with these experiments, as normal growth and development require the delivery of amino acids to particular cellular compartments at precise times. Supplementing exogenously cannot be expected to achieve such precision. Hence we interpreted trends in changes of phenotypes after supplementation as evidence of complementation.

asTDS plants displayed several developmental defects in particular tissues, namely delayed cotyledon opening, shortened pistils, stamens that converted into petals, and reduced trichome numbers (Fig. 2). TD promoter analysis of plants transformed with TD promoter:GUS constructs demonstrated that TD was specifically expressed in these tissues (Fig. 3), suggesting that TD's role in supplying Ile to these tissues was essential for normal development. This specific nutritional deficiency is expected to result in numerous non-specific developmental defects such as slowed growth, alterations in phyllotatic patterns, and reduced seed production. To establish associations between an observed phenotype and TD activity in asTDS plants, we attempted to complement the phenotype by growing plants on media supplemented with various TD substrates and products. Cotyledon opening in asTDS seedlings was accelerated but not fully restored by supplementing germination media with either α-KB or Ile (Figs. 4, S8). TD is highly expressed in cotyledons but not in roots, and the partial recovery of cotyledon opening in asTDS plants may be due to inefficient transport of α-KB and Ile from roots to cotyledons. Interestingly, the cotyledon-

opening phenotype in asTDS plants was strongly complemented when plants were grown on JA-Ile-supplemented media (Figs. 4, S8), a treatment that inhibited cotyledon opening in WT plants. Moreover, treatments with either JA or MeJA strongly inhibited both WT and asTDS plants. These results implicate JA-Ile in cotyledon opening and support the hypothesis that TD expression in cotyledons supplies the Ile pools required to form JA-Ile as well as to synthesize proteins for growth. However, supplementing JA-Ile did not complement the root-growth phenotype in the same way that supplementing Ile did (Figs. S5b, S7b), and hence the supply of Ile for processes other than the formation of JA-Ile must account for the short-root phenotype. In soybean seedlings, young rapidly dividing tissues such as plumule and stem hook were found to have higher JA levels, while lower levels were found in roots (Creelman & Mullet 1995). The strong inhibitory effect of JA, MeJA, and JA-Ile on root growth may reflect these internal gradients.

Just as TD-VIGS flowers had shortened pistils and petaloid anthers (Fig. 1), asTDS plants had shortened pistils, poor pollen production, petaloid anthers, and reduced trichome numbers (Fig. 2). These developmental defects have been associated with defects in JA signaling in other systems. For instance, mutants defective in JA biosynthesis or perception are male sterile in *Arabidopsis* (Park *et al.* 2002; Stintzi & Browse 2000; Xie *et al.* 1998) and female sterile in tomato (Li, Li & Howe 2001; Li *et al.* 2004). The pollen of a sterile mutant of tomato (*jasmonic acid-insensitive1* [*jai1*]), which is defective in JA signaling, has reduced viability, abnormal development of glandular trichomes, and no JA-regulated proteinase inhibitor (PI) proteins in reproductive tissues. This mutant is known to be defective in a tomato homolog of *CORONATINE-INSENSITIVE1* (*COI1*) that encodes a 592-amino acid protein containing an F-box motif and 16 leucine-rich repeat sequences, and is required for JA signaling in *Arabidopsis* (Li *et al.* 2004). The similarity of the pollen and trichome phenotypes between asTDS1 plants and *jai1* mutants suggests a common deficiency in JA signaling.

Delayed leaf senescence in asTDS plants is also related to JA signaling, which has recently been established in *Arabidopsis*. JA treatment accelerates senescence in WT *Arabidopsis* but does not induce precocious senescence in JA-insensitive *coi1* plants. JA concentrations increase in senescing leaves (He *et al.* 2002), but the flower buds of asTDS1 plants had higher JA and reduced JA-Ile levels compared to WT plants (Fig. 5b), suggesting that senescence may be signaled by JA-Ile or by other JA-amino acid conjugates other than JA alone. Hause et al. demonstrated that during flower development, different tissues had different ratios of JA, cis(+) 12-oxophytodienoic acid, JA-Ile, and its methyl ester, which suggests that these profiles play a specific role during flower development and stress responses (Hause *et al.* 2000). Moreover, JA-amino acid conjugates appear to play a role in pollen development, as large amounts of JA-Ile are found in pine pollen and in tomato floral organs (Hause *et al.* 2000; Knöfel & Sembdner 1995). In this work, we demonstrated that suppressing TD influences seedling growth, flower development, and leaf senescence. It is difficult to distinguish the effect of TD silencing on signaling via its role in JA-Ile biosynthesis from the effect that results from nutritional deficiencies, due to the difficulties of rescuing developmental phenotypes by exogenous supplementation experiments. To cleanly distinguish the jasmonate signaling from nutritional deficiency phenotypes will require genetic tools that more precisely inhibit the conjugation of Ile with JA. Research into the *Arabidopsis* JA-responsive gene (*JAR1*) equivalents in *Nicotiana* will be valuable in this regard. The *Arabidopsis* JAR1 was shown to adenylate JA prior to its conjugation with amino acids, of which JA-Ile was the most abundant (Staswick & Tiryaki 2004).

Taken together, these results suggest that TD supplies Ile for JA-Ile synthesis. While JA may be produced throughout seedling and flowering stages, Ile is synthesized from Thr by TD in particular tissues at particular times. The resulting Ile is conjugated with JA by *JAR1* and the resulting JA-Ile regulates plant development (Fig. 6).

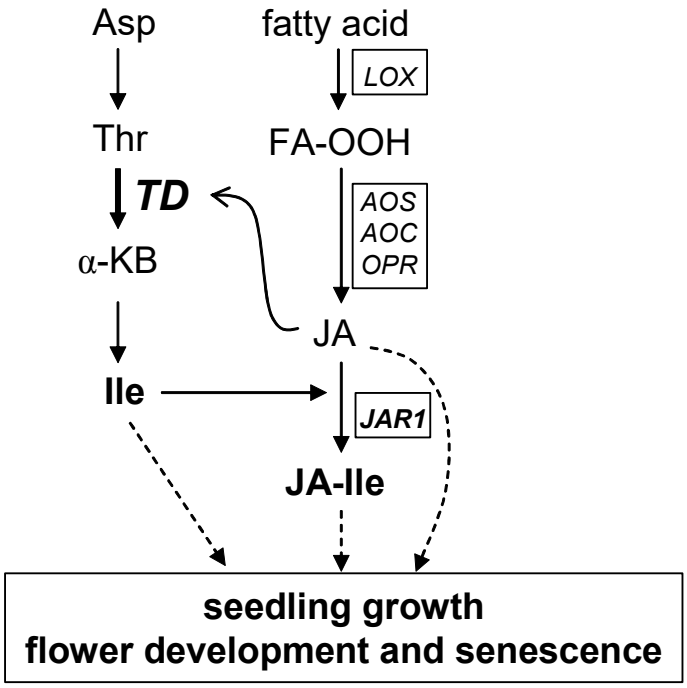


Figure 6. Role of TD in plant development. JA biosynthetic enzymes and JA-amino synthetase are represented in boxes. Dashed arrows represent signal transduction pathways. *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, 12-oxo-phytodienoic acid reductase; *JAR1*, JA-amino synthetase.

ACKNOWLEDGEMENTS

We thank Bernd Krock for assistance with the GC-MS and LC-MS analysis; Thomas Hahn for sequencing; Tamara Krügel and Michell Lim for the plant transformation; Lei Wang for the VIGS experiment; Emily Wheeler for editorial assistance. This work was supported by the Max Planck Society.

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SUPPLEMENTARY MATERIALS

Southern blot of genomic DNA

Genomic DNA was extracted from leaves as described previously (Richard 1997) and 10 μg of DNA was digested with *Eco*RI and blotted onto nylon membranes. To prepare the probe, plasmid pTD13 (GenBank accession no. AF229927) containing full-length cDNA of TD was cut with *Pst*I and gel-eluted using Geneclean Kit (BIO 101, Vista, CA, USA) and labeled with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little Chalfont, UK) and purified on G50 columns (Amersham-Pharmacia). Blots were washed after overnight hybridization at 42°C three times with 2xSSPE and one time with 2xSSPE / 2% SDS at 42°C for 30 min and analyzed on a phosphoimager (model FLA-3000; Fuji Photo Film, Tokyo, Japan).

Analysis of direct defense traits

Leaves growing at node 0 were analyzed by HPLC as described previously (Keinanen, Oldham & Baldwin 2001) with the following modification of the extraction procedure: approximately 100 mg frozen tissue was homogenized in 1 mL extraction buffer utilizing the FastPrep® extractions system (Savant Instruments, Holbrook, NY, USA). Samples were homogenized in FastPrep® tubes containing 900 mg lysing matrix (BIO 101, Vista, CA, USA) by being shaken at 6.0 m sec^{-1} for 45 seconds.

SUPPLEMENTARY DATA

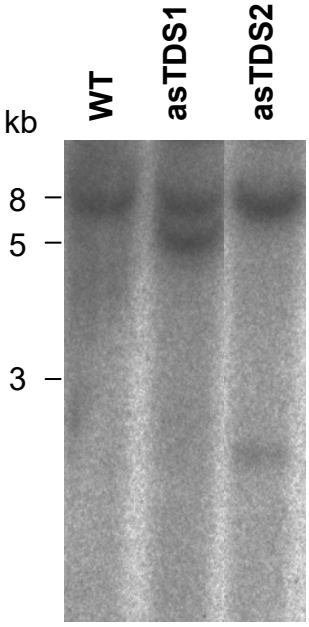

Figure S1. Southern blot of genomic DNA in WT, asTDS1, and asTDS2 plants. 10 µg of genomic DNA were digested with *Eco*RI and blotted onto nylon membranes. The blots were hybridized with a PCR fragment (1706 bp) of the region of TD used for the antisense construct. WT plants show one band and T_2 asTDS plants show two bands.

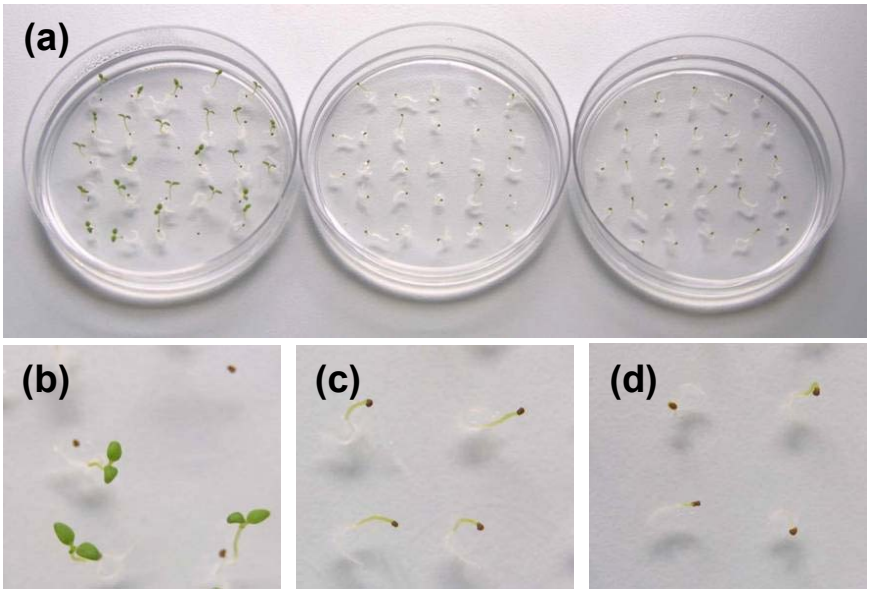

Figure S2. Phenotypic comparison between WT and asTDS plants. (a) 9-day-old seedlings of WT (left), asTDS1 (middle), and asTDS2 (right) plants. (b - d). Same magnification photographs of WT (b), asTDS1 (c), and asTDS2 seedlings (d) as in (a), showing opened and unopened cotyledons in WT and asTDS seedlings, respectively.

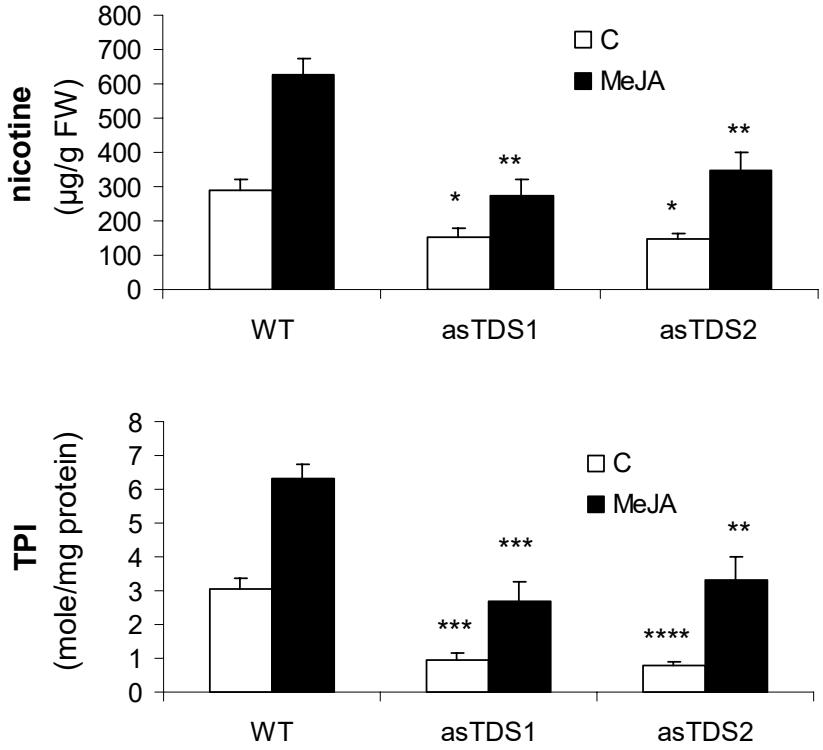

Figure S3. Mean (± SE) nicotine and TPI concentration of node 0 leaves, 4 days after leaves at nodes +1 and +2 were treated with either 20 µl of lanolin containing 150 µg of MeJA or 20 µl of pure lanolin (control) from 5 - 6 replicate WT and asTDS plants (asTDS1 and asTDS2). Stars represent significant differences between MeJA-treated WT and MeJA-treated asTDS plants and between control WT and control asTDS plants (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

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GAATTCGAAACCAAAGCTACAGGGTTCGATCGAACCCGCTCCCGGCACTCTAGCTCCGTC -1775
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                                  ↓+1
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TTCAAAAATTCTTCTATTTTCCTCTATCAAATATAGTACGATATGGAAGTTCTTTGTCAA   +85
GCCCCAGCTGGAAACTCAAATTTTGCCTGTAACCCCAAATTTACAGCAATTAGAACCAGA  +145
GCAATTTCTAGCAATGACACATTTAAAGTAATTTCCAGTACTGGCAACAACAAGAAAATG  +205
AAGGGCGCGATACGTACTTCGATTCCAAAACCATCAGCATTGCCATTGAAGGTCTCACAA  +265
TTATCTCCATCAGCTGATTCAATGCCTGTTCCAGCGTCTTTGC                   +308
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Figure S4. Nucleotide sequence of the TD promoter. Nucleotide position of TD sequence is indicated to the right of the sequence. The putative position of the ATG translational start codon is indicated in boldface type. The putative position of the transcription start site is indicated with an arrow and +1.

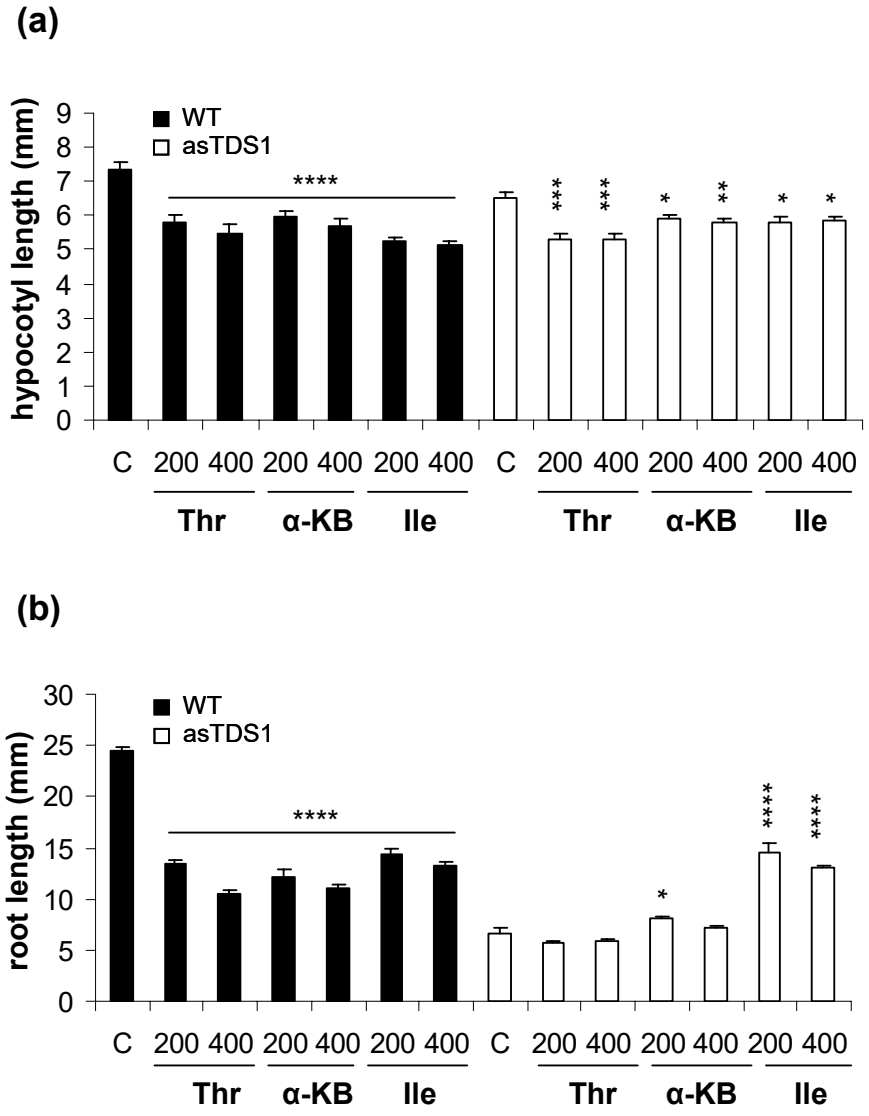

Figure S5. Effect of Thr, α-KB, and Ile on seedling growth in WT and asTDS1 seedlings. (a and b) Mean (± SE) hypocotyl length (a) and root length (b) of 10 replicate 15-day-old WT and asTDS1 seedlings. Seeds of WT and T_2 asTDS1 plants were grown on germination media (C) and germination media containing 200 or 400 μM of Thr, α-KB, or Ile. Stars represent significant differences between seedlings grown on germination media and seedlings grown on germination media containing Thr, α-KB, or Ile in WT and asTDS1 (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

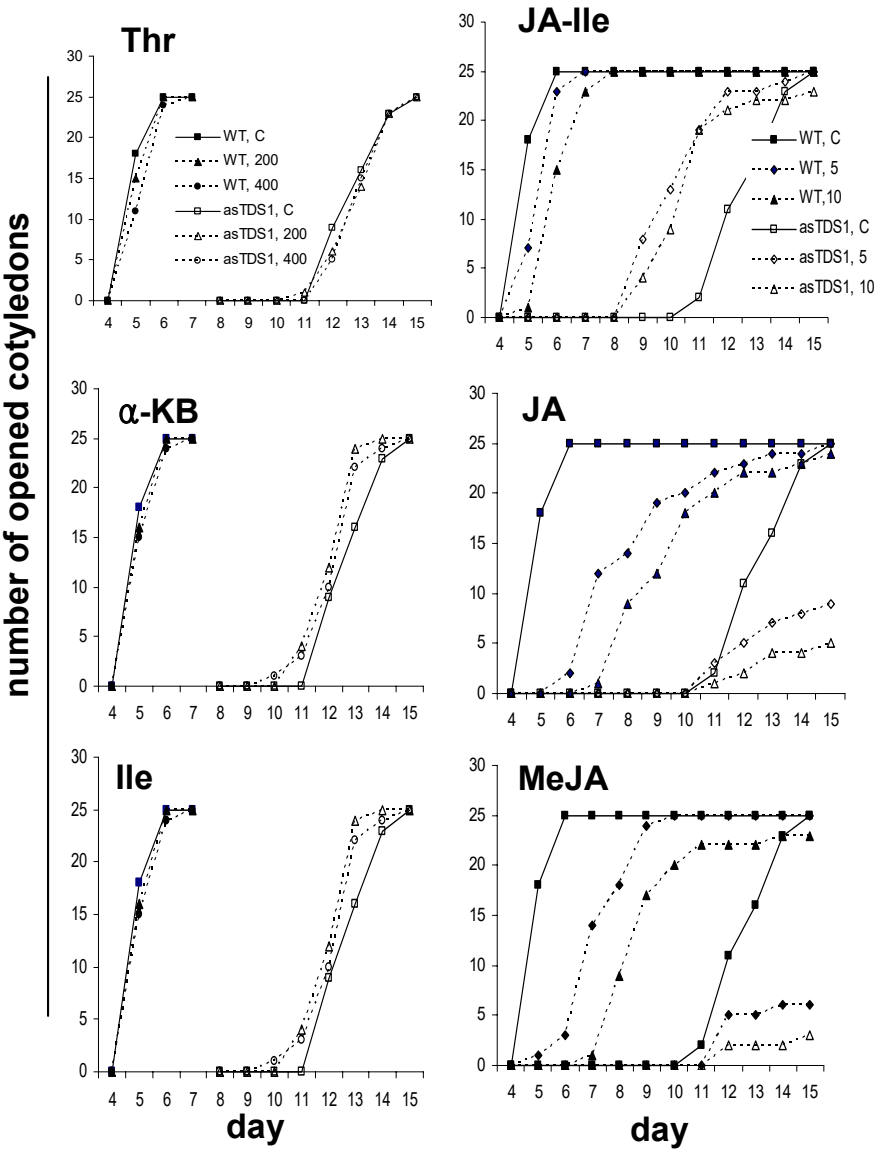

Figure S6. Effect of Thr, α-KB, Ile, JA, JA-Ile, and MeJA on cotyledon opening in WT and asTDS1 seedlings. Cotyledon opening was measured as the separation of a seed coat from cotyledons and subsequent splitting of cotyledons. The number of opened cotyledons was recorded 4 – 15 days after germination. WT and asTDS1 seeds (in replicates of 25) were grown on germination media (C), germination media containing 200 or 400 µM of Thr, α-KB, or Ile, and germination media containing 5 µM or 10 µM of JA, JA-Ile, or MeJA.

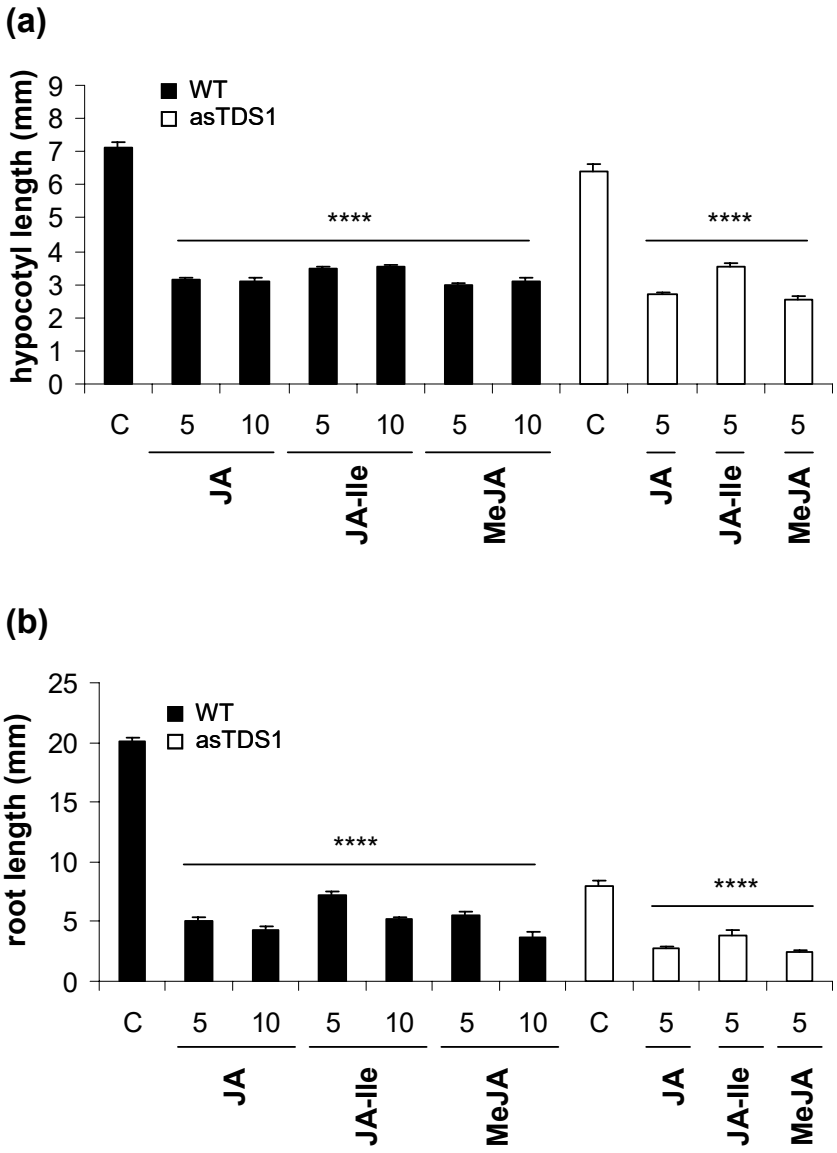

Figure S7. Effect of JA, JA-Ile, and MeJA on seedling growth in WT and asTDS1 seedlings. (a and b) Mean (± SE) hypocotyl length (a) and root length (b) of 7-10 replicate 15-day-old WT and asTDS1 seedlings. Seeds of WT and T_2 asTDS1 plants were grown on germination media (C) and germination media containing 5 or 10 µM of JA, JA-Ile, or MeJA. Stars represent significant differences between seedlings grown on germination media and seedlings grown on germination media containing JA, JA-Ile, or MeJA in WT and asTDS1 (unpaired t-test: ****, $P < 0.0001$).

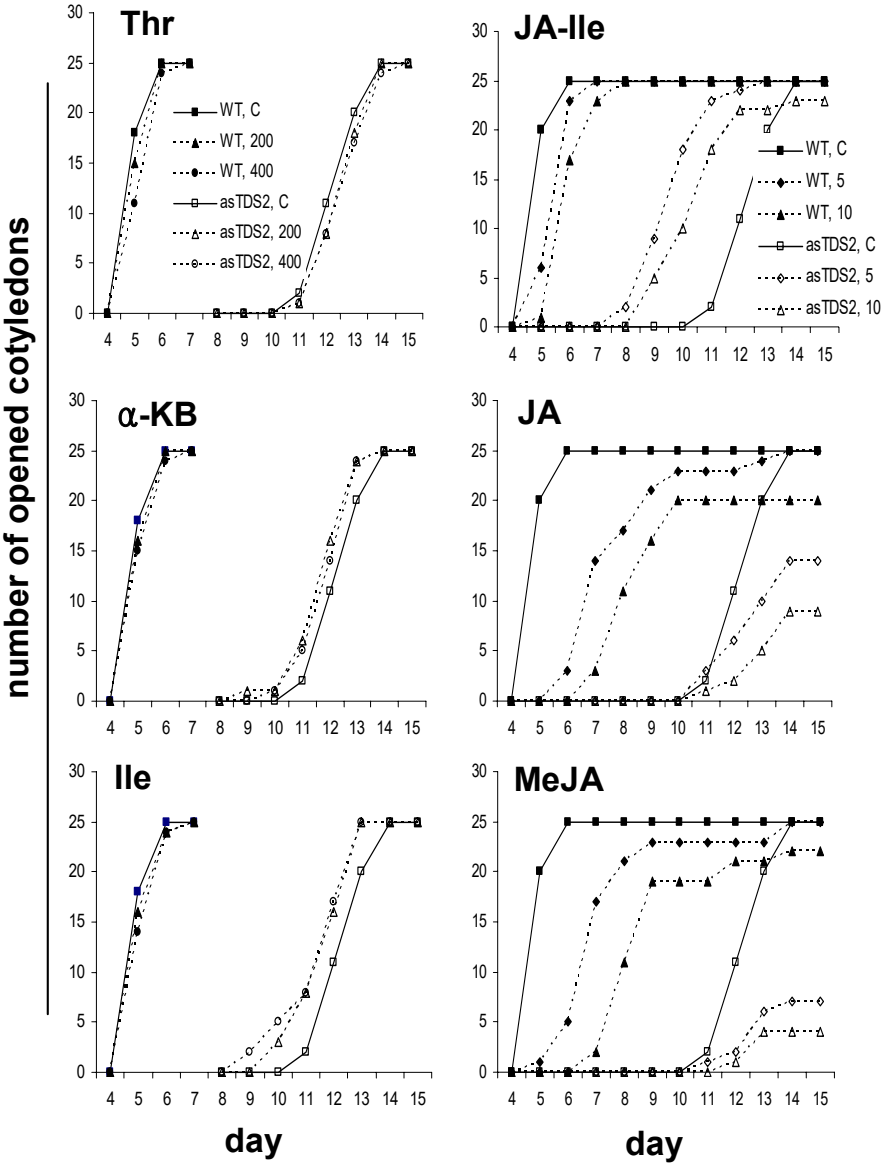

Figure S8. Effect of Thr, α-KB, Ile, JA, JA-Ile, and MeJA on cotyledon opening in WT and asTDS2 seedlings. Cotyledon opening was measured as the separation of a seed coat from cotyledons and subsequent splitting of cotyledons. The number of opened cotyledons was recorded 4 – 15 days after germination. WT and asTDS2 seeds (in replicates of 25) were grown on germination media (C), germination media containing 200 or 400 µM of Thr, α-KB, or Ile, and germination media containing 5 µM or 10 µM of JA, JA-Ile, or MeJA.

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Manuscript II

Reviewed: Plant Science (2006)

Isolation and characterization of the threonine deaminase promoter in *Nicotiana attenuata*

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Abstract

The enzyme encoded by the threonine deaminase (TD) gene catalyzes the conversion of threonine to α-keto butyrate in the biosynthesis of isoleucine. In *Nicotiana attenuata,* TD transcripts accumulate constitutively in cotyledons and flowers and are elicited in leaves by wounding, herbivore attack, and methyl jasmonic acid (MeJA) treatment. To understand TD's unique pattern of expression, we isolated a genomic clone of the TD gene from *N. attenuata* and characterized its promotor. T_2 transgenic plants, each harboring single copies of fusions of different parts of the 5' non-coding region to the β-glucuronidase reporter gene, demonstrated that the promoter was constitutively expressed in seedlings and flowers, and elicited in leaves by wounding or by MeJA treatment. Promoter deletion analysis defined the promoter regions capable of directing minimal expression in cotyledons and anthers as -142 to -31 bp, and in stigmas as -289 to -231 bp. Regions from -142 to -31 bp were found to be important for basal elicitation in leaves by both wounding and MeJA treatment. These promotor elements may prove valuable in biotechnological applications.

Keywords: threonine deaminase; wounding; promoter; β-glucuronidase

Abbreviations: GUS, β-glucuronidase; Ile, isoleucine; JA-Ile, jasmonic acid-isoleucine conjugate; MeJA, methyl jasmonic acid; TD, threonine deaminase.

1. **Introduction**

Threonine deaminase (TD) catalyzes the formation of α-keto butyrate from threonine, the first step in the biosynthesis of isoleucine (Ile). TD is inhibited by feedback from Ile [1]. The importance of TD in plant growth was demonstrated by isolating Ile-requiring auxotrophs of *Nicotiana plumbaginifolia* that lacked functional TD [2].

The physiological roles of TD in plants have not been fully established. However, the involvement of TD in plant development and defense against herbivore has been proposed (Kang and Baldwin, in review). TD was cloned as a result of its unusual up-regulation in tomato flowers. The first 80 amino acids have a putative transit peptide to transport TD into the chloroplast. Its mRNA expression is 500-fold higher in flowers than in leaves or roots [3,4]. Constitutive expression of TD in flowers were also observed in potato [5] and the native tobacco, *Nicotiana attenuata* [6]; Kang and Baldwin, in review). The effects of wounding, herbivore attack, or methyl jasmonic acid (MeJA) treatment on TD gene expression have been examined in tomato, potato, and *N. attenuata*. In tomato, TD in leaves was induced by MeJA treatment. Despite its high pre-existing expression level, TD in flowers was also induced by MeJA treatment [4]. In potato, TD transcripts increased in leaves in response to mechanical wounding or MeJA treatment [5]. In *N. attenuata*, TD transcripts increased in leaves in response to mechanical wounding, MeJA treatment, or attack by larvae of the Solanaceous specialist herbivore *Manduca sexta* [6,7]; Kang and Baldwin, in review).

Analysis of antisense TD transgenic plants (asTD plants) demonstrated that the ability of asTD plants to elicit direct defenses (e.g., nicotine and trypsin proteinase inhibitors) was impaired, making them more susceptible to *M. sexta* attack. Susceptibility resulted from reduced levels of the jasmonic acid-isoleucine conjugate (JA-Ile) that is elicited when larval oral secretions are applied to wounds during feeding (Kang and Baldwin, in review), suggesting that TD is involved in defense against herbivores. Moreover, unlike WT plants , asTD plants showed morphological

changes in seedlings and flowers , a response that is associated with reduced levels of α-keto butyrate, the first product of TD activity, and an imbalance of JA and JA-Ile (Kang and Baldwin, in review), suggesting that TD plays a role in development as well as defense.

In this study, we report the isolation and characterization of a TD genomic clone containing the promoter and full length of the open reading frame of the TD cDNA. We examined the developmental and inducible expression of the TD promoter fused to the β-glucuronidase (GUS) reporter gene. We show that TD:GUS fusions are constitutively expressed in seedlings and flowers, and induced by wounding or MeJA treatment. To define promoter regions important for tissue-specific and wounding-induced expression, a promoter deletion analysis was conducted.

2. Materials and methods

2.1. Plant materials and growth conditions

An inbred genotype of *Nicotiana attenuata* Torr. Ex Wats. (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae), originally collected from southwestern Utah in 1988, was transformed and used for all experiments. Seeds were sterilized and sown on germination media (Gamborg's B5 medium with minimal organics: [Sigma] and 0.6% [w/v] phytagel [Sigma]), and maintained in a growth chamber (Percival, Perry Iowa, USA) at 26ºC/16h hr 155 µm/s/m² light, 24ºC/8 hr dark as described previously [8]. Ten-day-old seedlings were planted into soil in Teku pots (Waalwijk, The Netherlands) and, once established, transferred to 1 L pots in soil and grown in the glasshouse at 26-28ºC, under 16 hr light supplemented by Philips Sun-T Agro 400 or 600W Na lights.

2.2. Chemical treatments

The leaf undergoing source–sink transition was designated as occupying node 0. For wound-treated plants, the older leaves at nodal position +1 were wounded by rolling a fabric pattern wheel over their surfaces to produce standardized puncture wounds. For MeJA-treated plants, leaves at position +1 were treated with 150 µg (0.625 µmole) of MeJA in 20 µl of lanolin paste as described previously [9].

2.3. Genomic clone of TD in N. attenuata

One hundred thousand plaque-forming units (pfu) of genomic DNA library (Lambda FIX®II Library; Stratagene, La Jolla, CA, USA) prepared from DNA isolated from *N. attenuata* leaves were plated, blotted, and screened according to the manufacturer's instructions. To prepare the probe, plasmid pDH14.2 (Genbank accession number; AW191811) containing a partial cDNA of TD was cut with *Eco*RI and *Spe*I, gel-eluted using Geneclean Kit (BIO 101, Vista, CA, USA), labeled with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little

Chalfont, UK), and subsequently purified on G50 columns (Amersham-Pharmacia). Blots were washed four times with 2x SSC, 0.1% SDS at 65°C for 30 min after hybridization at 65°C and analyzed by autoradiography. Initial positive clones were analyzed by PCR and two selected plaque-pure clones were sequenced on an ABI310 sequencer using the Big Dye terminator kit (Applied Biosystems, Darmstadt, Germany). These clones have the same TD sequences originally isolated from mRNA differential display [6].

2.4. Construction of the TD promoter-β-glucuronidase reporter gene fusion construct

The TD promoter region (1.9 kb) was isolated by PCR from the *N. attenuata* genomic clone (GenBank accession number; AY928105) using the primers 5'-CGGAATTCGAAACCAAAGCTACAGGGTTCGATC-3' and 5'-CATGCCATGGATCGTACTATATTTGATAGAGG-3', which contain an *Eco*RI and an *Nco*I site, respectively. The resulting PCR product was cloned into the pUC19 vector and sequenced. Next, the TD promoter region was cut from the pUC19 vector by *Eco*RI and *Nco*I digestion and inserted into the binary vector pCAMBIA1301, replacing the 35S promoter cauliflower mosaic virus so that the TD promoter directs the expression of the uidA (β-glucuronidase [GUS]) gene. This vector was called pTD1836. Using the pTD1836 vector as templates, other vectors were constructed by PCR or enzyme digestion. The following primers were used to amplify different sizes of TD promoter regions. For pTD1094, 5'-GCTCTAGATACGTACAAGCACGAATTTAAGATTTG-3' and 5'-CATGCCATGGATCGTACTATATTTGATAG- AGG-3', for pTD650, 5'-AATTTTAGAACGACGACTTCAGGGGT-3' and 5'-CATGCCATGGATCGTACTA TATTTGATAGAGG-3', For pTD289, 5'-GTCATGAAGCAACGATAAAGCTATCT-3' and 5'-ATGCCATGGATCGTACTATATTTGATAGAGG-3', for pTD231, 5'-GCTCTAGAAACAGCCGCTAATACTTGCA-3' and 5'-CATGCCATGGATCGTAC TATATTTGATAGAGG-3', and for pTD31, 5'-

CCATTGTATGTAGTATTTTACAAC-3' and 5'-CATGCCATGGATCGTACTATATTTGATAGAGG-3'. For pTD1094 and pTD231, resulting PCR fragments were cut with *Xba*I and *Nco*I and inserted into the pCAMBIA1301 cut with *Xba*I and *Nco*I, replacing the 35S promoter cauliflower mosaic virus. For pTD650, pTD289, and pTD31, resulting PCR fragments were treated with T4 polymerase, cut with *Nco*I, and inserted into the pCAMBIA1301 being cut with *Sma*I and *Nco*I, replacing the 35S promoter cauliflower mosaic virus. For pTD142, pTD1836 was cut with *Hind*III and *Nco*I, and the resulting fragment was inserted into the pCAMBIA1301 being cut with *Hind*III and *Nco*I, replacing the 35S promoter cauliflower mosaic virus. For a negative control, pCAMBIA1300, which does not contain the GUS gene, was used.

2.5. Plant transformation

The constructs were introduced into *A. tumefaciens* strain LBA4404, which was used to transform *N. attenuata* as previously described [8]. The progeny of homozygous plants were selected by hygromycin resistance screening. For all experiments, T_2 homozygous lines were used.

2.6. Histochemical and fluorometric β-glucuronidase activity

Enzymatic assays with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide were performed to localize the enzyme activity of the GUS [10]. Tissue samples were incubated at 37^{o}C in a modified GUS-staining buffer (80 mM sodium phosphate buffer [pH 7.0], 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 8 mM EDTA, 0.05% Triton X-100, 0.8 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, and 20% methanol). After overnight incubation, chlorophyll was extracted with 70% ethanol.

Four fully expanded, size-matched leaves from glasshouse-grown transgenic plants during their rosette stage were used for fluorometric GUS assays. Leaves were grounded into fine powder with liquid nitrogen and then vortexed with GUS

extraction buffer (50 mM $NaPO_4$ [pH 7.0], 10 mM β-mercaptoethanol, 10 mM Na_2EDTA [pH 8.0], 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100). The extracts were centrifuged for 10 min in a microcentrifuge at 4°C, and the supernatants were used for the GUS assay using 4-methyllumbelliferyl- β-D-glucuronide (MUG) as a substrate according to Jefferson (1987). MUG activity was expressed as fluorescence units pmole MU/mg protein/min. Protein concentrations were determined using Bradford reagent (BioRad).

3. Results

3.1. Structure of TD gene

An amplified genomic library of *N. attenuata* was screened with the previously described full length of TD cDNA clone pTD13 [6]. Screening about 4 x 10^5 recombinant plaques yielded two positive clones that were designated pTDG1 and pTDG2. Restriction fragment analysis combined with hybridization studies using probes corresponding to the 5'- and 3'-ends of the cDNA clone was used to map the promoter and TD-coding regions within the genomic clones. DNA sequences were obtained from two *Pst*I genomic fragments in pTDG1, comprising 5019 bp of the coding sequences, 5383 bp of the 5'-flanking sequences, and 2703 bp of the 3'-flanking sequences. The coding regions of TD contain 8 exons (435, 180, 474, 249, 72, 120, 135, and 141 bp, respectively) and 7 introns (1032, 457, 336, 205, 319, 124, and 740 bp, respectively) with consensus splice sites at the exon-intron boundaries (Fig. 1A). The exonic sequences are identical to the previously isolated full length of TD cDNA (pTD13).

3.2. Constitutive expression of the TD gene during seedling and flower development

We previously demonstrated that TD mRNA is constitutively expressed in floral buds [6] and that 1836 bp of TD promoter regions are sufficient to direct expression in cotyledons during seedling growth and in stigma and anthers during flower

development (Kang and Baldwin, in review). To test which regions are important for the expression of the TD gene, progressive 5' deletions of the promoter were fused to

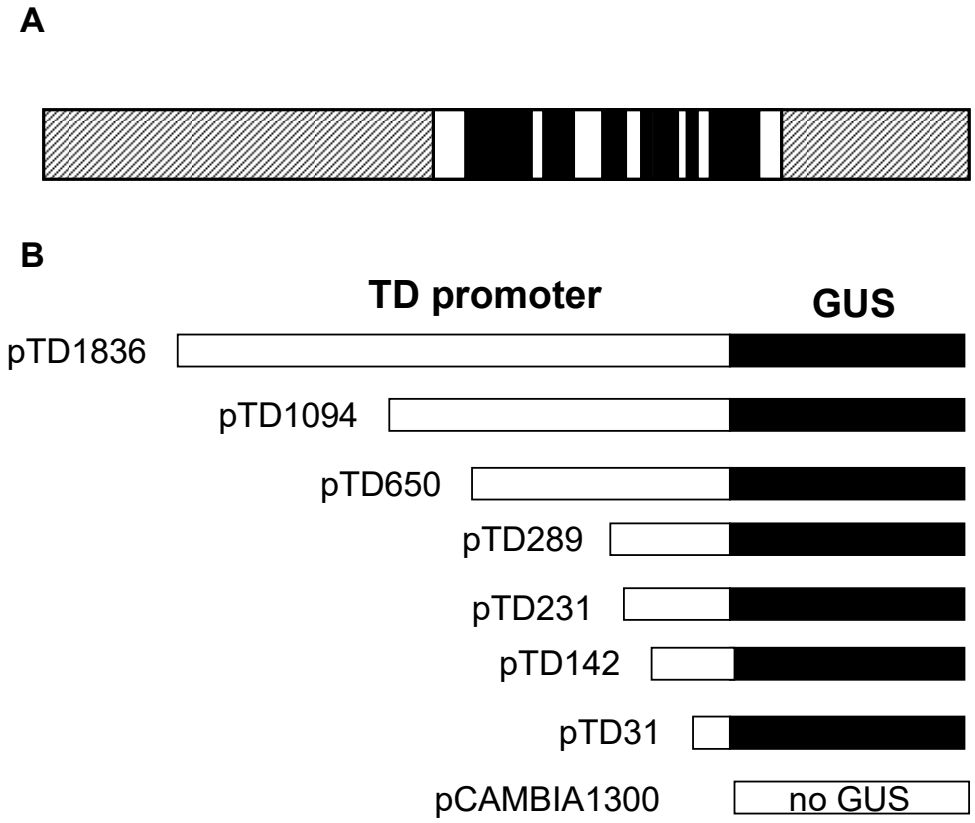
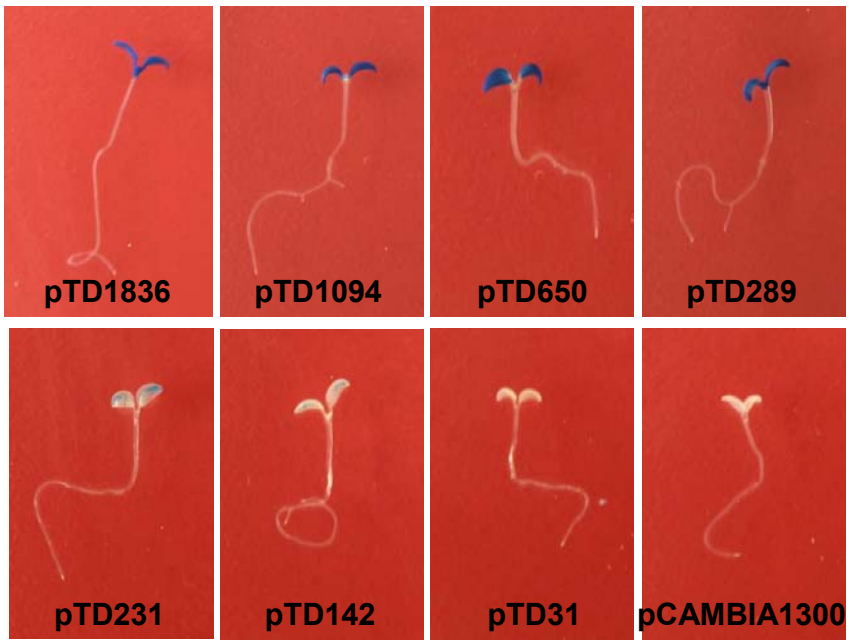


Fig. 1. Schematic representation of the TD genomic clone and the subsequent promoter fusions. (A) Organization of TD from *N. attenuata* (GenBank accession number; AY928105). The dashed boxes represent the 5' and 3' untranslated regions. White boxes represent exons while black boxes represent introns. Exon-intron sequences in the TD genomic clone were deduced by comparison to the *N. attenuata* TD cDNA clone (GenBank accession number; AF229927). (B) Diagrammatic representation of the constructs used for transformation in *N. attenuata*. Lengths of promoter deletion fragments are indicated from the 5' end to the transcription initiation site. The 5' end of the TD promoter was deleted to the end points indicated, with a 3' end at the ATG translational start codon. These deleted promoters were inserted into the binary vector pCAMBIA1301, replacing the 35S promoter cauliflower mosaic virus and used to transform *N. attenuata* plants. pCAMBIA1300, which does not contain GUS gene, was used as a negative control.

the GUS reporter gene (Fig. 1B). Homozygous T_2 transgenic plants containing each construct were obtained and more than 5 independent transgenic plants were analyzed for each construct. GUS staining in cotyledons was detected in all transgenic plants except those bearing pTD34 and pCAMBIA1300; the latter does not contain the GUS gene (Fig. 2A). The promoter regions from -1836 to -289 bp showed full color formation, the promoter regions from -231 to -142 bp showed weak and scattered color formation, and the promoter regions containing -31 bp didn't show color formation in cotyledons (Fig. 2A). As expected, the plants containing pCAMBIA1300, which does not contain the GUS gene, also didn't show color formation in cotyledons (Fig. 2A). These results indicate that the promoter regions from -289 to -231 bp contain the main strong enhancer elements and the promoter regions from -142 to -31 bp contain part of the sequences for cotyledon expression. In stigmas, the promoter regions from -1836 to -289 bp showed color formation; those containing -231 bp didn't (Fig. 2B). In anthers, the promoter regions from -1836 to -142 bp showed color formation and those containing -31 bp didn't (Fig. 2B). As these results demonstrate, elements in promoter regions from -289 to -231 bp and from -142 to -31 bp are necessary for TD expression in stigmas and anthers, respectively.

A



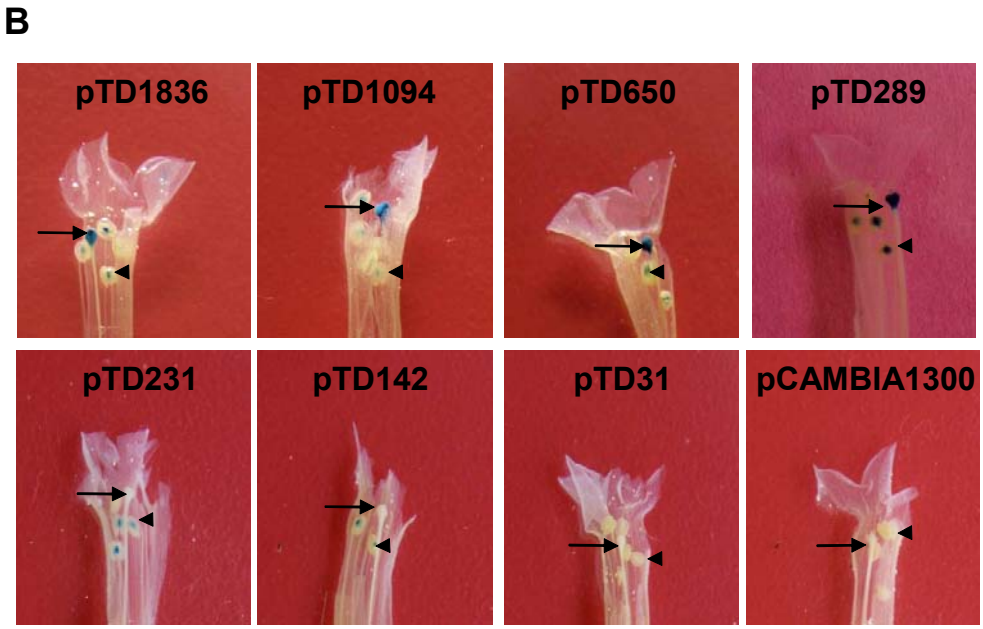


Fig. 2. Developmental patterns of expression directed by a series of deleted TD promoter:GUS fusion transgenic plants. (A and B) Histochemical GUS staining of seedlings (A) and flowers (B). Homozygous T_2 seedlings and flowers were incubated overnight with the GUS-staining buffer and washed with ethanol to remove chlorophyll. Arrow indicates stamen and arrowhead indicates stigma.

3.3. The TD gene is elicited in leaves by wounding or MeJA treatment

In addition to its constitutive expression in floral buds, TD mRNA was also elicited by mechanical wounding, herbivore attack, and MeJA treatment [6,7]. To further characterize which regions are important for the expression of the TD gene by wounding or MeJA treatment, leaves at the same rosette stage from transgenic plants bearing different constructs were wounded or treated with MeJA, and analyzed for GUS enzymatic activity using the fluorescence assay. In general, transgenic plants harboring longer promoters showed higher background levels of GUS activity compared to those with shorter promoters. When plants were wounded or treated with MeJA, all transgenic plants except those harboring pTD31 and pCAMBIA1300, the negative control vector, had higher GUS expression (1. 3 -2.5 times) compared to

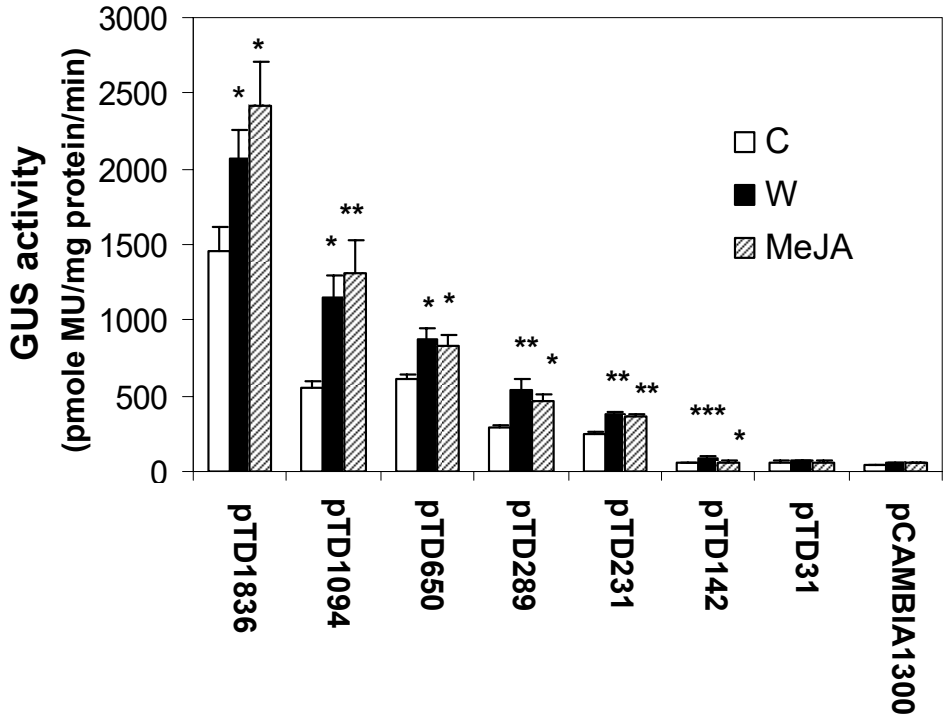


Fig. 3. Induction of GUS-reporter-gene activity directed by a series of deleted TD promoter:GUS fusion transgenic plants. Leaves from the same rosette-stage plants were harvested 24 hr after being wounded with a fabric pattern wheel (W) or treated with 20 µl of lanolin containing 150 µg of methyl jasmonic acid (MeJA), or left untreated (C). Error bars indicate the standard error (n =4). Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$: ***, $P < 0.001$).

untreated plants (Fig. 3; unpaired t-test, $P < 0.05$). These results indicate that the promoter regions from -142 to -31 bp are the minimal regions for wounding and MeJA induction.

4. Discussion

JA and its derivatives regulate the expression of a variety of genes involved in plants' defense responses to wounding and herbivore attack. We have previously shown that herbivore-attacked leaves in *N. attenuata* induce the accumulation of several transcripts, and their expression is tightly regulated by wounding and JA [6,11]. One of these transcripts was shown to encode TD. TD transcripts also have been shown to be expressed in flower buds [6]. In this report, we have isolated a genomic clone containing 5' sequences of the TD gene. Different lengths of 5'-upstream regulatory regions of this gene were fused to GUS reporter genes and transferred into *N. attenuata* plants. The analysis of TD promoter-GUS fusions clearly demonstrated that 1836 bp of TD promoter sequence directed GUS expression to cotyledons, stigmas, and anthers, indicating that sequences sufficient for tissue-specific expression are located within this region (Fig. 2).

Deletion analysis of 1846 bp regions of TD promoters identified two regions important for expression in cotyledons, stigmas, and anthers. To find putative cis-acting regulatory elements in TD promoter regions, we searched the PLACE database [12]. The cotyledon- and anther-responsive regions (from -142 bp to -31 bp) contain GCN4 (TGAGTCA) and GTGA motifs (Fig. 4). The GCN4 motif is known for endosperm-specific expression in rice [13] and the GTGA motif is known for regulating pollen expression in the late pollen gene of tobacco *g10*, which has homology to pectate lyase and is the putative homologue of the tomato pollen gene *lat56* [14]. The stigma-responsive regions (from -289 bp to -231 bp) contain GATA, GTGA, QELEMENTZMZM13 (AGGTCA), and W (TCACC/T) motifs. GATA motifs have been implicated in light-dependent control of GATA transcription factors in *Arabidopsis* and rice [15]. QELEMENTZMZM13 is known to confer pollen expression in maize gene ZM13 [16], which is the putative homologue of the tomato pollen gene *lat56*. W box is known to be involved in the regulation of the wound-induced expression of the ERF3 gene, which may be regulated by the WRKY

transcription factor [17]. WRKYs are involved in the regulation of various biological functions, including the sugar response, senescence, and the development of seed and trichomes [18-20], as well as defense against pathogen infection [21-23].

The analysis of TD promoter:GUS fusions also revealed that 1836 bp of TD promoter sequence was sufficient for wound and MeJA elicitation (Fig. 3). Further deletion analysis of 1846 bp regions of the TD promoter identified the minimal regions for wound and MeJA elicitation, which were located from -142 bp to -31 bp, beginning at the putative transcription start site (Fig. 4). These regions contain GT box (GTGTGCA) and CATG box (CATG), which are known to be involved in wounding and MeJA induction. In tomato, 192 bp of TD promoter were sufficient for an MeJA-induced response in leaves and flowers [24]. When the TD promoter of *N. attenuata* was compared to that of tomato, the sequences from -77 to -53 bp (gtgtgcatgaagttcccgtaactc) in *N. attenuata* were conserved in tomato from -68 to -44 bp (gtgtgcatgaagttcacgtaactc) excluding the single underlined nucleotide. These conserved regions contain putative MeJA-responsive elements (GT box and CATG box), suggesting that these cis-motifs are important for responsiveness to wounding and MeJA.

Recently, synthetic promoters that respond to pathogens have provided direct evidence that a range of pathogen-inducible cis-acting elements can mediate pathogen-inducible expression in plants [25]. Similarly, anther- and wound-specific cis-acting elements in TD promoter can be used in many applications, for example, as molecular markers to engineer crops with increased disease resistance or to develop male-sterile plants for the production of the F_1 hybrid seeds. The 289 bp of TD promoter are sufficient to drive GUS expression in cotyledons, stigmas, and anthers. Deletion analysis indicated that the regions from -142 bp to -31 bp are responsible for cotyledon- and anther-specific expression as well as for wound- and MeJA-induced expression, and that the regions from -289 bp to -231 bp are important for stigma-specific expression. Additional research will be needed to delineate the cis-elements from these regions.

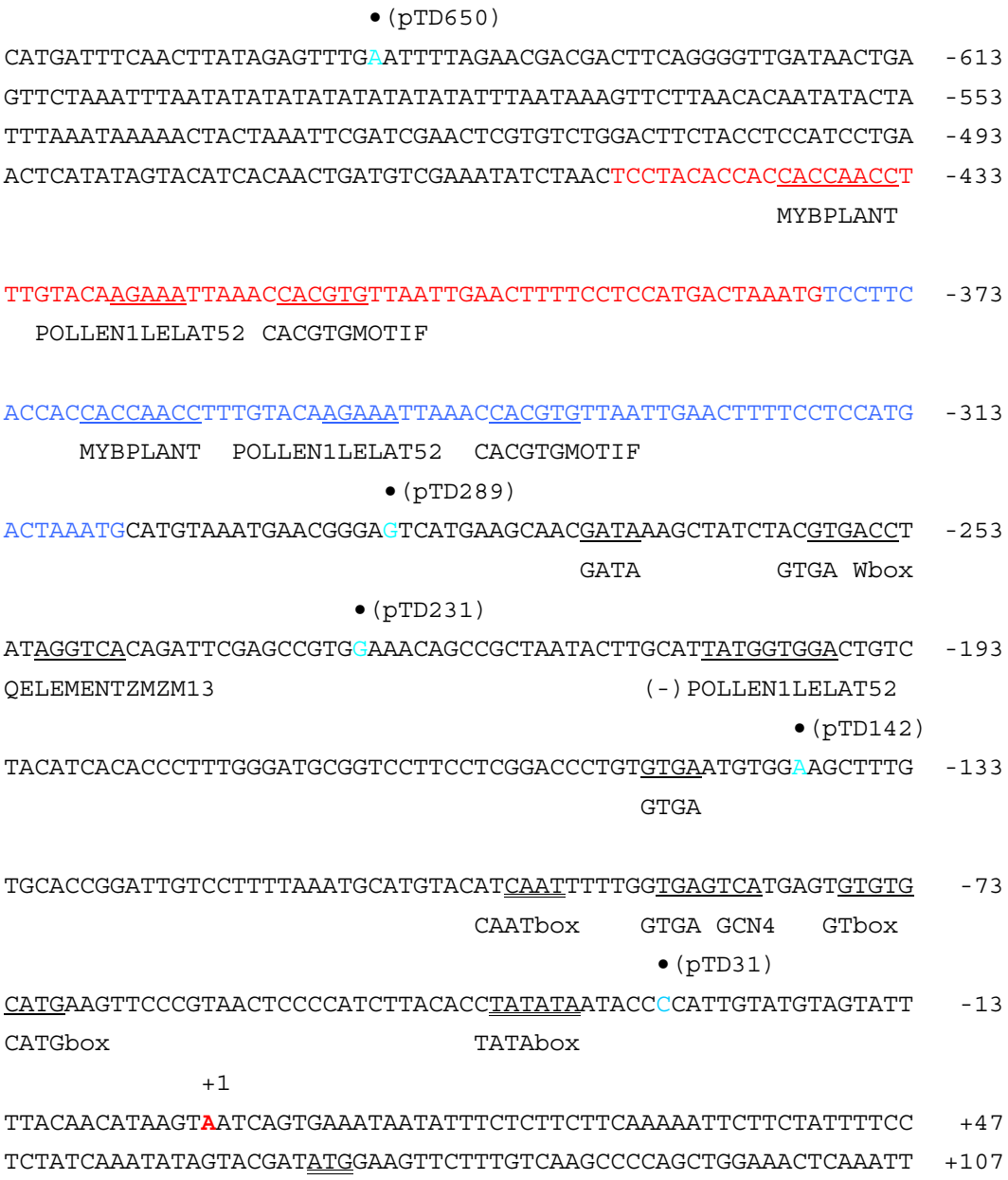


Fig. 4. Predicting cis-elements in TD promoter. Nucleotide position of TD sequence is indicated to the right of the sequence. Putative transcription start site is indicated with +1. Translational start codon and sequences with homology to the TATA box and CAAT box are underlined twice. Putative cis-elements are underlined once. Promoter position of plant transformation constructs is indicated with an arrow. Repeated sequences are indicated by red and blue colors.

Acknowledgments

We thank Tamara Kruegel and Michell Lim for the plant transformation, Emily Wheeler for editorial assistance. This work was supported by the Max Planck Society.

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Manuscript III

Reviewed: Plant Cell (2006)

Silencing Threonine Deaminase and *JAR1* Homologue in *Nicotiana attenuata* Impairs JA-isoleucine-Mediated Defense against the Specialist Herbivore, *Manduca sexta*

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Threonine deaminase (TD) catalyzes the conversion of threonine (Thr) to α-keto butyrate (α-KB) in isoleucine (Ile) biosynthesis but has an unusual pattern of expression in Solanaceous taxa: strong constitutive expression in flower buds and dramatic up-regulation in leaves after herbivore attack. We silenced TD by antisense expression in *Nicotiana attenuata* and selected lines with mildly (asTDM) and severely (asTDS) reduced TD transcripts and activity. asTDS plants were stunted, while asTDM plants had normal growth but were highly susceptible to *Manduca sexta* attack which was associated with impaired elicitation of direct defenses (nicotine and trypsin proteinase inhibitors [TPI]). Both herbivore susceptibility and attenuated defenses were associated with reduced levels of jasmonic acid-isoleucine (JA-Ile) that are elicited when larval oral secretions (OS) are applied to wounds. The lower elicited JA-Ile levels in asTD plants could be attributed to reduced TD activity at the wound site. Addition of $^{13}C_4$-Thr and $^{13}C_6$-Ile to OS-treated wounds revealed that Thr is converted to Ile and rapidly conjugated to JA to form JA-Ile at the wound. Adding JA-Ile to the wounds of asTDM and asTMS plants restored resistance, while eliciting TPIs and nicotine accumulation. Silencing TD and *JAR4*, the *Arabidopsis JAR1* homologue which is a JA-Ile conjugating enzyme, by virus induced gene silencing (VIGS) confirmed that TD and JAR4 play important roles in herbivore resistance by regulating JA-Ile production. These experiments demonstrate that herbivore-elicited TD supplies Ile and that JAR4 conjugates Ile to JA for JA-Ile-mediated defense elicitation.

INTRODUCTION

Threonine deaminase (TD) catalyzes the formation of α-keto butyrate (α-KB) from threonine (Thr), the first step in the biosynthesis of isoleucine (Ile). Regulation of TD activity by Ile was the first recognized instance of allosteric feedback regulation by the end-product of a biosynthetic pathway (Umbarger, 1956). The function of TD for Ile biosynthesis was demonstrated by analyzing the Ile auxotrophic mutant in *Nicotiana plumbaginifollia*, which has no detectable TD activity (Sidorov et al., 1981). When this mutant was transformed with the *Saccharomyces cerevisiae* ILV gene that encodes TD, the transformed lines could be grown on a medium without Ile (Colau et al., 1987). These results demonstrate that TD regulates Ile production and is indispensable for plant growth. However, TD's unusual expression pattern in Solanaceous plants suggests additional roles in development and herbivore defense.

For more than a decade, TD has been recognized as a reliable marker for wounding and JA elicitation in potato and tomato (Hildmann et al., 1992; Samach et al., 1995; Dammann et al., 1997). Wound-induced TD expression is mediated by ABA and jasmonate (JA) signaling in tomato plants (Hildmann et al., 1992), and in potato, protein phosphorylation is required for TD elicitation by JA. TD is also highly expressed in flowers and has a chloroplast transit peptide in the N-terminal region (Samach et al., 1991; Samach et al., 1995). A strong association between JA signaling and TD expression can be inferred from the synthesis of JA-amino acid conjugates.

JA synthesis begins in plastids. Here α-linolenic acid is oxygenated by lipoxygenase (LOX); converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC) before being exported to the peroxisome; and reduced by OPDA reductase (OPR3). JA is produced after three consecutive β-oxidation steps in the peroxisome (Li et al., 2005). JA can be subsequently methylated to its volatile counterpart, methyl jasmonic acid (MeJA), or conjugated with various sugars and amino acids (Sembdner and Parthier, 1993; Sembdner et al., 1994). Recently, an *Arabidopsis* gene (*JAR1*) involved in JA responsiveness was shown to adenylate JA prior to its conjugation with amino acids, of which the JA-isoleucine conjugate was the most abundant (Staswick et al., 2002; Staswick and Tiryaki, 2004).

N. attenuata is a particularly useful system in which to study herbivore-resistance responses. Not only is it well established that JA signaling mediates

herbivore resistance in the field (Baldwin, 1998; Kessler and Baldwin, 2001; Kessler et al., 2004), but also, the direct and indirect defense traits with which JA signaling influences herbivore resistance are known (Halitschke et al., 2004; Steppuhn et al., 2004). The responses of *N. attenuata* to one particular herbivore, the Solanaceous specialist *Manduca sexta*, are particularly well understood. The attacked plant reorganizes its wound response when 8 fatty acid amino acid (FACs) conjugates, present in the herbivore's oral secretions and regurgitants (OS), are introduced into plant wounds during feeding. The reorganization begins with a dramatic JA burst in the attacked leaves (Schittko et al., 2000), which alters the expression of numerous genes and the accumulation and release of secondary metabolites (Halitschke et al., 2000; Kahl et al., 2000; Halitschke et al., 2001; Halitschke et al., 2003; Roda et al., 2004). Silencing the expression of the specific lox that supplies the fatty acid hydroperoxides for JA biosynthesis in *N. attenuata* (NaLOX3), reduces the OS-elicited JA burst and all associated changes in the plant's resistance traits (Halitschke and Baldwin 2003; Kessler et al. 2004).

Many of *N. attenuata*'s herbivore-responsive genes have been identified by cDNA differential display, subtractive hybridization, and cDNA-amplified fragment-length polymorphism display (Halitschke et al., 2001; Hermsmeier et al., 2001; Schittko et al., 2001; Halitschke et al., 2003; Hui et al., 2003; Voelckel and Baldwin, 2003). These genes have been spotted into microarrays and their expression behavior has been analyzed in response to various environmental stresses (Halitschke et al., 2003; Hui et al., 2003; Izaguirre et al., 2003; Voelckel and Baldwin, 2003; Lou and Baldwin, 2004; Voelckel and Baldwin, 2004). In these experiments, TD expression was consistently found to correlate with elicited herbivore resistance. TD was cloned by DDRT-PCR display, found to be encoded by a single gene and strongly elicited by Northern blot analysis only when plants were attacked by *M. sexta* larvae, mechanically wounded, or treated with MeJA; tobacco mosaic virus (TMV) or *Agrobacterium tumefaciens* infection, ethylene or methyl salicylate treatment did not elicit TD expression (Hermsmeier et al., 2001). Wounding and OS-elicitation dramatically increases TD expression, not only in the wounded leaf but also in distal non-wounded leaves that are phyllotactically connected by common orthostichies (Schittko et al., 2001). Wound-induced expression of TD is dramatically reduced in *N. attenuata* plants transformed with NaLOX3 in an antisense orientation, demonstrating that TD elicitation requires JA signaling (Halitschke and Baldwin, 2003). These

observations suggest that TD may be involved in plant defense against herbivore attack as well as in plant growth.

To examine the effect of TD on defense responses, we first expressed 1.3 kb of the NaTD in an antisense orientation. Transformed lines were readily characterized as having one of two growth phenotypes: 1) plants with severely reduced TD expression and activity, and stunted growth and development (asTDS plants), and 2) plants with mildly reduced TD expression and activity but otherwise with wild type (WT) growth and development patterns (asTDM plants). Since plant-herbivore interactions are difficult to interpret in plants that are severely stunted in their growth and development, we additionally silenced TD with a Virus Induced Gene Silencing (VIGS) system optimized for *N. attenuata* (Saedler and Baldwin, 2004), to silence TD in WT plants. We additionally clone *NaJAR4*, the *Arabidopsis JAR1* homologue in *N. attenuata*, and demonstrate that TD supplies the Ile required for JA-Ile conjugate formation and conjugates Ile to JA to mediate defense signaling and resistance to *M. sexta* larvae.

RESULTS

We used DDRT-PCR to monitor changes in transcripts accumulation in *N. attenuata* plants exposed to folivory by first-instar larvae of *M. sexta*. Among the characterized herbivore-responsive genes, TD was one of the highly expressed genes (Hermsmeier et al., 2001). Further analysis with cDNA arrays demonstrated that TD is also strongly up-regulated by attack from other herbivore species. TD mRNA in WT plants is strongly elicited after attack from *M. sexta* larvae (62 fold) and when puncture wounds to leaves are treated with *M. sexta* OS (Figure S1). Attack from other leaf-chewing insect herbivores (*Heliothis virescens* and *Spodoptera exigua*) as well as a species that feeds by lacerating and flushing cells (*Tupiocoris notatus*) also strongly elicits TD transcript accumulation (18-41 fold: Figure S1), suggesting that TD may be involved in plant defense.

To examine the function of TD, we produced transgenic plants expressing TD in an antisense orientation. T_2 homozygous plants from independently transformed lines, each harboring a single copy of the transgene as verified by segregation analysis for antibiotic resistance and Southern blot analysis (Figure S2), were analyzed. Transformed lines were readily characterized as having one of two growth phenotypes: 1) plants with severe reduction in TD expression and activity, and retarded growth (asTDS plants, Figure 1A), 2) plants with mild reductions in TD expression and activity but with growth and development patterns that were indistinguishable from WT plants (asTDM plants, Figure 1A). All lines were also analyzed for levels of defense-related secondary metabolites such as trypsin proteinase inhibitors (TPI) and nicotine.

Mild Silencing of TD Transcripts and α-KB Accumulation Dramatically Impairs Herbivore Resistance without Influencing Plant Growth or Development

Northern blot analysis of TD mRNA expression demonstrated that MeJA treatment elicited dramatic and sustained increases in TD mRNA in leaves. When compared to levels in WT plants, TD mRNA in both asTDM lines was reduced by 50% 1 hr after MeJA treatment and by 30% 24 hr after MeJA treatment; TD mRNA in asTDS1 plants was reduced by 90% 1 hr after MeJA treatment and by 95% 24 hr after MeJA treatment. As expected, antisense-oriented TD mRNA was found only in the asTD

lines (Figure 1B). Silencing the expression of TD transcripts translated into large changes in TD activity, which was assayed by measuring α-KB, the product

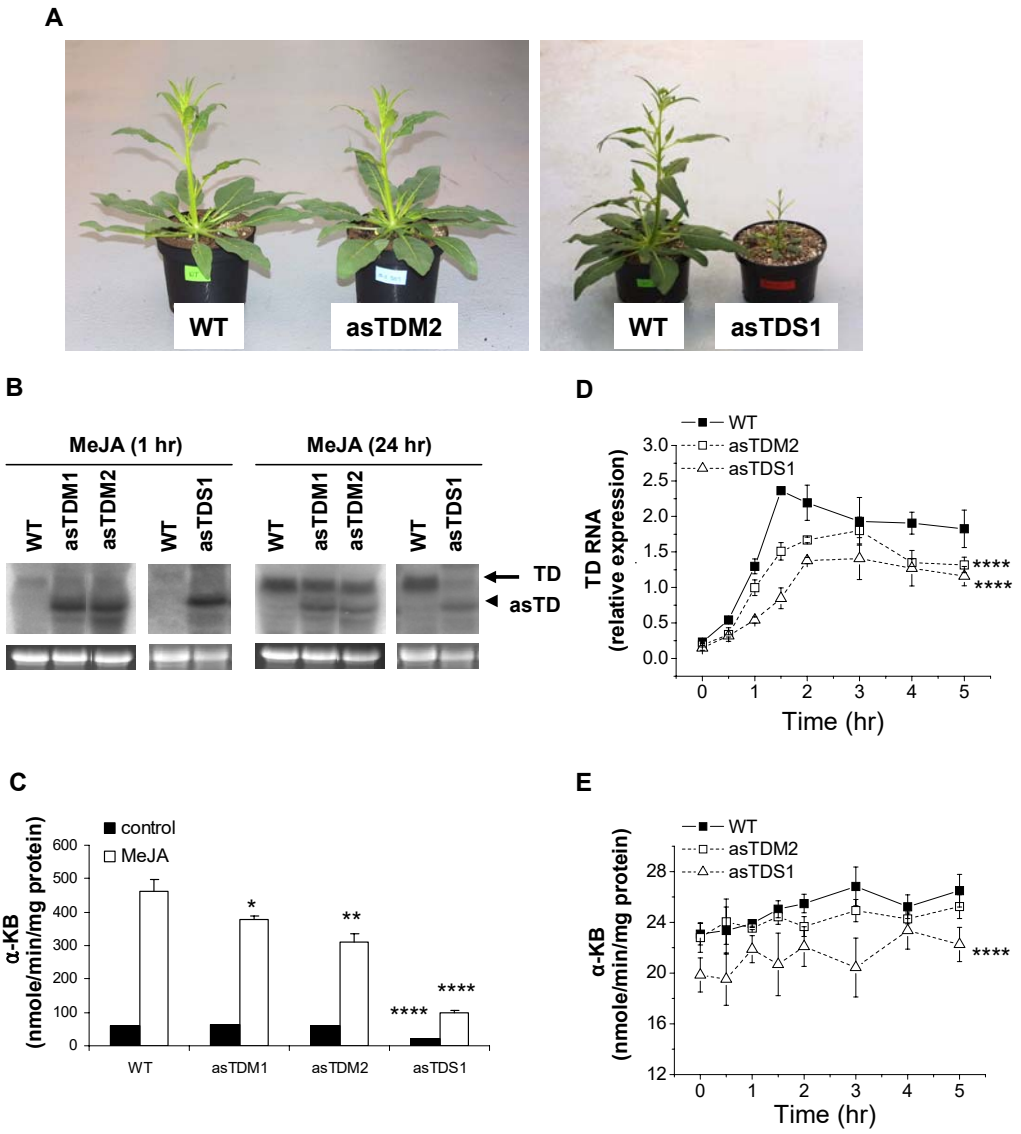


Figure 1. Antisense Suppression of Wound- and MeJA-Induced TD in asTD Plants.
(A) WT (55-day old), asTDM2 (55, 57-day old, left and right panels, respectively), and asTDS1 (85-day old) plants. Note that asTDM2 plants are morphologically indistinguishable from WT plants but asTDS1 plants are severely stunted in their growth and display numerous morphological differences from WT plants.
(B) Accumulation of TD transcripts in a pooled sample of 4 replicate node +1 leaves, which were treated with 20 µl of lanolin containing 150 µg of MeJA and harvested after 1 or 24 hr from WT and 3 independently transformed T_2 asTD plants (asTDS1,

asTDM1, and asTDM2). Arrow indicates endogenous TD RNA (TD) and arrow head indicates anti-sense TD RNA (asTD). Ethidium bromide-stained 18S rRNA is shown as a loading control.

(C) Mean (± SE) α-KB concentration of 4 replicate node +1 leaves, which were treated with 20 µl of lanolin containing 150 µg of MeJA and harvested after 24 hr from WT and 3 independently transformed T_2 asTD plants (asTDS1, asTDM1, and asTDM2). Stars represent significant differences between MeJA-treated WT and MeJA-treated asTD plants (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

(D) Accumulation of TD transcripts in leaves at node +1, which were wounded with a fabric pattern wheel and the resulting wounds were immediately treated with 20 µl of deionized water in 3 replicate WT, asTDS1, and asTDM2 plants. The transcripts were analyzed by real-time PCR as mean (± SE) of 3 replicate leaves in arbitrary units from a calibration with 5x dilution series of cDNAs prepared from RNA samples extracted 1 hr after wounding. Stars represent significant differences between WT and asTD plants (two-way ANOVA, Fisher's PLSDs, ****, $P < 0.0001$).

(E) Mean (± SE) α-KB concentration in leaves at node +1, which were wounded with a fabric pattern wheel and the resulting wounds were immediately treated with 20 µl of deionized water in 3 replicate WT, asTDS1, and asTDM2 plants. Stars represent significant differences between WT and asTD plants (two-way ANOVA, Fisher's PLSDs, ****, $P < 0.0001$).

of TD. Before MeJA treatment, TD activities in WT and asTDM plants were similar (Figure 1C; unpaired t-test, $P \leq 0.546$), but TD activities in asTDS1 plants were significantly reduced compared to WT plants (Figure 1C; unpaired t-test, $P < 0.0001$). TD activity increased eight fold in WT plants 24 hr after MeJA elicitation (Figure 1C). When asTD plants were elicited, the increases in asTDM1, asTDM2, and asTDS1 plants were significantly reduced by 19, 33, and 80%, respectively, compared to that of WT plants (Figure 1C; unpaired t-test, $P \leq 0.0498$).

When plants are treated with MeJA in a lanoline paste, plants are continuously elicited as the MeJA slowly diffuses into the plant (Zhang et al., 1997). To examine the effects of a more subtle elicitation treatment, plants were wounded once with a

fabric pattern wheel and TD mRNA expression was analyzed by real-time PCR. TD mRNA attained maximum values in WT plants 1.5 hr after wounding and slowly waned thereafter. Levels in both asTDM2 and asTDS1 plants were significantly lower compared to WT plants (Figure 1D; Fisher's PLSD, $P < 0.0001$). Wounding slightly increased the production of α-KB and the accumulation of α-KB in asTDM2 was reduced but did not differ significantly compared to that in WT (Figure 1E; Fisher's PLSD, $P = 0.243$), but the accumulation of α-KB in asTDS1 was significantly reduced compared to that in WT plants (Figure 1E; Fisher's PLSD, $P < 0.0001$).

To determine whether the mild suppression of TD transcripts and activity observed in the asTDM lines influenced plant growth and competitive ability, we synchronized the germination and growth of the different lines and grew them individually in 2L pots or in competition with each other in 2L pots and measured stalk elongation, which previous experimentation has revealed to accurately measure competitive ability and relative fitness (Glawe et al., 2003). No differences in stalk elongation among the lines were observed when plants were grown singly or in competition with WT plants (Figure S3).

To determine whether TD is involved in plant defense, we measured the performance of the insect herbivore (*M. sexta* larvae) that is responsible for the largest losses in leaf area from *N. attenuata* plants growing in nature (Baldwin, 1998). asTDS1 plants were severely stunted in their growth and differed from WT plants in leaf developmental traits (Figure 1A), which would confound comparisons of

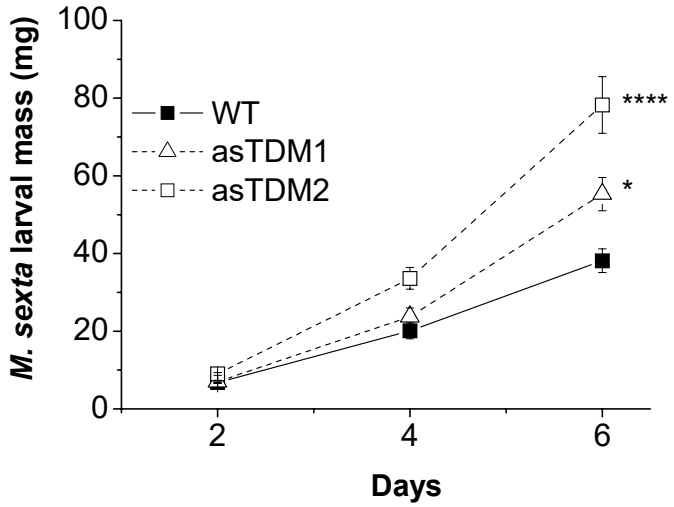


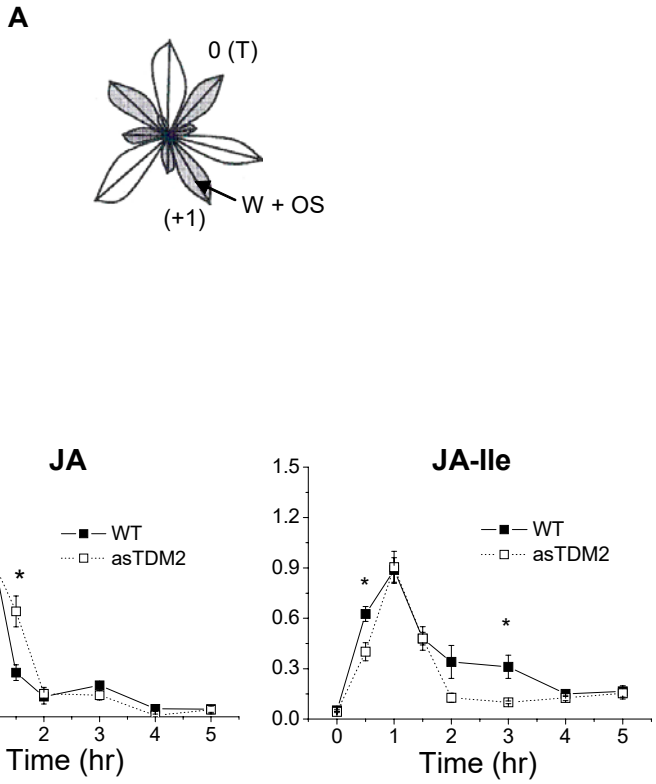
Figure 2. Silencing of TD Reduces Herbivore Resistance.
Data represent mean (± SE) mass of *M. sexta* larvae after 2, 4, and 6 days of feeding on 7 replicate WT and 2 lines of T_2 asTD plants (asTDM1 and asTDM2). A freshly eclosed *M. sexta* larva was placed on the fully-expanded node +2 of each plant. Stars represent significant differences between WT and asTD plants (repeated measurement ANOVA, Fisher's PLSDs, *, $P < 0.05$; ****, $P < 0.0001$).

herbivore performance on WT and asTDS1 plants. Therefore, we first compared herbivore performance on WT and the morphologically indistinguishable asTDM lines. When one freshly eclosed *M. sexta* larvae was placed on the source-sink transition leaf of each of seven replicate plants of each genotype, larvae gained significantly more mass on plants of both asTDM lines than they did on WT plants. By day 6, they had almost doubled their mass on asTDM2 plants compared to larvae on WT plants (Figure 2; repeated measurement ANOVA, $F_{2, 36} = 15.988$; $P = 0.0001$; PLSD ≤ 0.0485). These results demonstrate that while mild reductions in TD expression and activity does not influence growth, even under intense intra-specific competition (Figure S3), mild reductions in TD expression severely impair resistance against an adapted herbivore.

TD Silencing Impairs Jasmonate Signaling and Elicited Direct defenses in asTDM Plants

Recent research in *N. attenuata* has demonstrated that silencing of the LOX required for JA biosynthesis (NaLOX3) also silences the inducible nicotine and TPI defenses and increases *M. sexta* larval performance (Halitschke and Baldwin, 2003). Moreover, JA is known to be conjugated to several amino acids *in vitro* and JA-Ile is the most abundant JA-amino acid conjugates in *Arabidopsis* seedlings (Staswick and Tiryaki, 2004). Since TD is involved in Ile synthesis, we examined whether the effect of silencing TD on herbivore performance could be attributed to JA signaling via JA-Ile synthesis or turnover. To determine whether *M. sexta* OS elicits a rapid increase and decline in JA-Ile pools when added to puncture wounds as it does for JA, leaves at node +1 from WT and asTDM2 plants were wounded and treated with OS (Figure 3A), and analyzed by LC-MS from four independently treated plants from each

genotype at each harvest time. As expected, a dramatic JA burst was elicited within 30 min, which reached maximum levels at 1 hr, and declined rapidly after 1.5 hr in treated WT leaves (Figure 3B). Similar responses were observed in JA-Ile pools in treated WT leaves (Figure 3B). Compared to WT plants, the JA burst was similar in asTDM2 plants but waned more slowly than that in WT plants 1.5 hr after elicitation (Figure 3B; unpaired t-test, $P = 0.0120$). The OS-elicited JA-Ile burst in asTDM2 plants was less than that of WT plants, with pools being significantly lower (36 and 68%,) than those of WT at 0.5 hr and 3 hr, respectively (Figure 3B; unpaired t-test, $P \leq 0.0237$). Given that these are subtle changes in the timing of very dynamic jasmonate response and that the aspects of the JA and JA-Ile bursts which are relevant for signal transduction remain unknown, we tested the hypothesis that increasing Ile pools at the wound site could restore the diminished accumulation of a jasmonate-elicited direct defense (TPI) in *N. attenuata*.



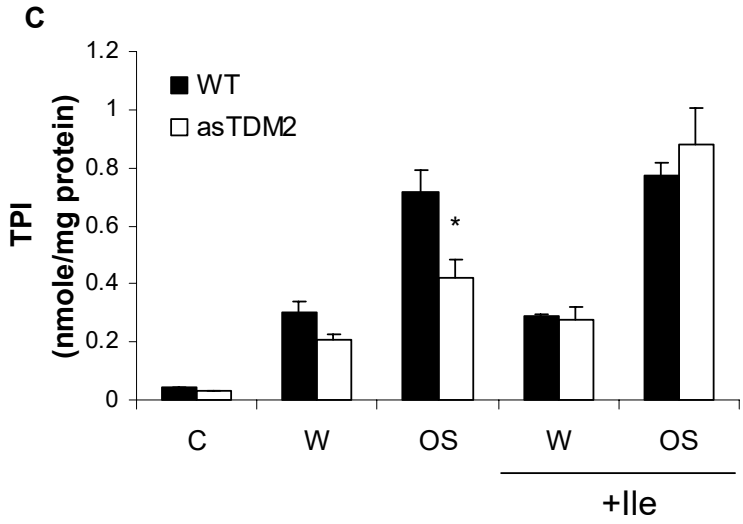


Figure 3. TD Silencing Alters JA and JA-Ile Accumulations and Impairs OS-elicited TPI Activity.

(A) Numbering of leaf position in rosette-stage *N. attenuata*. The leaf undergoing the source–sink transition (T) was designated as growing at node 0. The treated leaf growing at node +1, which is older by one leaf position than the source-sink transition leaf, was wounded with a fabric pattern wheel, and the resulting puncture wounds (W) were immediately treated with 20 µl of *M. sexta* oral secretions (OS). The treated leaves were harvested to measure JA and JA-Ile.

(B) Mean (± SE) JA and JA-Ile concentration in leaves of 4 replicate WT and asTDM2 plants. Stars represent significant differences between members of a pair harvested at a given time point (unpaired t-test: *, $P < 0.05$).

(C) Mean TPI activity (± SE) in WT and asTDM2 plants of three replicate node +1 leaves which were harvested 3 days after they were wounded and treated with 20 µl of the following solutions: deionized water (W) or *M. sexta* OS supplemented with 0.625 µmole of isoleucine (+ Ile). Leaves from control plants (C) were left unwounded and untreated. Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$).

OS-treatment of puncture wounds in WT plants results in a 2.4-fold increase in TPI activity compared to wounding alone (Figure 3C). The wound-induced

accumulation of TPI activity in asTDM2 plants was 31% lower than that in WT plants (Figure 3C; unpaired t-test, $P = 0.0911$) and the OS-induced accumulation of TPI in asTDM2 plants was 41% lower than that in WT plants (Figure 3C; unpaired t-test, $P = 0.0386$). These results suggest that diminished TPI levels are the causal reason for the increase in performance of *M. sexta* larvae feeding on asTDM2 plants. When Ile was added to water or OS before being applied to the puncture wounds, the TPI elicitation in asTDM2 plants was restored to levels found in WT plants (Figure 3C). The restoration of TPI activity by Ile supplementation at the wound site in asTDM plants could be either due to the restoration of the biosynthetic needs of TPI production or its signaling.

JA-Ile is Produced at the Wound-Site and Is Limited by Ile Pools in asTDM Plants

To determine whether JA-Ile is synthesized from JA and Ile at the wound site, node +1 leaves from WT and asTDM2 plants were wounded and immediately treated with OS containing 0.625 µmole $^{13}C_4$-Thr or $^{13}C_6$-Ile (Figure 4A). Four replicate plants were harvested for each treatment and harvest time to measure the elicited kinetics of unlabeled JA-Ile, ^{13}C-labeled JA-Ile, and JA by LC-MS analysis (Figure 4B). Significant quantities of ^{13}C-labeled JA-Ile were detected when either $^{13}C_4$-Thr or $^{13}C_6$-Ile was applied, demonstrating that $^{13}C_4$-Thr was rapidly converted to Ile at the wound site and used to synthesize ^{13}C-labeled JA-Ile. As expected, Thr was less efficiently incorporated into JA-Ile than Ile (Figure 4B). asTDM2 plants were marginally less efficient in incorporating $^{13}C_4$-Thr into JA-Ile than were WT plants 0.5 hr after elicitation (Figure 4B; unpaired t-test, $P = 0.0183$), which reflects the lower TD activity of these plants. This experiment also demonstrated that the conjugation capacity of an elicited leaf is limited by substrate availability. In WT plants, addition of Thr to an OS-elicited wound reduced the maximum JA values by approximately 5.5 nmole/g FW (compare $^{13}C_4$-Thr treatments: 6 nmole/g FW in Figure 4B with OS elicited values of 11.5 nmole/g FW: Figure 3B). Addition of the more efficiently incorporated amino acid, Ile, reduced JA values even further (to 4 nmole/g FW: Figure 4B). In summary, this experiment demonstrates that Thr is rapidly converted into Ile, which is subsequently used for JA-Ile synthesis from an OS-elicited JA burst. The experiment also demonstrates that supplementing the Ile pool at the wound site can dramatically draw down the free JA pools during an OS-

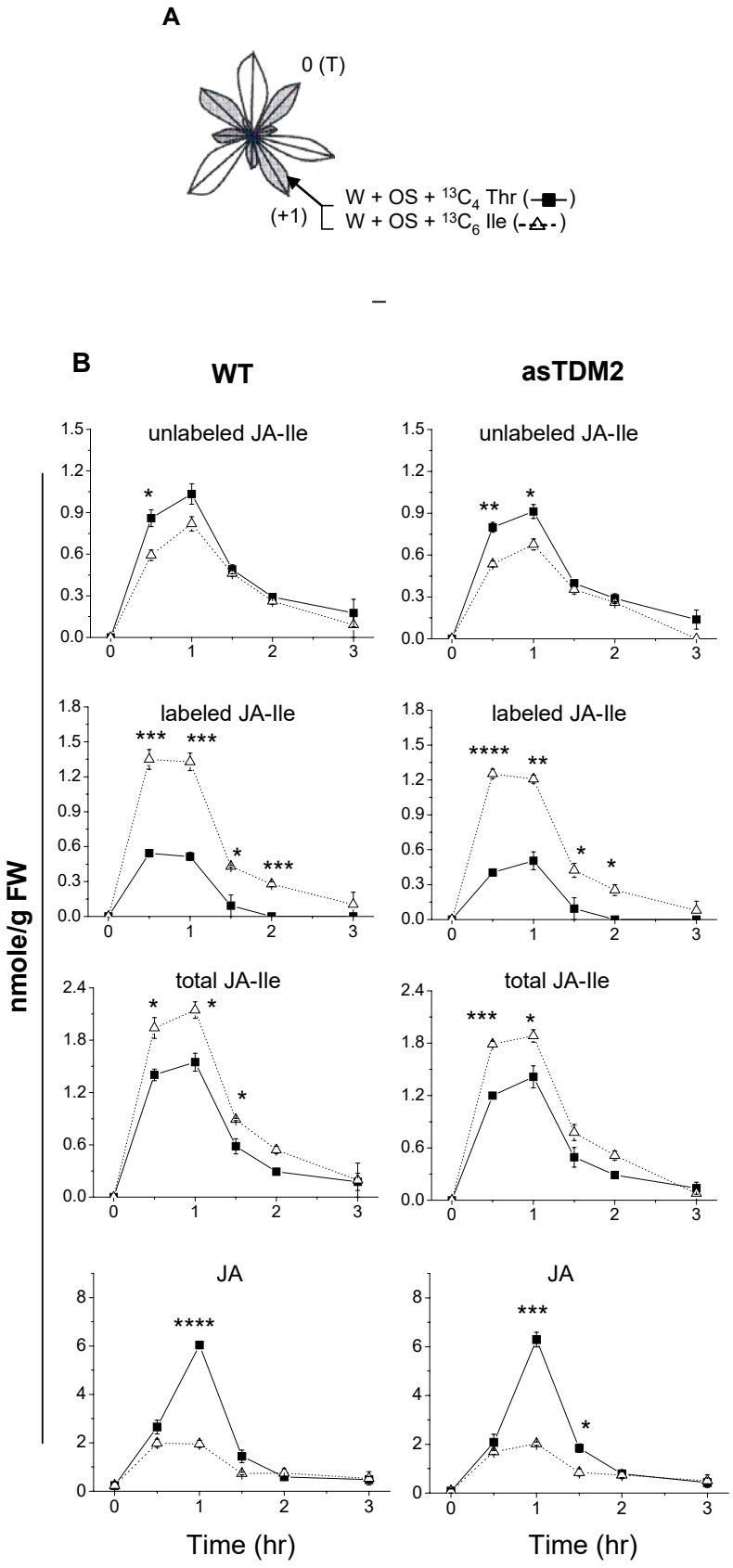

A
0 (T)
(+1)
W + OS + $^{13}C_4$ Thr (–■–)
W + OS + $^{13}C_6$ Ile (-△-)
B
WT
asTDM2
unlabeled JA-Ile
labeled JA-Ile
total JA-Ile
JA
nmole/g FW
Time (hr)

Figure 4. Addition of ^{13}C-Labeled Thr and Ile to an OS-Elicited Wound Results in the Rapid Synthesis of ^{13}C-JA-Ile and Depletion of JA at the Wound Site.
(A) Node +1 leaves were wounded with a fabric pattern wheel, and the resulting puncture wounds (W) were immediately treated with 20 µl of *M. sexta* OS containing 0.625 µmole of $^{13}C_4$ labeled threonine ($^{13}C_4$ Thr) or $^{13}C_6$ labeled isoleucine ($^{13}C_6$ Ile).
(B) Mean (± SE) JA-Ile, ^{13}C-labeled JA-Ile, and JA of node +1 leaves from 3 replicate WT and asTDM2 plants. Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

elicited JA burst. To more clearly characterize the effect of silencing TD in asTDM plants on JA-Ile formation, an additional experiment was conducted in which the supply of free JA was made non-limiting for the conjugation reaction.

To determine whether JA-Ile synthesis is limited by the amount of free JA at the wound site, node +1 leaves from WT and asTDM2 plants were wounded and treated with 0.625 µmole JA (Figure 5A). When only wounded, JA-Ile pools increased within 30 min and waned to control values by 1.5 hr in both WT and asTDM2 plants. Levels of JA-Ile did not differ between WT and asTDM2 plants in plants whose wounds were treated only with water. However, when wounds were supplemented with JA, plants sustained a dramatically elevated JA-Ile pools for 2.5 hr (Figure 5B), demonstrating that the level of JA regulates the level of JA-Ile. Under these experimental conditions, when JA is not limiting, clear differences between asTDM2 and WT plants in their ability to produce JA-Ile were readily discerned: the amount of JA-Ile in asTDM2 plants was significantly lower than that in WT plants (Figure 5B; two-way ANOVA, $F_{1,16} = 9.768$; $P = 0.0065$). When $^{13}C_6$ Ile alone or in combination with JA was supplied to the wound, the levels of JA-Ile did not differ between WT and asTDM2 plants (Figure 5B; two-way ANOVA, $F_{1,16} = 0.999$; $P = 0.3324$). These results demonstrate that the supply of the Ile limits JA-Ile synthesis in asTDM2 plants compared to WT plants, and that the JA-Ile conjugation enzyme in asTDM2 plants is as active as it is in WT plants. These experiments also demonstrate that the JA and JA-Ile bursts that erupt when *M. sexta* OS is introduced into a leaf wound can be simulated by adding JA to a wound. Addition of JA to wounds results

in a JA-Ile burst that is sustained at higher levels in WT plants than in asTDM plants due to their diminished supplies of Ile.

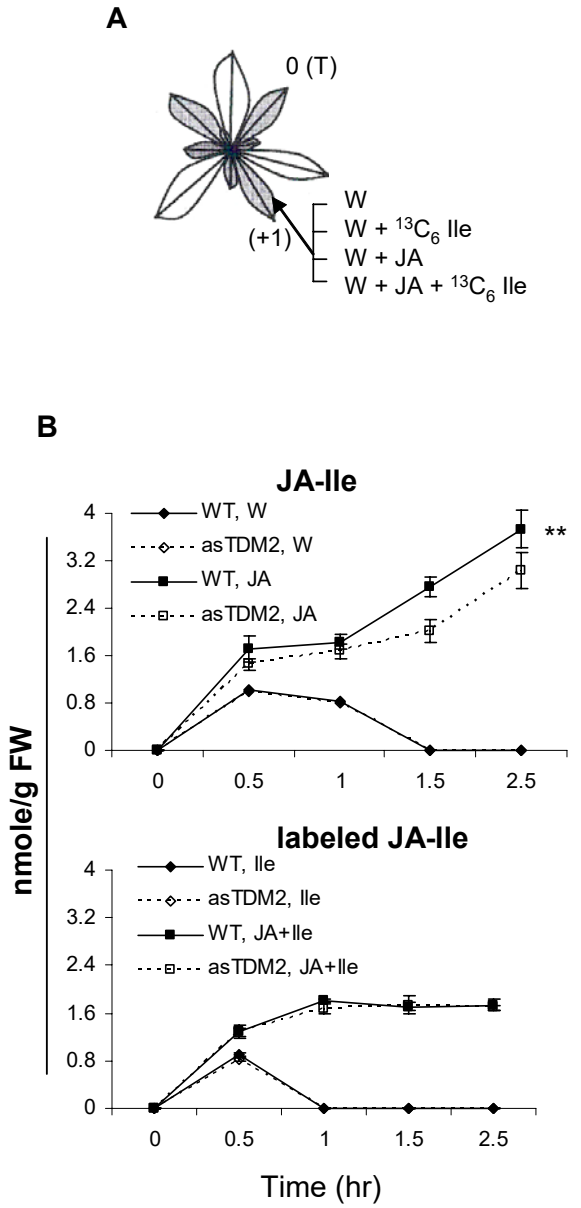


Figure 5. Addition of JA to Wounds Creates a Sustained JA Burst at the Wound Site (without OS) and Reveals Clear Differences between asTDM2 and WT Plants in the Accumulation of JA-Ile.

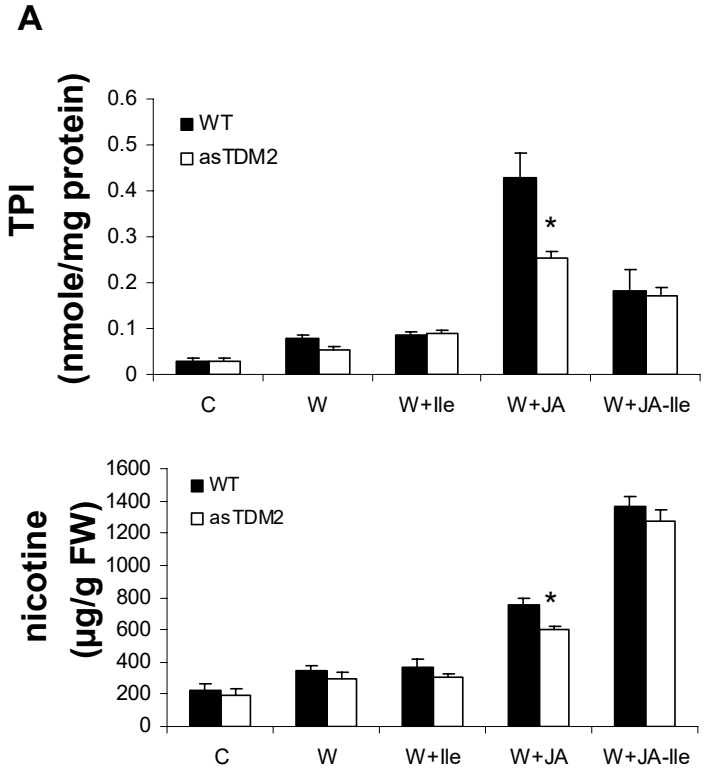
(A) Node +1 leaves were wounded with a fabric pattern wheel, and the resulting puncture wounds (W) were immediately treated with 20 µl of water containing 0.625 µmole of $^{13}C_6$ labeled isoleucine ($^{13}C_6$ Ile), 20 µl of water containing 0.625 µmole of

JA (JA), or 20 µl of water containing 0.625 µmole of JA and 0.625 µmole of $^{13}C_6$ labeled isoleucine (JA + $^{13}C_6$ Ile).
(B) Mean (± SE) JA-Ile and ^{13}C-labeled JA-Ile of leaves from 3 replicate WT and asTDM2 plants. Stars represent significant differences between WT and asTDM2 plants (two-way ANOVA: Fisher's PLSDs, **, $P < 0.01$).

Supplementing asTDM Plants with JA-Ile Restores Direct Defenses and Herbivore Resistance

After discovering that asTDM2 plants had reduced levels of JA-Ile (Figure 5B), we were interested in determining whether JA-Ile could elicit direct defenses and whether the herbivore resistance of asTDM2 plants could be restored by JA-Ile treatment. To address the first question, we wounded four replicate of WT and asTDM2 plants; treated the wounds with Ile, JA, or JA-Ile; and measured their TPI and nicotine responses. The compounds were added to wounds in aqueous solutions, because these water-soluble compounds are unable to transverse the leaf cuticle when applied in a lanolin paste. The addition of JA or JA-Ile to wounds of both WT and asTDM2 plants significantly increased TPI to levels higher than when plants were only wounded (Figure 6A; unpaired t-test, $P \leq 0.0035$). When plant wounds were treated with JA, a treatment demonstrated to produce sustained differences in endogenous JA-Ile levels between WT and asTDM2 plants (Figure 5B), elicited TPI activity in asTDM2 plants was 40 % lower than that of WT plants (Figure 6A; unpaired t-test, $P = 0.0361$). When plants were treated with JA-Ile, the induced TPI responses did not differ between WT and asTDM2 lines (Figure 6A; unpaired t-test, $P = 0.8197$), though they were significantly lower than those elicited in WT plants by JA treatment. Similar results were observed for the nicotine responses (Figure 6A). Treatment with JA or JA-Ile resulted in higher levels of nicotine than when plants were only wounded (Figure 6A; unpaired t-test, $P \leq 0.0022$). Accumulations of nicotine in response to JA treatment in asTDM2 plants were 19% lower than those in WT plants (Figure 6A; unpaired t-test, $P = 0.0396$), while the responses to JA-Ile treatment did not differ between asTDM2 and WT plants (Figure 6A; unpaired t-test, $P = 0.4214$). However, unlike levels of TPI, the levels of nicotine elicited in plants treated with JA-Ile were much higher than those in plants treated with JA (Figure 6A).

Levels of chlorogenic acid in JA or JA-Ile treatment did not differ from levels in the untreated control, and though levels of diterpene glycosides were similarly elicited by JA or JA-Ile treatment, levels did not differ between asTDM2 and WT plants (Figure S4) demonstrating that these secondary metabolites are not differentially elicited by JA and JA-Ile. Differences in the ability of JA and JA-Ile to elicit nicotine and TPI may reflect different rates of absorption in treated leaves or their transport within the plant. For example, JA may be more readily absorbed in the treated leaf to effect changes in TPI production, while JA-Ile may be more readily transported from the treated leaves to the nicotine-producing roots. Most important, the elicited nicotine and TPI responses, which were significantly lower in JA-treated asTDM2 plants than in WT plants, did not differ between WT and asTDM2 plants when plants were treated with JA-Ile. These results demonstrated that JA-Ile could restore the direct defense responses of asTDM2 plants to those of WT plants; the next step was to determine if resistance against *M. sexta* larvae could be similarly restored.



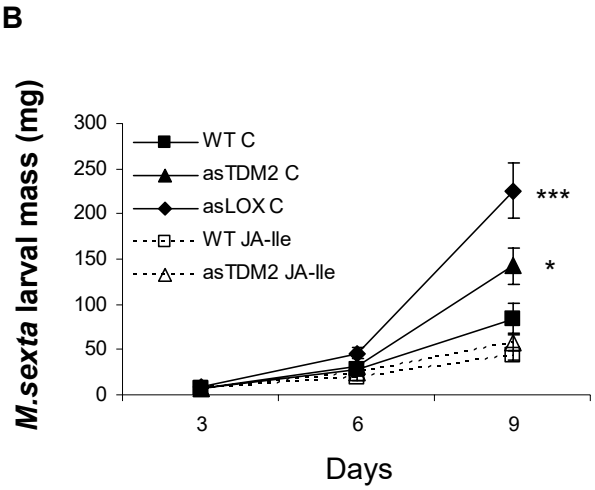


Figure 6. Addition of JA to Wounds Results in Lower TPI and Nicotine Accumulation in asTDM2 Plants Compared to WT Plants but JA-Ile Additions Result in Similar TPI Responses and Restores Herbivore Resistance.

(A) Mean (± SE) TPI and nicotine of leaves growing at node +1, 3 days after they were wounded and treated with 20 µl of the following solutions in 3 replicate WT and asTDM2 plants: deionized water (W), isoleucine (W+Ile), jasmonic acid (W+JA), or jasmonic acid-isoleucine conjugate (W+JA-Ile) all at 0.625 µmole. Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$).

(B) Mean (± SE) mass of *M. sexta* larvae after 3, 6, and 9 days of feeding on 16 replicate WT and two lines of T_2 transgenic plants (asTDM2 and asLOX3). Leaves were treated with 0.625 µmole JA-Ile or left untreated (C). Stars represent significant differences between untreated WT and untreated 2 lines of T_2 transgenic plants in 9 days (unpaired t-test: *, $P < 0.05$; ***, $P < 0.001$). asLOX3 plants, which are largely defenseless due to their impaired jasmonate signaling (Halitschke and Baldwin, 2003), were included as a "positive control" for herbivore resistance.

We measured the performance of the *M. sexta* larvae on WT, asTDM2, and a genotype of *N. attenuata* plants (asLOX plants), in which *NaLOX3*, the lipoxygenase gene supplying fatty acid hydroperoxides for JA biosynthesis, was silenced by antisense expression. asLOX plants have lower levels of JA and dramatically reduced

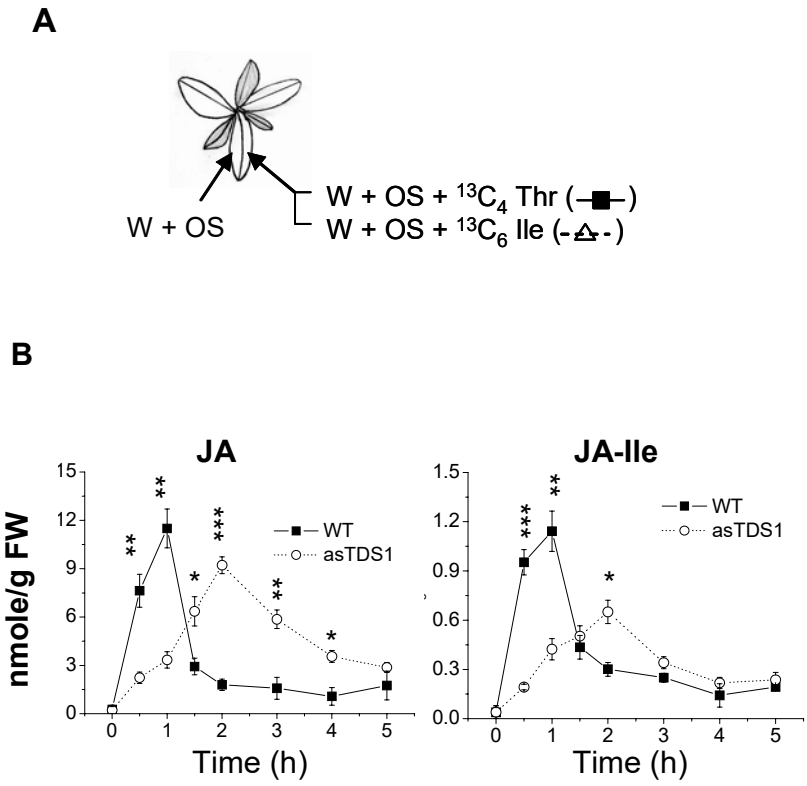
levels of the direct defenses, nicotine and TPI, and are dramatically impaired in their herbivore resistance (Halitschke and Baldwin, 2003). These "defenseless" plants were included in the analysis to gauge the degree to which herbivore resistance had been impaired in the asTDM lines. At day 9, *M. sexta* larvae fed on untreated asTDM2 and asLOX plants gained 68% and 166% more mass than those fed on WT plants, respectively (Figure 6B; unpaired t-test, $P \leq 0.041$). Treating asTDM plants with JA-Ile fully restored the plant's resistance; larvae fed on JA-Ile-treated asTDM plants attained masses that were statistically indistinguishable from those fed on JA-Ile elicited WT (Figure 6B; unpaired t-test, $P = 0.19$). In summary, these results demonstrate that JA-Ile is a potent elicitor of direct defenses, particularly TPI and nicotine, and that treatment of asTDM2 plants with JA-Ile can restore this line's resistance against *M. sexta* larvae. Since we now understood the traits responsible for the defects in herbivore resistance associated with TD silencing, we were ready to examine herbivore resistance in the developmentally challenged asTDS plants.

Jasmonate Signaling in asTDS1 Plants

To determine the effect of OS on JA and JA-Ile elicitation in asTDS1 plants, WT and asTDS1 plants were wounded and treated with OS, and analyzed from four independently treated plants from each genotype at each harvest time (Figure 7A). The OS-elicited changes in JA and JA-Ile pools in asTDS1 plants did not resemble the bursts observed in either WT or asTDM plants. Both JA and JA-Ile pools waxed and waned slowly, attaining maximum values at 2 hr (Figures 7B). The integrated JA levels in asTDS1 plants (approx 33.66nmole/g FW/5 hrs; Figure 7B) were 18% higher than those in WT plants (approx 28.53nmole/g FW/5 hrs; Figure 7B). The integrated JA-Ile levels in asTDS1 plants (approx 2.61nmole/g FW/5 hrs; Figure 7B) were 25% lower than those in WT plants (approx 3.46nmole/g FW/5 hours; Figure 7B). The lower Ile pools of asTD plants may account for the slower decline of the JA burst and the lower levels of JA-Ile observed in these plants.

The analysis of the asTDS1 plants clearly demonstrated that JA-Ile synthesis is limited by the Ile produced by TD at the wound site. asTDS1 plants were wounded and treated with OS containing 0.625 μmole $^{13}C_4$-Thr or $^{13}C_6$-Ile, and four replicate plants were harvested for each treatment to measure the elicited kinetics of unlabeled JA-Ile, ^{13}C-labeled JA-Ile, and JA (Figure 7A). When $^{13}C_4$-Thr was applied to asTDS1 plants, ^{13}C-labeled JA-Ile was only detected at 0.5 hr (Figure 7C). However,

when $^{13}C_6$-Ile was applied to asTDS1 plants, ^{13}C-labeled JA-Ile was continuously produced at lower levels compared to those of WT plants (Figures 4B, 7C), demonstrating that silencing TD expression could be directly associated with reductions in the conversion of Thr to Ile and its subsequent incorporation into JA-Ile.



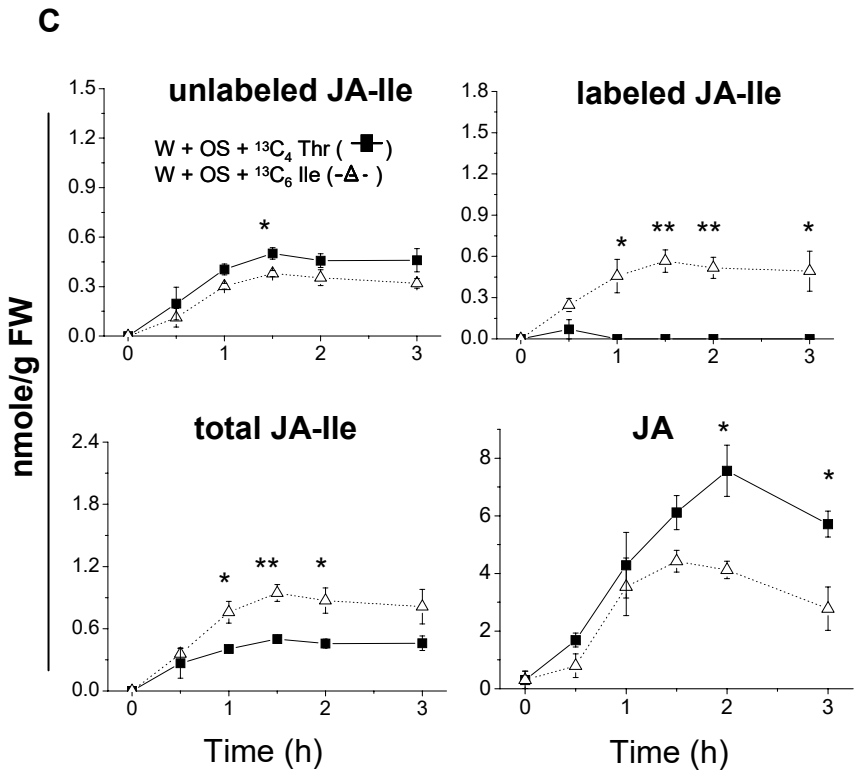


Figure 7. Accumulation of JA and JA-Ile in Response to OS Elicitation, and Addition of ^{13}C-Labeled Thr and Ile to OS-Elicited Wounds in Leaves of asTDS1 Plants.

(A) Leaves were wounded with a fabric pattern wheel, and the resulting puncture wounds (W) were immediately treated with 20 µl of *M. sexta* oral secretions (OS), 20 µl of *M. sexta* OS containing 0.625 µmole of $^{13}C_4$ labeled threonine ($^{13}C_4$ Thr) or $^{13}C_6$ labeled isoleucine($^{13}C_6$ Ile).

(B) Mean (± SE) JA and JA-Ile concentrations in leaves of 4 replicate WT and asTDS1 plants. Leaves were wounded and treated with *M. sexta* OS.

(C) Mean (± SE) JA-Ile, ^{13}C-labeled JA-Ile, and JA concentration in leaves of 3 replicate WT and asTDS1 plants. Leaves were wounded and treated with *M. sexta* OS containing 0.625 µmole of $^{13}C_4$ Thr or $^{13}C_6$ Ile. Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$).

Supplementing asTDS Plants with Ile Restores TPI Activity and Herbivore Resistance

OS-treatment of puncture wounds in WT plants results in a dramatic increase in TPI activity compared to wounding alone (Figure 3C). In asTDS1 plants, OS treatment of wounds did not significantly increase TPI activity compared to water-treated wounds (Figure 8A; unpaired t-test, $P = 0.719$), suggesting that severe nutritional deficiencies inhibited TPI production in these plants. However, adding Ile to the puncture wounds did not significantly increase TPI activity of wounded plants (Figure 8A; unpaired t-test, $P \geq 0.5787$). In contrast, adding Ile to OS before being applied to the puncture wounds increased TPI activity significantly (Figure 8A; unpaired t-test, $P = 0.0159$) compared to that in OS-treated asTDS1 plants.

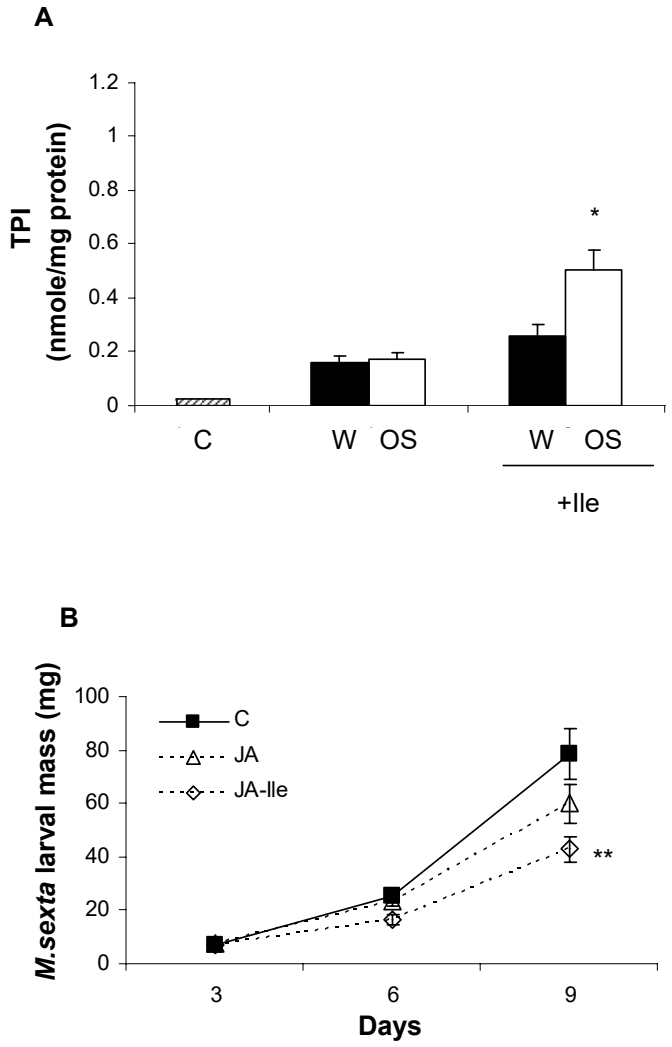


Figure 8. Effect of Ile on OS-Elicited TPI Activity and Response of *M. sexta* to JA and JA-Ile in asTDS1 Plants.

(A) Mean (± SE) TPI activity in asTDS1 plants. Three replicate leaves were harvested 3 days after they were wounded and treated with 20 µl of the following solutions: deionized water (W) or *M. sexta* OS supplemented with 0.625 µmole of isoleucine (+ Ile). Leaves from control plants (C) were left unwounded and untreated. Stars represent significant differences between OS-treated asTDS1 plants supplemented with Ile and only OS-treated asTDS1 plants (unpaired t-test: *, $P < 0.05$).

(B) Mean (± SE) mass of *M. sexta* larvae after 3, 6, and 9 days of feeding on 16 replicate asTDS1 plants. Leaves were treated with 0.625 µmole of JA, JA-Ile or left untreated (C). Stars represent significant differences between untreated and JA-Ile treated plants in 9 days (unpaired t-test: **, $P < 0.01$).

To determine if JA-Ile treatment could elicit induced resistance in asTDS plants, we measured the performance of the *M. sexta* larvae on JA and JA-Ile treated asTDS1 plants. *M. sexta* larvae fed on JA-treated plants gained less mass compared to those fed on untreated plants but the difference was not significant at day 9 (Figure 8B; unpaired t-test, $P = 0.134$). When fed on JA-Ile treated plants however, the larvae gained significantly less mass (45%) compared to those fed on untreated plants at day 9 (Figure 8B; unpaired t-test, $P = 0.0027$). These results demonstrate that even in plants with severely silenced TD that suffer from severe nutritional deficiencies, Ile is conjugated to JA at the wound site to mediate defense signaling. Supplementing wounds with JA-Ile restores a modicum of induced resistance in these severely growth-impaired plants.

Suppression of TD and JAR4 by VIGS Impairs Jasmonate Signaling and Herbivore Resistance

To further examine whether JA-Ile is the signal molecule that elicits herbivore resistance, we cloned the *Arabidopsis JAR1* homologue (*Na*JAR4*;*Genebank accession number xxxxxx *)* from *N. attenuata* using RT-PCR. To investigate whether *NaJAR4* encodes the enzyme conjugating amino acids to JA in *N. attenuata*, we collected amino acid sequences of JAR-like proteins using *Na*JAR4 as a query.

Phylogenetic analysis revealed these proteins clustered into 3 groups. *Na*JAR4 and *At*JAR1 cluster together with three other functionally unknown proteins (Figure S5) which share more than 60% amino acid identity (Figure S6), suggesting that they share similar functions as JAR1, conjugating amino acid to JA (Staswick et al., 2002; Staswick and Tiryaki, 2004). The other JAR family members, *At*GH3.1, *At*GH3.2, *At*GH3.5 and *At*GH3.17, which conjugate amino acids to IAA (Staswick et al., 2005), all clustered together in a separate group. Southern blotting revealed that *Na*JAR4 is a single copy gene in the *N. attenuata* genome (Figure S7). These results suggested that JAR4 is a good candidate for the JA conjugating enzyme in *N. attenuata*.

To determine whether JAR4 mRNA is elicited by wounding or OS-treatment of wounds, plants were wounded with a fabric pattern wheel, treated with water or OS, and *Na*JAR4 mRNA accumulation was analyzed by quantitative real-time PCR (qRT-PCR). In response to wounding alone, JAR4 mRNA levels increased within 30 min, reached maximum levels at 1.5 hr, and declined after 3 hr (Figure 9). Similar patterns of transcript accumulation were observed in OS-treated WT leaves but levels waned more slowly and did not return to control levels by 12 hr (Figure 9).

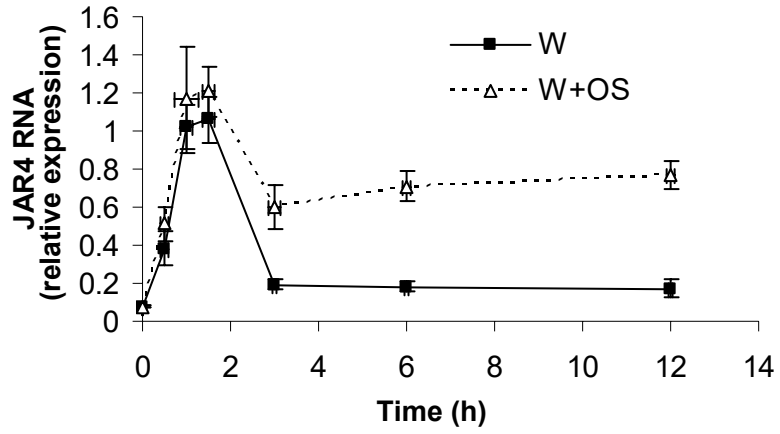
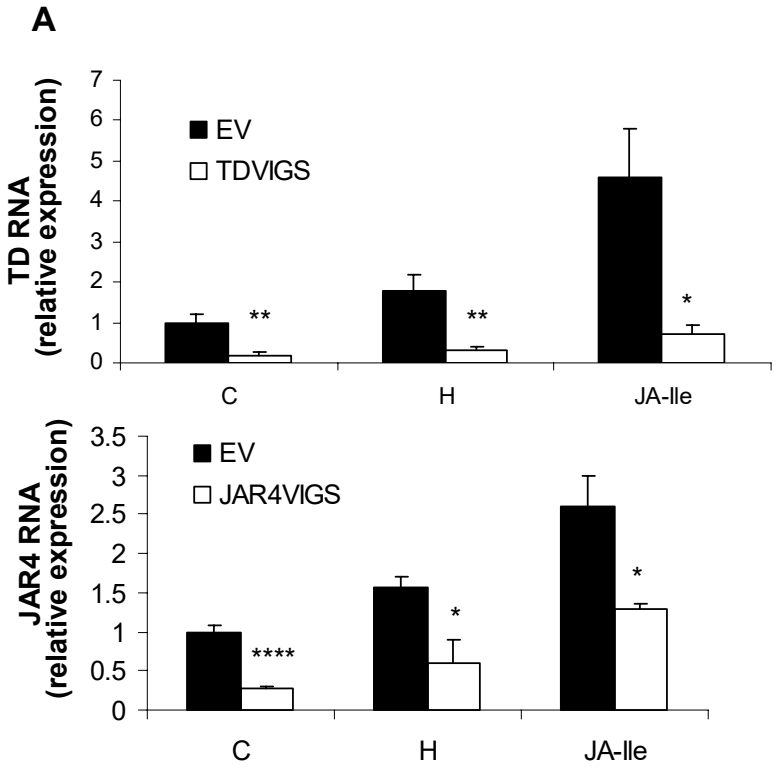


Figure 9. Accumulation of JAR4 Transcripts After Elicitation by Wounding and OS Treatment.

Leaves at node +1, which were wounded with a fabric pattern wheel and the resulting wounds were immediately treated with 20 µl of deionized water (W) or treated with *M. sexta* OS in 5 replicate WT plants. The transcripts were analyzed by real-time PCR

as mean (± SE) of 5 replicate leaves in arbitrary units from a calibration with 5x dilution series of cDNAs prepared from RNA samples extracted 1hr after wounding.

To determine whether JAR4, as well as TD, is involved in eliciting herbivore resistance, we used virus-induced gene silencing (VIGS) system optimized for *N. attenuata* (Saedler and Baldwin, 2004) to silence *Na*JAR4 and *Na*TD mRNA separately in WT plants. To monitor the progress of VIGS, we silenced phytoene desaturase (PDS), a gene which oxidizes and cyclizes phytoene to α-and β-carotene, which are subsequently converted into xanthophylls of the antenna pigments of the photosystems of plants, results in visible bleaching of green tissues. When the leaves of PDS-silenced plants began to bleach (6 weeks after germination, Figure S8), leaves of TD-silenced (TDVIGS), JAR4-silenced (JAR4VIGS), and empty vector inoculated (EV) plant were elicited by wounding and treating the puncture wounds with JA-Ile,



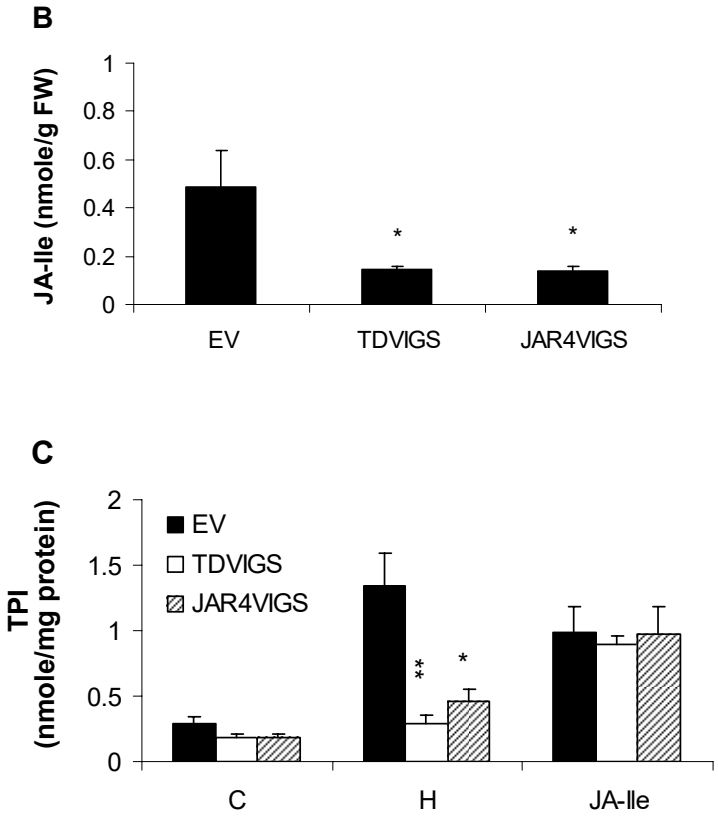


Figure 10. Silencing of TD and JAR4 by VIGS Reduces Transcript and JA-Ile Accumulations, TPI activity, Which Is Restored by JA-Ile Treatment.

(A) Suppression of TD and JAR4 transcripts in VIGS plants. Plants were inoculated with *Agrobacterium* harboring TRV constructs containing an empty vector (EV), a 335 bp *N. attenuata* TD fragment (TDVIGS), or a 292 bp *N. attenuata* JAR4 fragment (JAR4VIGS). 14 days after inoculation, leaves were wounded with a fabric pattern wheel and the resulting wounds were treated with 0.625 µmole of JA-Ile and subsequently harvested 1 hr after JA-Ile treatment (JA-Ile), or after 12 days of *M. sexta* larvae feeding (H), or from untreated plants (C). The transcripts were analyzed by real-time PCR as mean (± SE) of 5 replicate leaves in arbitrary units from a calibration with 5x dilution series of cDNAs prepared from EV control RNA samples. Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

(B) Mean (± SE) JA-Ile concentrations in leaves of 4-5 replicate EV, TDVIGS, and JAR4VIGS plants. 14 days after inoculation, leaves were harvested 1 hr after they

were wounded and treated with 20 µl of *M. sexta* oral secretions. Stars represent significant differences between EV and VIGS plants (unpaired t-test: *, $P < 0.05$). **(C)** Mean (± SE) TPI activity of 5 replicate EV, TDVIGS, and JAR4VIGS plants. 14 days after inoculation, leaves were wounded, treated with 0.625 µmole of JA-Ile and harvested 3 days after JA-Ile treatment (JA-Ile) or after 12 days of *M. sexta* larvae feeding (H), or from untreated (C) plants. Stars represent significant differences between members of a pair (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$).

other plants were left untreated and on other plants, a freshly hatched *M. sexta* larvae was placed on each plant. Real-time PCR analysis confirmed that suppression of TD and JAR4 by VIGS (Figure 10). TD RNA levels in TDVIGS plants were 20, 17, and 16 % of those in EV control plants when plants were untreated, attacked by *M. sexta* larvae, or treated with JA-Ile (Figure 10A; unpaired t-test, $P \leq 0.035$). JAR4 RNA levels in JAR4VIGS plants were 27, 38, and 49 % of those in EV plants when plants were untreated, attacked by *M. sexta* larvae, or treated with JA-Ile (Figure 10A; unpaired t-test, $P \leq 0.032$). The analysis JA-Ile pools 1 hr after OS elicitation in the VIGS plants, demonstrated that both TD and JAR4 are comparably important in supporting the JA-Ile burst; elicited JA-Ile levels in TDVIGS and JAR4VIGS plants were 30 and 29 % of those in EV plants (Figure 10B; unpaired t-test, $P \leq 0.042$). Neither the addition of JA nor the addition of Thr to the wound site of either elicited TDVIGS or JAR4VIGS plants could restore the JA-Ile accumulation observed in EV plants (Figure S9; unpaired t-test, *P's* < 0.0054). As WT plants do, VIGS plants also showed increased TPI levels when attacked by *M. sexta* larvae or treated with JA-Ile (Figure 10C). When plants were attacked by *M. sexta* larvae, elicited TPI activity in TDVIGS and JAR4VIGS plants were 22 and 35 % of that in EV plants (Figure 10C; unpaired t-test, $P \leq 0.023$), demonstrating that both TD and JAR4 are involved in TPI elicitation. When plants were treated with JA-Ile, the induced TPI responses in TDVIGS and JAR4VIGS plants were restored to those of EV plants (Figure 10C; unpaired t-test, $P \geq 0.627$), demonstrating that TPI responses were not affected by VIGS inoculation and that treatment of JA-Ile to TD and JAR4 silenced plants restored elicited TPI activity.

As demonstrated by asTD transgenic plants, TDVIGS and JAR4VIGS plants were also susceptible to *M. sexta* larval attack compared to EV plants. When *M. sexta* larvae were placed on untreated leaves, larvae gained significantly more mass on both TDVIGS and JAR4VIGS plants than they did on EV plants. By day 6, their masses were already twice those of larvae on EV plants (Figure 11, C; unpaired t-test, $P \leq 0.021$). By day 9, larvae fed on TDVIGS and JAR4VIGS plants had 80 % more weight than those fed on EV plants (Figure 11, C; unpaired t-test, $P \leq 0.040$). When *M. sexta* larvae were placed on JA-Ile-treated leaves, larvae fed on TDVIGS and JAR4VIGS plants attained masses that were statistically indistinguishable from those fed on EV plants at both day 6 and day 9 (Figure 11, JA-Ile; unpaired t-test, $P \geq 0.209$), demonstrating that JA-Ile could restore herbivore resistance in TDVIGS and JAR4VIGS plants.

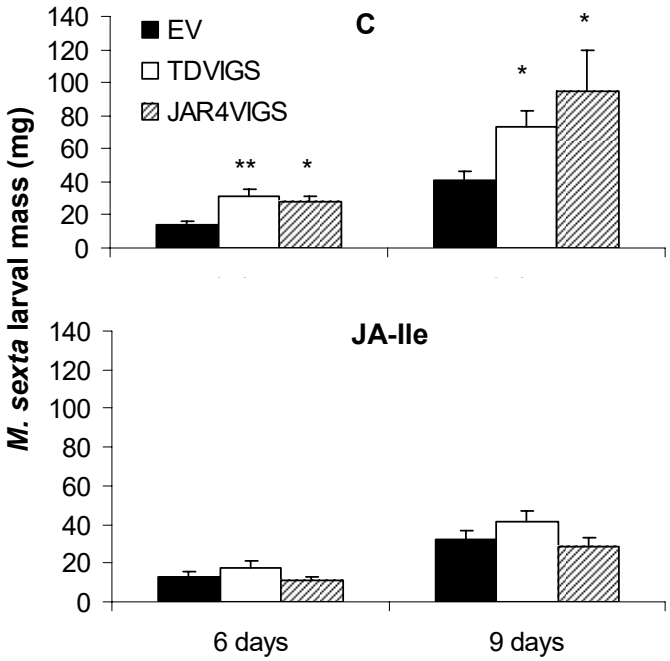


Figure 11. Silencing of TD and JAR4 by VIGS Reduces Herbivore Resistance Which Is Restored by the Addition of JA-Ile.

Mean (± SE) mass of *M. sexta* larvae after 6 and 9 days of feeding on 11-12 replicate plants, each inoculated with *Agrobacterium* harboring TRV constructs containing either an empty vector (EV), a 335 bp *N. attenuata* TD fragment (TDVIGS), or a 292 bp *N. attenuata* JAR4 fragment (JAR4VIGS). 14 days after inoculation, leaves were

wounded and treated with 0.625 µmole of JA-Ile (JA-Ile) or left untreated (C). Stars represent significant differences between EV and TDVIGS or EV and JAR4VIGS (unpaired t-test: *, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

Due to the discovery more than two decades ago of the genes responsible for the biosynthesis of amino acids, plant biologists were able to determine which were essential for growth and development. In an attempt to improve the nutritional value of cereal crops, which have low levels of lysine and Thr, biologists have focused attention to the essential amino acids, Thr, Lys, Met, and Ile which are synthesized via a common pathway (Azevedo et al., 1997). TD catalyzes the conversion of Thr to α-KB, the first committed step in Ile biosynthesis (Umbarger, 1978). The research presented here highlights the challenges of disentangling the multiple roles that amino acid biosynthesis plays in plants and the ability to analyze subtle phenotypes when working with a plant for which the determinants of ecological performance are well understood.

TD's role in herbivore resistance was discovered with the transformants (asTDM) in which TD expression was mildly silenced. These plants had completely normal growth phenotypes, even under stringent competition regimes, but their resistance to herbivores was impaired, allowing us to understand TD's unusual transcriptional behavior in response to wounding, herbivore attack, and jasmonate elicitation (Hildmann et al., 1992; Halitschke et al., 2001; Hermsmeier et al., 2001; Schittko et al., 2001). The susceptibility of asTDM plants to attack from *M. sexta* larvae was associated with reduced levels of two inducible direct defenses: TPIs and nicotine. Previous research has demonstrated that silencing either of these defenses in *N. attenuata* plants dramatically increases the susceptibility of plants to attack from *M. sexta* larvae and increases larval performance (Steppuhn et al., 2004; Zavala et al., 2004a; Zavala et al., 2004b). Moreover, both of these direct defenses are elicited by JA signaling (Halitschke et al., 2004). The kinetics of the JA and JA-Ile bursts induced by larval elicitors were found to be subtly altered in asTDM plants. This observation led to the discovery of the dramatic ability of JA to be conjugated with Ile at the wound site, and that herbivory-elicited TD activity supplied the Ile required for the formation of JA-Ile. Supplementing wounds in asTDM plants with Ile restored the WT kinetics of the JA-Ile burst and the elicitation of the direct defenses. Treating asTDM plants with JA-Ile restored the plant's ability to elicit the direct defenses and thereby the resistance of asTDM plants to attack from *M. sexta* larvae. These results

highlight the dynamic role that JA-Ile plays in defense signaling and suggest that subtle changes in the kinetics of JA and JA-Ile accumulation after herbivore attack can profoundly affect defense elicitation.

JA-Ile's role as a defense signal was confirmed in the analysis of the asTDS plants, in which all of the subtle changes in defense signaling observed in asTDM plants were exaggerated. In these slow-growing lines, the OS-elicited JA and JA-Ile bursts observed in WT plants were much slower and smaller and addition of Ile to OS-elicited wounds substantially increased the JA-Ile levels (Figure 7). The OS-elicited JA-Ile production was restored by adding Ile (but not Thr) to OS-treated wounds and JA-Ile treatment effectively restored herbivore resistance (Figure 7). Hence while the developmental defects of asTDS plants prevented direct comparisons of herbivore resistance with WT plants, some of defensive deficiencies of asTDS plants could be complemented by Ile or JA-Ile treatments. The ability to complement these defensive deficiencies in plants suffering from severe nutritional deficiencies underscores the importance of JA-Ile in defense signaling. The VIGS experiments in *N. attenuata* confirmed that TD is involved in Ile synthesis and JA-Ile conjugation is limited by the supply of Ile in wounded tissues.

It has long been known that JA is metabolized to its volatile counterpart, MeJA, and numerous conjugates with O-glucosides, hydroxylation, and amino acid (Sembdner and Parthier, 1993; Sembdner et al., 1994). The glycosylated forms and amino acid derivatives have been viewed as mere conjugates of JA which may be important for hormone homeostasis. Because all applied conjugates could be de-esterified to JA, JA and JA conjugates were thought to all have the same effect (Schaller et al., 2004). However, recent reports have demonstrated that JA conjugates have their own activities. Transgenic *Arabidopsis* plants which constitutively express an S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) exhibited constitutive expression of jasmonate-responsive genes, including *VSP* and *PDF1.2*. Furthermore, the transgenic plants showed enhanced resistance against the virulent fungus *Botrytis cinerea* (Seo et al., 2001). Studies that have applied synthetic JA-amino acid conjugates to plants suggest that the spheres of activity within JA-amino acid conjugates differ widely. For example, treatment of barley leaves with JA-Ile elicits jasmonate-induced protein (JIP) without isoleucine cleavage from JA (Kramell et al., 1997). JA-Ile, JA-phenylalanine, JA-leucine conjugates elicit accumulation of the flavonoid phytoalexin, sakuranetin, in rice (*Oryza sativa*) leaves,

but JA-tryptophan does not (Tamogami et al., 1997). However, it had not been previously appreciated that JA conjugates had elicitor-induced dynamics that were comparable to those of JA, and that subtle changes in these dynamics were associated with large changes in defense function.

The pioneering work of Staswick and colleagues has demonstrated that an *Arabidopsis* JA responsive gene (*JAR1*) adenylates JA's carboxyl group, and that adenylated JA is actively conjugated with various amino acids, of which Ile is quantitatively the most important (Staswick et al., 2002; Staswick and Tiryaki, 2004). The mutant defective in JAR1 (*jar1-1*) exhibits decreased resistance to the soil fungus *Pythium irregulare* (Staswick et al., 1998), implying that JA-Ile is involved in pathogen resistance. The analysis of JAR4VIGS plants demonstrated that *Na*JAR4 is involved in JA-Ile conjugation (Figure 10) and that JAR4VIGS plants are susceptible to the attack of *M. sexta* (Figure 11), indicating that JA-Ile is involved in herbivore defenses in *N. attenuata*. Further analyses of other JA-amino acid conjugates at the attack site will be required to determine if other JA-conjugates are equally as dynamically elicited and whether these conjugates are also eliciting specific developmental and defense responses in the plant.

Reduced levels of JA-Ile in asTD plants resulted in reduced accumulations of TPI and nicotine. Treatment of plants with JA and JA-Ile elicited different TPI and nicotine responses, which may be due to different absorption and transport rates or to different elicitation activities of these chemicals. The JA-Ile burst can account for approximately 13% of the elicited JA burst (Figure 3B). The smaller quantities of JA-Ile compared to JA may be due more to a rapid metabolism of JA-Ile to unknown structures than to the conversion of JA to JA-Ile. Alternatively, JA may be converted to MeJA or other conjugates. The rapid declines in JA and JA-Ile may be due to their binding to putative receptor(s), which remain to be identified. Identification of the JA receptor(s), when it occurs, will be a breakthrough that will clarify the structural basis for activity differences among JA conjugates as well as the information content of these dynamic metabolites.

The role of JA in systemic signaling was recently demonstrated in an elegant set of reciprocal grafting experiments. Li and coworkers grafted the JA biosynthetic mutant (*spr-2*), known to be defective in fatty acid desaturase required for JA biosynthesis (Li et al., 2003), with the JA response mutant (*jai-1*), known to be defective in a homolog of the *Arabidopsis* coronatine-insensitive1 (*COI1*) gene (Xie

et al., 1998) in different combinations, and analyzed the wound-induced expression of the proteinase inhibitor II gene (Li et al., 2002). Their results demonstrated that the JA biosynthetic pathway was required for production of the long-distance signal, suggesting that JA or related compounds derived from the octadecanoid pathway function as systemically transmitted signals in tomato. In various *Nicotiana* species, nicotine synthesis in the roots is activated by leaf wounding. In *N. attenuata,* this systemic response is known to require JA signaling (Halitschke and Baldwin, 2003). JA-Ile, in comparison to JA, elicits dramatically more nicotine (Figure 6A). This suggests that JA-Ile as the long-distance signal which elicits nicotine in roots. However, JA-Ile was not detected in roots when leaves were treated with OS, JA, or JA-Ile (data not shown), indicating that subsequent metabolites of JA-Ile or unknown molecules elicited by JA-Ile may be the long-distance signals.

To summarize, we propose the following model to account for TD's role in defense signaling. When attacked by herbivores, plants produce JA and activate TD in the attacked tissues. Ile synthesized from Thr by TD is conjugated with JA by JAR1.

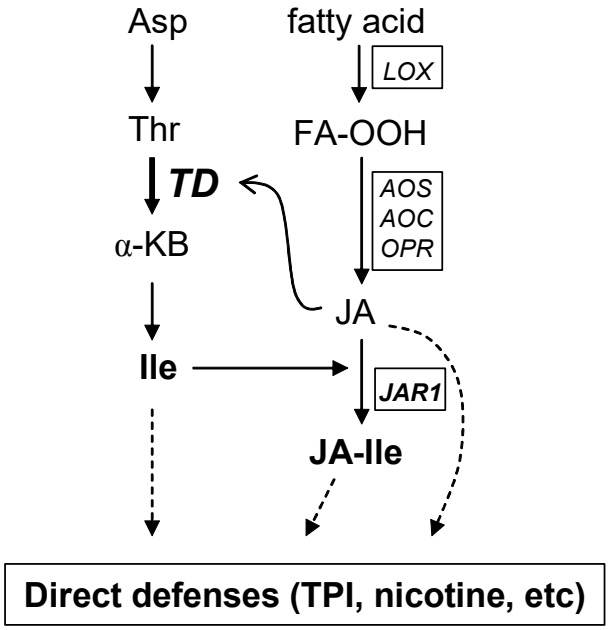


Figure 12. Proposed Role of TD in JA-Mediated Defense Signaling.
JA biosynthetic enzymes and JA-amino synthetase are boxed. Dashed arrows represent signal transduction pathways. *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, 12-oxo-phytodienoic acid reductase; *JAR1*, JA-amino synthetase.

The resulting JA-Ile elicits the accumulation of the direct defenses, TPI and nicotine (Figure 12). TD may be playing additional defensive roles after the plant is ingested by herbivores. A recent proteomics analysis of the midgut contents of *M. sexta* larvae fed on tomato, revealed that one of the abundant proteins was TD, lacking its regulatory domain which would allow for efficient degradation of Thr without being inhibited by Ile (Chen et al., 2005). This result suggests that TD could function defensively by limiting amino acid supply for herbivore growth in addition to supplying Ile to JA for JA-Ile conjugation.

METHODS

Plant Materials and Growth Conditions

An inbred genotype of *Nicotiana attenuata* Torr. Ex Wats. (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae), originally collected from southwestern Utah in 1988, was transformed and used for all experiments. Seeds were sterilized and germinated as described previously (Krügel et al., 2002). Ten-day-old seedlings were planted into soil in Teku pots (Waalwijk, The Netherlands) and, once established, transferred to 1 L pots in soil and grown in the glasshouse at 26-28ºC, under 16 h light supplemented by Philips Sun-T Agro 400 or 600W Na lights.

Chemical Treatments

The leaf undergoing the source–sink transition (T) was designated as growing at node 0. *M. sexta* larval oral secretions (OS) were collected with teflon tubing connected to a vacuum and stored under argon at -80 ^{o}C. For OS-treated plants, the leaf growing at node +1, which is older by one leaf position than the source-sink transition leaf, was wounded by rolling a fabric pattern wheel over the leaf surface to produce standardized puncture wounds. Immediately after wounding, the wounds were treated with 20 µl of water; OS at a 1:5 dilution with water; OS containing 0.625 µmole of L-threonine, L-isoleucine, $^{13}C_4$ labeled threonine, or $^{13}C_6$ labeled isoleucine. Leaves from JA- or JA-Ile-treated plants were wounded with a fabric wheel and directly treated with 0.625 µmole of JA or JA-Ile. Leaves from MeJA-treated plants were treated with 150 µg (0.625 µmole) of MeJA in 20 µl of lanolin paste as described previously (Halitschke et al., 2000).

Generation and Characterization of asTD Transgenic Lines

For the plant transformation vector, 1349 bp portion of the *N. attenuata* TD cDNA resident on plasmid pTD13 (Hermsmeier et al., 2001) was PCR amplified using primers 5'-GCGGCGCCATGGCATAGGTCCCACAAGTTCGC-3' and 5'-GCGGCGGGTCACCTGGAAGTTCTTTGTCAAGCC-3'. The obtained 1.4 kb PCR fragment was cut with *Bst*EII and partially cut with *Nco*I. The resulting 1.4 kb

fragment was cloned in pNATGUS3 (Krügel et al., 2002) and digested with the same enzymes, resulting in plant transformation vector pNATTD1 (10.1 kb), which contained in its T-DNA a 1.4 kb fragment of *Na*TD in an antisense orientation under the control of the 35S promoter of the cauliflower mosaic virus. The *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure and the transformation vector are previously described (Krügel et al., 2002). Progeny of homozygous plants were selected by NTC resistance screening, and screened for the desired phenotype, namely, reduced MeJA-induced α-KB accumulation. For all experiments, T_2 homozygous lines, each harboring a single insertion which was confirmed by southern blot analysis (Figure S2), or WT plants were used.

JAR4 Full Length cDNA Isolation

A cDNA fragment was obtained by RT-PCR from total RNA isolated from WT plants 60 minutes after source leaves had been wounded with a fabric pattern wheel. The primers were designed from the conserved regions of *At JAR1* and *Le BTO13679* cDNA sequences. The forward primer was 5'-TTCACCTATTCTTACTGG-3', the reverse primer was 5'-ACATTACTAGACAGTATTTGGA-3'. Full length cDNA was isolated using GeneRacer Kit (Invitrogen, USA) according to manufacturer's instructions. The 5' primer and 5' nested primer were 5'-AGAACACCTTCCC TTATATTGGTCACAA-3' and 5'-ACTTAAGGAAATAGTGGTAATAGGCTTT-3', respectively. The 3' primer was 5'-AAAGTGAATGCAATTGGAGCACTTGA-3'.

Phylogenetic Analysis and DNA Sequence Alignment

For the analysis of gene trees, JAR4-related plant sequences were identified by BlastP searches (P value < 10^{-40}) of the nonredundant translated database using *N. attenuata* JAR4 as the query sequence. Phylogenetic relationships were determined using the neighbor-joining method with bootstrap analysis (1000 replicates). Sequence alignment was performed with ClustalW using the DNASTAR MegAlign software. The tree was displayed with TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Generation and Characterization of VIGS Plants

PCR was used to generate TD and JAR4 fragments from *N. attenuata* in antisense orientations with the following primer pairs: TD forward primer; 5'- GCGGCGGGA TCCGCACCAAATGGCTCAACTCC-3', TD reverse primer; 5'- GCGGCGGTC GACGTCATGCCTGTTACCACACC-3', JAR4 forward primer; 5'-GCGGCGGT CGACGTAATATTTGGCCCTGATTTCC-3', JAR4 reverse primer; 5'-GCGGCGG GATCCAATTGCTTAACCGGCTG-3'. The obtained TD (335 bp) and JAR4 (292 bp) PCR fragment were digested with *Bam*HI and *Sal*I. The resulting fragments were cloned into the pTV00 vector digested with the same enzymes. The pTV00 vector is a 5.5 kb plasmid with an origin of replication for *E. coli* and *A. tumefaciens* and a gene for kanamycin resistance (Ratcliff et al., 2001). *A. tumefaciens* (strain GV3101)-mediated transformation procedure was previously described (Saedler and Baldwin, 2004). Phytoene desaturase (PDS) gene was used to monitor the progress of VIGS and inform the start of experimental treatments. Plant growth and inoculation conditions were as previously described (Saedler and Baldwin, 2004).

Nucleic Acid Blot Analysis

Extraction of total RNA and Northern blot analysis was performed as previously described (Winz and Baldwin, 2001). Genomic DNA was extracted from leaves as described previously (Richard, 1997) and 10 μg of DNA digested with *Eco*RI and blotted onto nylon membranes. To prepare the probe, plasmid pTD13 (GenBank accession number; AF229927) containing full-length cDNA of TD was cut with *Pst*I and gel-eluted using Geneclean Kit (BIO 101, Vista, CA, USA), labeled with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little Chalfont, UK), and purified on G50 columns (Amersham-Pharmacia). After overnight hybridization, blots were washed three times with 2xSSPE at 42°C and one time with 2xSSPE / 2% SDS at 42°C for 30 min, and analyzed on a phosphoimager (model FLA-3000; Fuji Photo Film Co., Tokyo, Japan).

Real Time PCR Assay

Total RNA was extracted with TRI Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions, and cDNA was prepared from 200 ng total RNA

with MultiScribe™ reverse transcriptase (Applied Biosystems). The primers and probes specific for *Na*TD and *Na*JAR4 mRNA expression detection by qPCR were as follows: TD forward primer; 5'-TAAGGCATTTGATGGGAGGC-3', TD reverse primer; 5'-TCTCCCTGTTCACGATAATGGAA-3', JAR4 forward primer; 5'-ATGCCAGTCGGTCTAACTGAA-3', JAR4 reverse primer; 5'- TGCCATTGTGGA ATCCTTTTAT-3', ECI forward primer; 5'-AGAAACTGCAGGGTACTGTTGG-3', ECI reverse primer; 5'-CAAGGAGGTATAACTGGTGCCC-3', FAM labeled TD probe; 5'-TTTTTAGATGCTTTCAGCCCTCGTTGGAA-3', FAM labeled JAR4 probe; 5'- CAGGTCTGTATCGCTATAGGCTCGGTGATGT-3', FAM labeled ECI probe; 5'-CGTCAAAATTCTCCACTTGTTTCAACTGT-3'. The assays using a double dye-labeled probe were performed on an ABI PRISM® 7700 Sequence Detection System (qPCR™ Core Kit, Eurogentec) with *N. attenuata* sulfite reductase (ECI) for normalization and according to the manufacturer's instructions with the following cycle conditions: 10 min 95°C; 40 cycles: 30 sec 95°C, 30 sec 60°C.

TD Activity Measurement

Leaves were homogenized in 2 volumes of extraction buffer (100 mM Tris buffer [pH 9], 100 mM KCl, and 10 mM β-mercaptoethanol) and centrifuged at 15,000g for 15 min at 4°C. TD activity was assayed by incubating the enzyme with substrate and determining the quantity of α-KB formed. The α-KB was estimated by modifying the method described by (Sharma and Mazumder, 1970). 100 µl of protein extract was added to the same volume of reaction buffer (40 mM $_{L}$-threonine, 100 mM Tris buffer [pH 9], and 100 mM KCl). After incubation at 37°C for 30 min, 160 µl of 7.5% trichloracetic acid was added to stop the reaction and the protein precipitate was removed by centrifugation at 10,000g for 2 min. The α-KB was determined by adding 400 µl of 0.05% dinitrophenylhydrazine to the sample solution. After incubation at room temperature for 10 min, 400 µl of 4N sodium hydroxide was added to the sample solution and mixed well. After incubation at room temperature for 20 min, the absorbance of the sample solution was read at 505 nm in spectrophotometer (model Ultraspec®3000; Pharmacia Biotech, Cambridge, England).

***M. sexta* Performance**

Leaves at nodes +1 and +2 were wounded and treated with JA, JA-Ile, or left untreated. For the effect of TD on *M. sexta* larval mass in untreated and JA and JA-Ile

treated transgenic and WT plants, freshly hatched larvae (North Carolina State University, Raleigh, NC, USA) were placed on 7-16 replicate leaves at node 0 on individual plants, 3 days after treatment. Larval mass was measured 2, 4, and 6 days or 3, 6 and 9 days after larvae were allowed to feed on the plant. In the experiments with VIGS plants, freshly hatched larvae were placed on 12 replicate leaves (on separate plants), 3 days after elicitation. Larval mass was measured 6 and 9 days after larvae began feeding.

Analysis of Direct Defense Traits

Nicotine, chlorogenic acid, and diterpene glycoside were analyzed by HPLC as described previously (Keinanen et al., 2001) with the following modification of the extraction procedure: approximately 100 mg frozen tissue was homogenized in 1 mL extraction buffer utilizing the FastPrep® extractions system (Savant Instruments, Holbrook, NY, USA). Samples were homogenized in FastPrep® tubes containing 900 mg lysing matrix (BIO 101, Vista, CA, USA) by shaking at 6.0 m sec^{-1} for 45 seconds.

Trypsin protease inhibitor (TPI) activity was analyzed by radial diffusion activity assay as described previously (Van Dam et al., 2001).

JA and JA-Ile Measurement

Leaves were harvested and immediately frozen in liquid nitrogen. Samples were homogenized in 3 volumes of extraction buffer (acetone: 50 mM citric acid, 7:3 [v/v]). Samples were centrifuged at 13,000 rpm for 15 min at 4 ^{o}C and supernatants were transferred to a new tube. The pellet was re-extracted with extraction buffer. The combined supernatants were evaporated to dryness in a heating block and the remaining aqueous phase was extracted 3 times with 1 mL ether. The ether layer was evaporated completely and the residue dissolved in acetonitrile. The samples were separated by an Agilent LC1100 HPLC system (Agilent, Waldbronn, Germany) with degasser, binary pump, autoinjector, column thermostat, and detected by a DAD coupled to a LCQ DECA XP mass spectrometer (Thermo-Finnigan, Egelsbach, Germany). Mobile phase A consisted of 0.5% acetic acid in water and mobile phase B of 0.5% acetic acid in acetonitrile. The mobile phase gradient was increased linearly from 20% B (initial value) to 50% B at 16 min, held constant at 50% for 25 min, and subsequently increased linearly to 100% B at 30 min. The mobile phase flow was 0.7

mL/min and the injection volume 30 µL. The stationary phase was a Luna 5µ C18 column (250 x 4.60 mm, 5 µm particle size, Phenomenex, Aschaffenburg, Germany). The MS conditions were as follows: APCI Ion source: 500°C vaporizer temperature; 275°C capillary temperature; 10 µA discharge current; sheath gas: nitrogen, 50 (arbitrary units); auxiliary gas; nitrogen, 30 (arbitrary units). Three MS/MS ion acquisition segments were programmed as follows: 1) 10 – 17.5 min: m/z 155 @ 28 negative polarity for 2-chloro benzoic acid (CBA, internal standard); 2) 17.5 – 21.5 min: m/z 211 @ 23 positive polarity for JA. The third segment (21.5 – 30 min) contained the following 3 scan events: A) m/z 324 @ 30 positive polarity for endogenous JA-Ile; B) m/z 328 @ 30 positive polarity for synthetic JA-Ile derived from $^{13}C_4$-L-Thr (Cambridge Isotope Laboratories, Andover, Massachusetts, USA); C) m/z 330 @ 30 positive polarity for synthetic JA-Ile derived from $^{13}C_6$-L-Ile (Cambridge Isotope Laboratories, Andover, Massachusetts, USA). Standard curves were constructed with known quantities of Ile, JA, and JA-Ile and used for quantification of those chemicals in samples. The MS/MS spectra of JA and JA-Ile are given in Figure S10.

To estimate the JA and JA-Ile responses, we integrated the amount which was produced in each leaf from 0 hr to 5 hr.

ACKNOWLEDGEMENTS

We thank Bernd Krock for unflagging assistance in LC-MS analysis, and for the synthesis of the JA-Ile; Thomas Hahn and Klaus Gase for sequencing; Tamara Krügel and Michell Lim for the plant transformation; Anke Steppuhn for assistance in the HPLC analysis; Dominik Schmidt for assistance in the real-time PCR analysis; Rayko Halitschke for help with data analysis and for the first sequences of *Na*JAR4; Emily Wheeler for editorial assistance. This work was supported by the Max-Planck-Gesellschaft.

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Supplemental Materials and Methods

Southern Blot of Genomic DNA

Genomic DNA was extracted from leaves as described previously (Richard, 1997). 10 µg of DNA digested with *Eco*RI and blotted onto nylon membranes. To prepare the probe, plasmid pTD13 (GenBank accession number; AF229927) containing full-length cDNA of TD was cut with *Pst*I and gel-eluted using Geneclean Kit (BIO 101, Vista, CA, USA) and labeled with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little Chalfont, UK) and purified on G50 columns (Amersham-Pharmacia). Blots were washed after overnight hybridization at 42°C three times with 2xSSPE and one time with 2xSSPE / 2% SDS at 42°C for 30 min and analyzed on a phosphoimager (model FLA-3000; Fuji Photo Film Co., Ltd. Tokyo, Japan).

Competition Experiments

To examine whether suppression of TD affects growth, we used a competition design optimized for the detection of subtle differences in growth performance. Two same-aged seedlings of similar size and appearance were transplanted 7 cm apart in 2 L pots in a glasshouse as described in (Glawe et al., 2003). We measured stalk length for the duration of stalk elongation (20 days) during flowering of 15 replicate asTDM and WT plants which are grown in individual 2 L pots or grown in the same 2 L pots with a size-matched competitor.

Microarray Analysis

Pooled leaf samples were ground under liquid nitrogen and total RNA was extracted with TRI Reagent (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. Isolation of mRNA and cDNA synthesis was performed as previously described (Halitschke et al., 2003). To examine the TD expression in WT plants, cDNA derived from untreated WT plants was labeled with Cy5, and cDNA of herbivore- or OS-treated WT plants was labeled with Cy3 fluorescent dye, according to the procedure previously described (Halitschke et al., 2003). The labeled samples were hybridized to the microarray (789 50-mer oligonucleotides spotted onto an epoxy-coated glass slide; Quantifoil Microtools, Jena, Germany), according to the

published procedure (Halitschke et al., 2003). An Affymetrix 428™ Array Scanner (Affymetrix, Santa Clara, CA, USA) was used to scan the hybridized microarrays with sequential scanning for Cy5- cDNA and then for Cy3- labeled cDNA at a maximum resolution of 10 μm/pixel with a 16-bit depth. The data analysis followed the method previously described (Halitschke et al., 2003).

Supplemental Figures

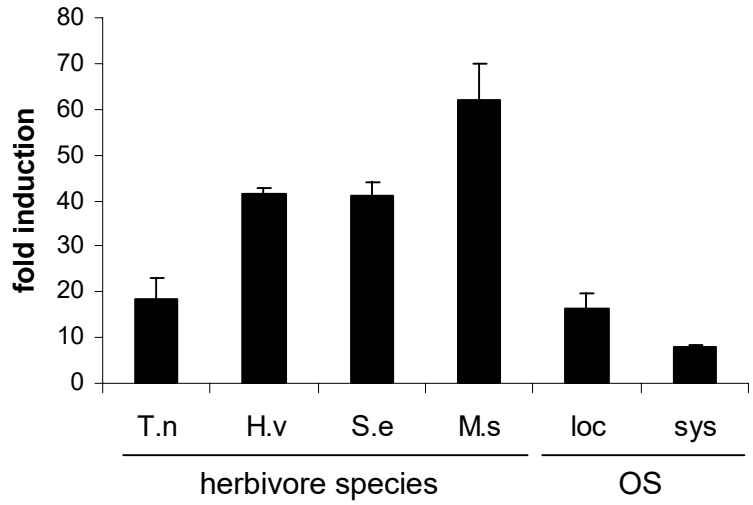

Figure S1. Expression of TD in *N. attenuata* Plants Attacked by Insect Herbivores. Data represent mean (± SE) expression ratios of TD in wild type *N. attenuata* plants from cDNA microarray analysis with four replicate TD oligo-nucleotides. Leaves attacked by the following herbivores (T.n, *Tupiocoris notatus*; H.v, *Heliothis virescens*; S.e, *Spodoptera exigua*; M.s, *Manduca sexta*) were harvested 24 hr after herbivores started feeding from 10 replicate plants for each herbivore species. *M. sexta* OS was added to puncture wounds, and the treated (loc) and untreated systemic (sys) leaves were harvested 1 hr after OS treatment from 10 replicate plants. Each microarray was hybridized with Cy3- labeled cDNA generated from plants that were treated with herbivore- or OS- elicited plants against Cy5-labeled cDNA generated from leaves from untreated plants.

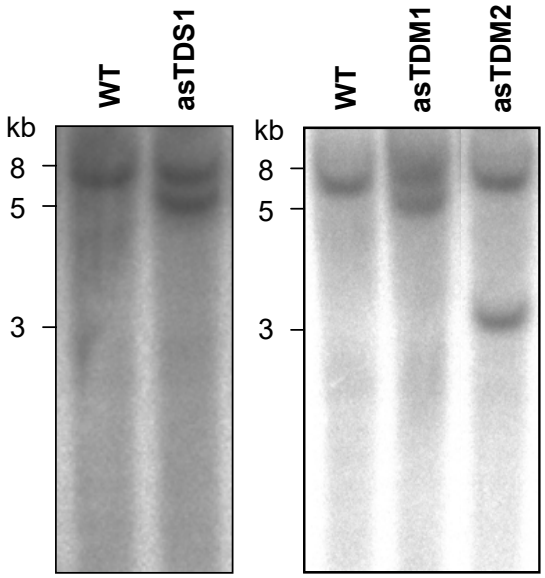

Figure S2. Southern Blot of Genomic DNA in WT and asTD Plants.
10 μg of genomic DNA were digested with *Eco*RI and blotted onto nylon membranes. The blots were hybridized with a PCR fragment (1706 bp) of the region of TD used for the antisense construct. WT plants show one band and 3 independently transformed T_2 asTD plants (asTDS1, asTDM1, and asTDM2) show two bands.

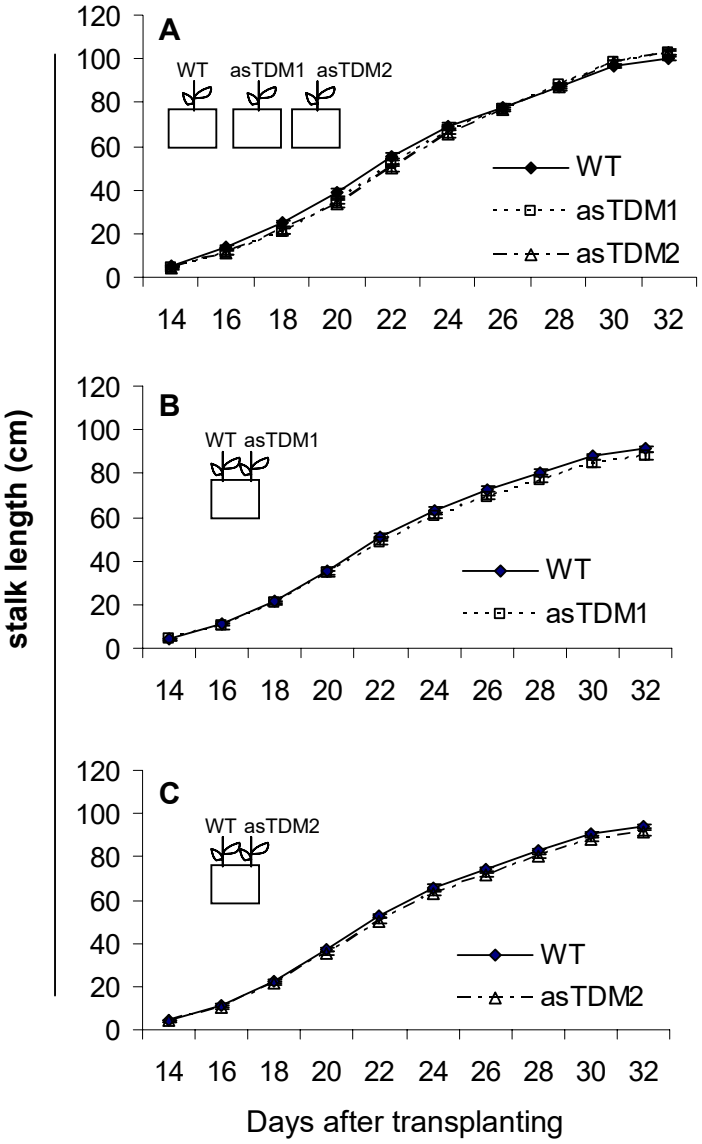

Figure S3. Comparison of growth rates of WT and T_2 asTDM plants grown in individual pots or competing with each other in the same pot.

(A) to **(C)** Mean (± SE) stalk length of 15 replicate WT and asTDM plants (asTDM1 and asTDM2) grown in an individual 2 L pot (A) or in the same 2 L pot in the competition (B and C), starting on the day with measurable stalk growth for 19 subsequent days.

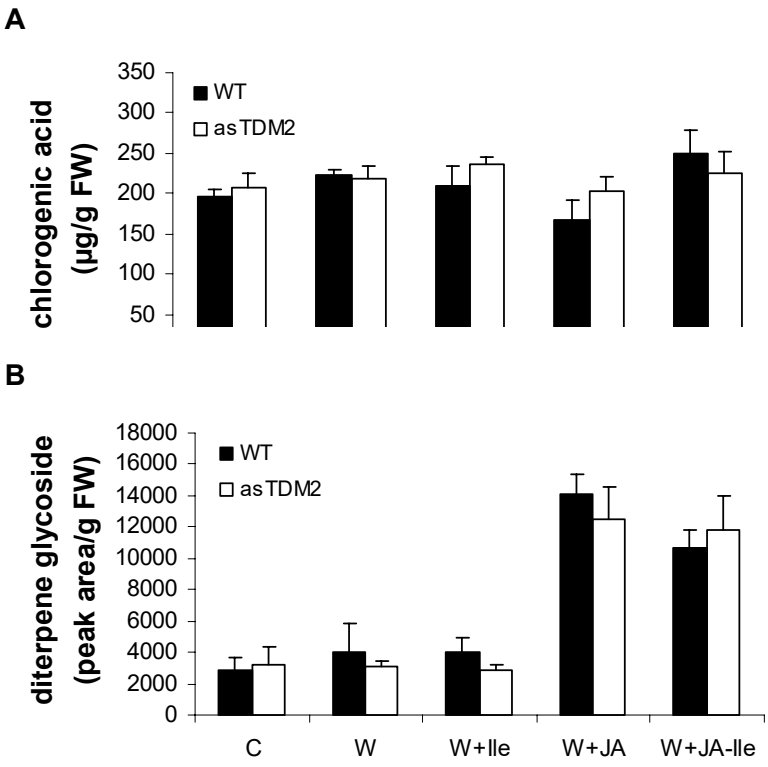

Figure S4. Chlorogenic acid and Diterpene Glycoside concentrations elicited by JA and JA-Ile treatments to leaves in WT and asTDM2 plants.

(A) and **(B)** Mean (± SE) chlorogenic acid (A) and diterpene glycoside concentration (B) of leaves growing at node +1, 3 days after they were wounded and treated with 20 µl of the following solutions from 3 replicate WT and asTDM2 plants: deionized water (W), isoleucine (W+Ile), jasmonic acid (W+JA), or jasmonic acid-isoleucine conjugate (W+JA-Ile) all at 0.625 µmole. Control plant leaves (C) were left unwounded and untreated.

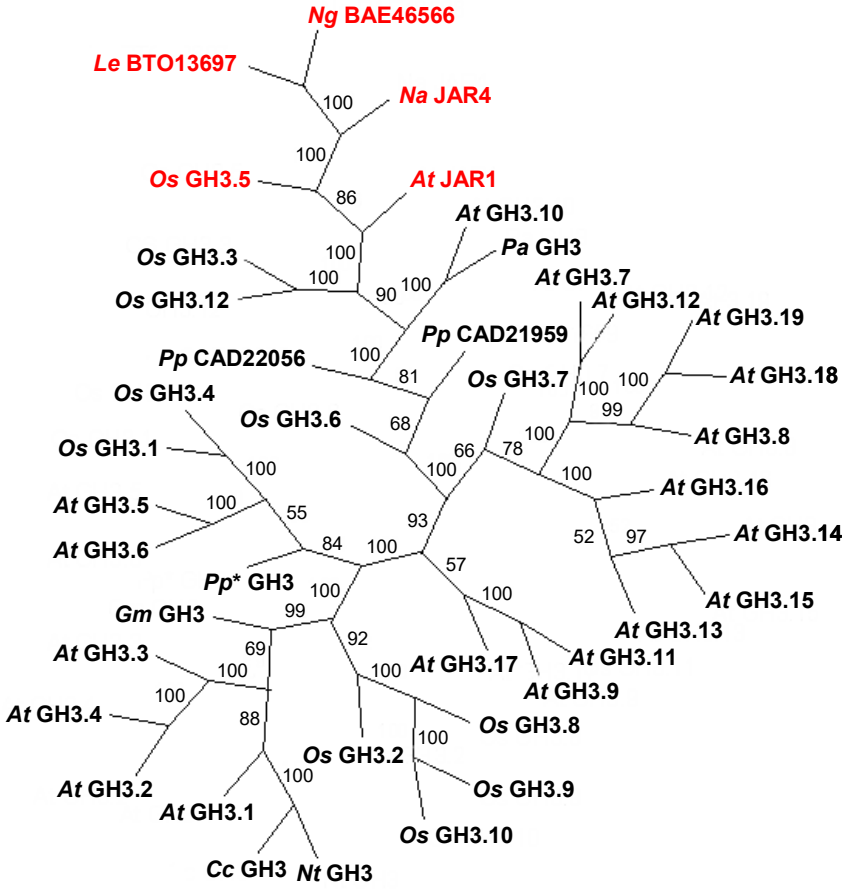

Figure S5. Gene Tree of the JAR Family.

A ClustalW phylogeny was constructed using the deduced sequences of amino-acid encoded by *JAR* genes. Proteins are identified by two-letter genus/species designations followed by the gene name or database accession number for the nucleotide sequence from which the protein was translated. At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Na, *Nicotiana attenuata*; Ng, *Nicotiana glutinosa*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Nt *Nicotiana tabacum*; Gm, *Glycine max*; Pp*, *Pinus pinaster*; Pa, *Pringlea antiscorbutica*; Cc, *Capsicum chinense*.

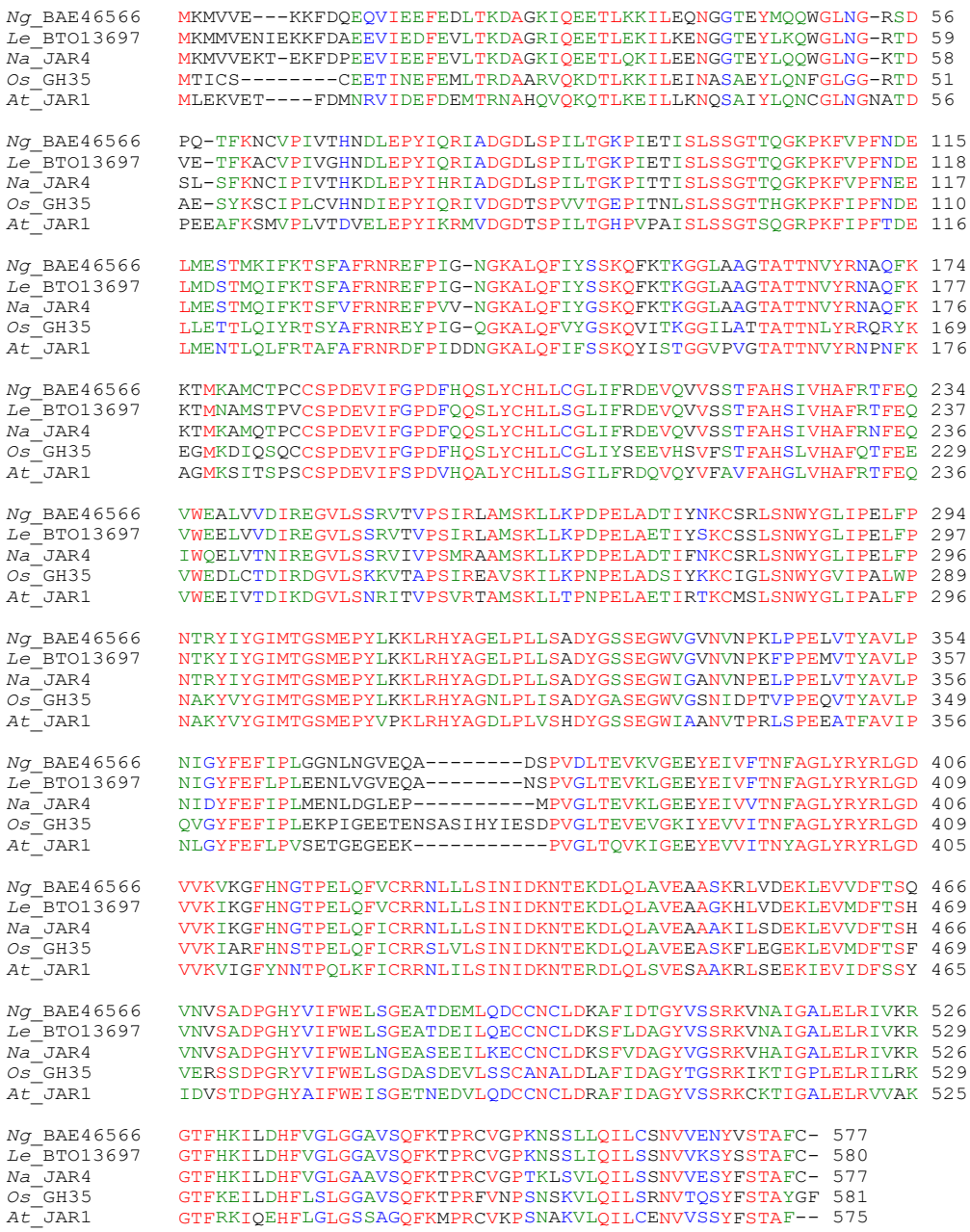

Figure S6. Alignment of Deduced Amino Acid Sequences of JARs from *Nicotiana attenuata* (*Na* JAR4), *Arabidopsis thaliana* (*At* JAR1), *Lycopersicon esculentum* (*Le* BTO13697), *Oryza sativa* (*Os* GH3.5), *Nicotiana glutinosa* (*Ng* BAE46566). Amino acid identity is indicated by red and similarity by green or blue.

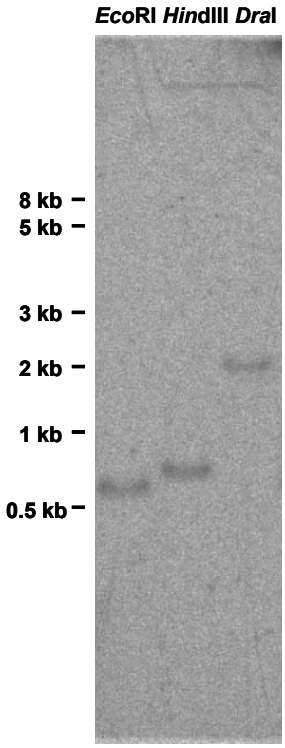

Figure S7. Southern Blot of *JAR4* in WT Plants.

10 µg genomic DNA were digested with *Eco*RI, *Hin*dIII and *Dra*I and blotted onto nylon membrane. The blots were hybridized with a PCR fragment (288 bp) of the *JAR4* coding region.

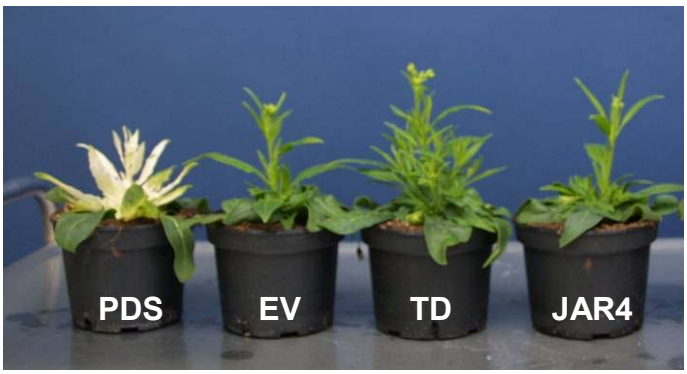

Figure S8. Portrait of VIGS Plants during Stalk Elongation Stage.
PDS–silenced plants began to bleach at 5 weeks after seed germination. All experiments with the VIGS plants were performed when PDS-silenced plants showed beaching in most rosette-stage leaves (6 weeks after seed germination--2 weeks after inoculation with VIGS constructs). Picture was taken 6 week after seed germination. Plants were inoculated with *Agrobacterium* harboring TRV constructs containing a 160 bp PDS fragment (PDS), an empty vector (EV), a 335 bp *N. attenuata* TD fragment (TDVIGS), or a 292 bp *N. attenuata* JAR4 fragment (JAR4VIGS).

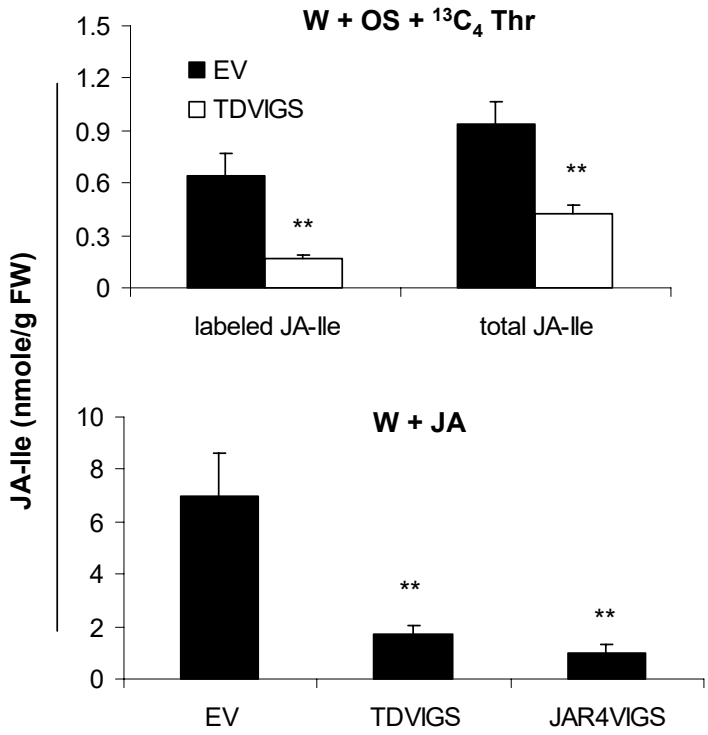

Figure S9. Silencing of TD and JAR4 by VIGS Dramatically Reduces Elicited JA-Ile Accumulation.

Mean (± SE) JA-Ile concentrations in leaves of 4-5 replicate EV, TDVIGS, and JAR4VIGS plants. 14 days after inoculation, leaves were harvested 1 hr after they were wounded and treated with 20 µl of *M. sexta* oral secretions (OS) containing 0.625 µmole of $^{13}C_4$ labeled threonine (W + OS + $^{13}C_4$ Thr), or 20 µl of water containing 0.625 µmole of JA (W + JA). Stars represent significant differences between EV and VIGS plants (unpaired t-test: **, $P < 0.01$).

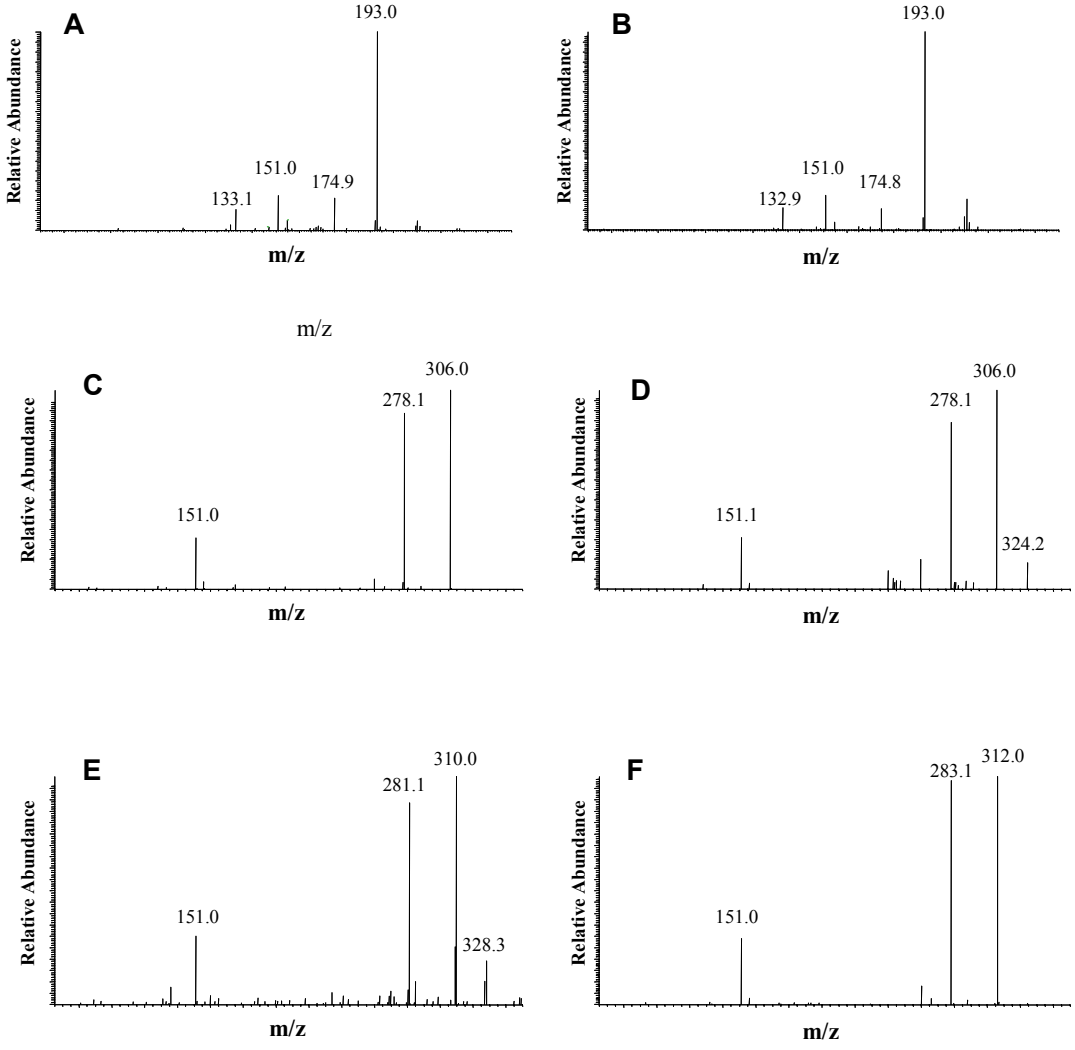

Figure S10. Analysis of JA and JA-Ile conjugates by LC/MS.
(A) and **(B)** ms/ms spectra of JA from standard (A) and leaves treated with *M. sexta* OS (B). **(C)** to **(F)** ms/ms spectra of JA-ILE from standard (C), ms/ms spectra of natural JA-ILE from leaves treated with *M. sexta* OS (D), ms/ms spectra of isotope labeled JA-ILE from leaves treated with *M. sexta* OS and $^{13}C_4$ threonine (E), and ms/ms spectra of isotope labeled JA-ILE from leaves treated with *M. sexta* OS and $^{13}C_6$ isoleucine (F).

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3. Discussion

In undamaged plants, TD accumulated in cotyledons during seedling growth and in young floral buds, sepals, anthers, stigmas, and trichomes during flowering (Manuscripts I and II). Similar accumulations were found in tomato, where the peak enzyme activity was found in sepals from buds and flowers (Samach et al., 1995), and in potato where transcripts were expressed only in bud tissue (Hildmann et al., 1992). TD also had a specific response to insect attack: TD transcripts strongly accumulated after herbivory and application of MeJA to leaves (Manuscript III), and not at all after inoculation with tobacco mosaic virus, *A. tumefaciens*, or exposure to abscic acid, ethylene, and methyl salicylate (Hermsmeier et al., 2001). The conservation of catalytic domains, exon-intron boundaries, and putative plastid targeting sequences (Samach et al., 1991) in combination with the similar developmental and induced expression of TD in potato, tomato, and *N. attenuata* provide strong evidence that the functions of TD are conserved in Solanacous plants.

The role of TD in development

Understanding the pathways that control amino acid synthesis in plants has significance with regard to basic research on the control of metabolic pathways as well as practical implications. Plant mutants in amino acid biosynthetic enzymes have shown that the synthesis of amino acid in vivo affects numerous processes, including photorespiration, hormone biosynthesis, and plant development. Thus, although they are products of primary metabolism, amino acids also control diverse aspects of plant growth and development (Coruzzi and Last, 2000).

To examine the effect of TD on plant development and defense responses, we suppressed TD using two different methods: we expressed *Na*TD in an antisense orientation and we used the virus-induced gene silencing (VIGS) method (Manuscripts I and III). In transgenic plants, transformed lines were readily characterized as having one of two growth phenotypes: 1) plants with mild reductions in TD expression and activity but otherwise wild type (WT) growth and development patterns (asTDM plants), and 2) plants with severe reduction in TD expression and activity, and retarded growth (asTDS plants). The observation that transgenic plants with strongly silenced TD (asTDS) have morphological differences in young seedlings and flowers compared to WT plants demonstrates the involvement of TD in

development. The lower levels of isoleucine and different levels of JA and JA-Ile in asTDS floral buds compared to those in WT plants suggest that TD is involved in development by regulating isoleucine and JA levels (Manuscript I).

Jasmonates and octadecanoids can modulate various developmental processes in plants, most prominently, the inhibition of germination and seedling development, flower development, and the promotion of leaf senescence (Creelman and Mullet, 1997; Wasternack and Hause, 2002). The expression of genes coding for enzymes of JA biosynthesis, the elevation of JA levels, and the expression of JA-regulated genes were found to correlate with distinct developmental processes or plant organogenesis. Further insight into the function of jasmonates was provided by the characterization of mutants that are JA-insensitive or JA-deficient (Table 1).

Table 1. Phenotypes of JA-related mutants of *Arabidopsis thaliana*.

	Type	Morphological phenotype	Expression of JA-responsive gene	Sensitivity to insects
fad	Deficient	Male-sterile	Reduced	Increased
opr3	Deficient	Male-sterile		
coi1	Insensitive	Male-sterile	Reduced	Increased
jar1	Insensitive	WT	Reduced	
jin1/jin4	Insensitive	WT	Reduced	WT
cet1-9	Constitutive	Stunted growth	Increased	
cev1	Constitutive	Stunted growth	Increased	
cex1	Constitutive	Stunted growth	Increased	

Abbreviations: *fad*, fatty acid desaturation (McConn and Browse, 1996); *opr3*, 12-oxophytodienoic acid reductase (Stintzi and Browse, 2000); *coi1*, coronatine-insensitive (Feys et al., 1994; Xie et al., 1998); *jar1*, jasmonate-resistant (Staswick et al., 1992); *jin1*, *jin4*, jasmonate-insensitive (Berger et al., 1996); *cet1-9*, constitutive expression of thionins (Hilpert et al., 2001); *cev1*, constitutive expression of VSP (Ellis and Turner, 2001); *cex1*, constitutive expression (Xu et al., 2001); WT, wild-type phenotype.

Seedling growth and development Jasmonates exert both inhibitory and promoting effects on seed germination. JA and MeJA inhibit the germination of non-dormant seeds and stimulate the germination of dormant seeds. In soybean, JA levels in late stages of seed development are higher than in developing seeds shortly after anthesis. After imbibition, even the relatively high level of jasmonates in dry seeds increases, but this level declines during later stages of seedling development. The increase of JA levels in early developmental stages of soybean seedlings correlates with seed reserve mobilization, suggesting that JA synthesis is a consequence, rather than a trigger, of germination (Creelman and Mullet, 1997). In all seedlings analyzed so far, elevated JA levels and concomitant gene expression took place in tissues which are known to act as sink tissues.For example, during germination of soybean and *Arabidopsis*, elevated levels of JA occur in the hypocotyl hooks of these seedlings (Staswick, 1994). Another effect of JA on seedling development is the inhibition of root growth (Yamane et al., 1982). This feature was used to isolate JA-insensitive mutants by screening for plants with less sensitivity to MeJA in comparison to the wild type *Arabidopsis* (Staswick et al., 1992; Xie et al., 1998). JA-responsive gene expression is lacking or reduced in leaves of these mutants.

One of the dramatic differences between WT and asTDS1 plants was the timing of cotyledon opening during seedling growth on germination media. Cotyledon opening was highly delayed in asTDS seedlings. The cotyledon-opening phenotype in asTDS plants could be complemented by growing plants on Ile or JA-Ile supplemented media, suggesting that TD expression in cotyledons supplies the Ile pools required to form JA-Ile from JA as well as to synthesize proteins for seedling growth (Manuscript I).

Flower development Flower development is the best-studied developmental process in which an essential role for JA and octadecanoids has been shown. For a long time it was assumed that JA had a role in flower development, because of its relatively high levels in developing plant reproductive tissues (Creelman and Mullet, 1997; Wasternack and Hause, 2002). Jasmonates have also been shown to occur in mature pollen (Yamane et al., 1982; Miersch et al., 1998). In pine pollen, a high amount of JA amino acid conjugates was detected, but its function is unknown (Knoefel and Sembdner, 1995). In tomato flowers, OPDA, JA, and their amino acid conjugates and methyl esters accumulate to levels far exceeding those of leaves

(Hause et al., 2000). In addition, OPDA, JA, and their derivatives occur in a characteristic ratio within distinct flower organs (Hause et al., 2000). Another putative role of jasmonates in flowers is related to male fertility, as shown by mutant analysis with *A. thaliana*. The coronatin- and jasmonate-insensitive mutant *coi1* cannot produce viable pollen (Xie et al., 1998). JA-deficient mutants in *Arabidopsis* are also defective in pollen development (Table 1). Interestingly, asTDS plants had short stigmas, poor pollen production, petaloid anthers, and reduced trichome numbers. The higher levels of JA and lower levels of JA-Ile in asTDS plants compared to those in WT plants suggest that phenotypic changes in asTDS flowers are due to an imbalance of JA and JA-Ile (Manuscript I).

The tissue-specific oxylipin signature within flower organs may result in differential gene expression. Indeed, a number of JA-induced genes are specifically expressed within flowers. Among them are those coding for vegetative storage proteins (Benedetti et al., 1995), defense-related proteins such as proteinase inhibitor II (Lorberth et al., 1992), a dioxygenase (Lantin et al., 1999), the leucine amino peptidase A (Chao et al., 1999), and threonine deaminase (Samach et al., 1995; Hermsmeier et al., 2001; Manuscript I). These genes are preferentially expressed in reproductive organs that are attractive targets for pathogens and insects. The increased expression of defense-related genes might be biologically significant if it leads to constitutive defense against pathogens and insects. Reduced levels of trypsin proteinase inhibitor (TPI) in asTDS flower buds hint that TD may be involved in defense as well as development (Manuscripts I and III).

Senescence One of the most striking phenotypes in asTDS plants is the delayed senescence of leaves and flowers (Manuscript I). Recently the role of JA in leaf senescence has been examined by analyzing the JA-insensitive mutant *coi1* (He et al., 2002). Exogenous application of JA caused premature senescence in attached and detached leaves in wild-type *Arabidopsis* but failed to induce precocious senescence in *coil* plants, suggesting that the JA-signaling pathway is required for JA to promote leaf senescence. JA levels in senescing leaves are 4-fold higher than in non-senescing ones, because of a subset of genes encoding isozymes for JA biosynthesis which are differentially activated during leaf senescence (He et al., 2002). Analysis of the *coi1* suppressor1 (*cos1*) recessive mutant demonstrated that *cos1* mutation restored the *coi1*-related phenotypes, including defects in JA signaling, senescence, and plant

defense responses (Xiao et al., 2004). The *cos1* gene was found to encode lumazine synthase, a key component in the riboflavine pathway that is essential for many critical cellular processes. However, the flower buds of asTDS plants had higher JA and lower JA-Ile levels compared to WT plants (Manuscript I). This result suggests that senescence may be signaled by JA-Ile or other JA-amino acid conjugates rather than by JA itself. Identification of jasmonate receptor(s) will clarify which jasmonates are signal molecules.

The role of TD in defense

Challenged by a variety of abiotic and biotic stresses, plants need to discriminate between different environmental challenges to optimize the allocation of their resources to growth, defense, and reproduction. Because phytophagous insects display a great diversity of feeding modes and life histories, plants may use the chemical and physical attributes of herbivory to distinguish their attackers. The differential activation of distinct sets of genes or gene products in response to these various challenges is referred to as specificity. The plant must be able to recognize the type of challenge and to translate that into distinct signals to elicit specific responses (Karban and Baldwin, 1997). To elucidate herbivore-responsive genes, first we initiated the molecular characterization of the *N. attenuata-M. sexta* interaction, analyzing the insect-responsive transcriptome of *N. attenuata* by mRNA differential display (DDRT-PCR). The “ask-the-plant-approach” via DDRT-PCR allowed us to isolate those genes that we didn’t prddict would be elicited or repressed by herbivore attack. The analysis demonstrated that TD was one of the herbivore-induced genes. TD was strongly elicited only when plants were attacked by *M. sexta* larvae, mechanically wounded, or treated with MeJA; tobacco mosaic virus (TMV) or *Agrobacterium tumefaciens* infection, ethylene, or methyl salicylate treatment did not elicit TD expression (Hermsmeier et al., 2001), suggesting that TD is involved in herbivore defense and regulated by JA signaling.

A wound-induced increase in JA accumulation has been found in a large number of plant species and seem to be ubiquitous (Sembdner and Parthier, 1993). Additionally, amplification of the wound-induced JA accumulation in response to herbivore feeding (JA burst) has been observed in the wild tobacco plants *Nicotiana*

sylvestris (Baldwin et al., 1997) and *N. attenuata* (Schittko et al., 2000; Ziegler et al., 2001). There are several indications that jasmonates are involved in plant-insect interactions in *N. attenuata* as well as other species. The *Arabidopsis fad3-2 fad7-2 fad8* mutant abolishes JA and octadecanoid intermediate synthesis and is more susceptible to fungal gnat larvae (*Bradysia impatiens*) (McConn et al., 1997). *Arabidopsis* mutants that have an impact on JA sensitivity (*jin1, jar1, jar4,* and *coi1*) have been identified. Some are impaired in their resistance to several fungal pathogens (Staswick et al., 1998; Thomma et al., 1998; Vijayan et al., 1998); some are impaired in their resistance to herbivores, for example, the tomato (*jai1*) mutant, homolog of *Arabidopsis coi1*, vis-à-vis tobacco hornworm larvae (*Manduca sexta*) (Li et al., 2004). Transgenic plants in *N. attenuata* that down-regulate JA synthesis or have JA-regulated genes (trypsin proteinase inhibitor [TPI], putrescine-N-methyl transferase) impair resistance to *M. sexta* attack (Halitschke and Baldwin, 2003; Steppuhn et al., 2004; Zavala et al., 2004a). Experiments with antisense transgenic plants containing *N. attenuata* lipoxygense3 (as-*lox3*), which is involved in JA synthesis, demonstrated that the as-*lox3* reduced the wound- and herbivore-elicited increases in JA (Halitschke and Baldwin, 2003). The reduced JA levels decreased plant resistance and increased the performance of *M. sexta* larvae feeding on as-*lox3* plants compared with larvae feeding on WT *N. attenuata* plants. This reduction in resistance correlated with the reduced expression of two direct defenses: nicotine accumulation and TPI activity (Halitschke and Baldwin, 2003). In WT plants, *M. sexta* attack and wounding with application of *Manduca* oral secretions (OS) and regurgitants elicited increased expression of defense-related genes. In *M. sexta*-attacked as-*lox3* plants, the defense-related transcripts, including genes coding for TPI and TD, decreased compared with the expression in WT plants, demonstrating that the LOX3-mediated octadecanoid pathway regulates TD genes.

To study the effect of TD on defense responses, asTDM plants, which have mild reductions in TD expression and activity but otherwise exhibit WT growth and development patterns, were used (Manuscript III). Suppression of TD decreased plants' resistance and increased the performance of *M. sexta* larvae feeding on asTDM plants compared with larvae feeding on WT plants (Manuscript III). This enhanced performance of larvae correlated with reductions in induced TPI and nicotine accumulations. Reduced levels of TPI in asTDM plants could be restored by Ile

treatment, suggesting that Ile is needed for TPI production as mere protein building elements or for unknown signal molecules.

There are some clues that Ile is involved in jasmonate signaling. One is from the *Arabidopsis*-JA-responsive gene (*JAR1*). The *JAR1* adenylates JA's carboxyl group and adenylated JA is actively conjugated with various amino acids, of which Ile is quantitatively the most important (Staswick et al., 2002; Staswick and Tiryaki, 2004). The mutant *jar1* exhibits decreased resistance to the soil fungus *Pythium irregulare* (Staswick et al., 1998). Second, treatment of barley leaves with synthetic JA-Ile elicits jasmonate-induced proteins without isoleucine cleavage from JA (Kramell et al., 1997). Analysis of asTDS floral buds also supports the hypothesis that TD is involved in JA signaling. asTDS floral buds contained higher levels of JA and lower levels of JA-Ile and TPI compared to WT floral buds (Manuscript I). This observation led us to examine whether the effect of silencing TD on herbivore performance could be attributed to JA signaling via JA-Ile synthesis or turnover. When leaves from WT plants were wounded and treated with *M. sexta* OS, a dramatic JA and JA-Ile burst was elicited. Compared to WT plants, JA production was delayed and JA-Ile was reduced in asTD plants (Manuscript III). The isotope-labeled amino acid feeding experiment demonstrated that Thr is rapidly and efficiently converted into Ile and used for JA-Ile conjugation at the wound site. When plants' wounds were treated with JA or JA-Ile, both WT and asTDM2 plants significantly increased TPI and nicotine to levels higher than when plants were only wounded (Manuscript III). When plants' wounds were treated with JA, elicited TPI activity and nicotine levels in asTDM2 plants were lower than those in WT plants. However, when plants were treated with JA-Ile, the induced TPI and nicotine levels did not differ between WT and asTDM2 plants (Manuscript III), suggesting that JA-Ile synthesized from JA and Ile is a potent signaling molecule for direct defense. JA-Ile's role as a defense signal was also confirmed in the analysis of the asTDS plants. OS-elicited JA and JA-Ile bursts observed in WT plants were much slower and smaller. The OS-elicited TPI production was restored by adding Ile to OS-treated wounds. The performance of the *M. sexta* larvae on JA and JA-Ile treated asTDS1 plants further demonstrated that JA-Ile is a signal molecule. When fed on JA-treated plants, the larvae gained less mass compared to those fed on untreated plants but the difference was not significant. When fed on JA-Ile treated plants however, the larvae gained significantly less compared to those fed on untreated plants (Manuscript III). The direct evidence that

JA-Ile is a signal molecule for plant defense against herbivory is from the characterization of *Arabidopsis JAR1* homologue in *N. attenuata*. The suppression of *Na*JAR4, the *Arabidopsis JAR1* homologue by the virus-induced gene silencing (VIGS) system, clearly showed that JA-Ile is involved in herbivore defenses. The analysis of TD-VIGS plants also clearly demonstrated that TD can supply isoleucine to synthesize JA-Ile (Manuscript III).

In addition to JA-Ile, a bewildering variety of metabolites are formed *in planta*. O-glycosylated derivatives of 11-OH and 12-OH-JA have been detected in plant extracts as well as methyl, glucosyl, and gentobiosyl esters and amide-linked amino acid conjugates of the C1 carboxyl (Wasternack and Hause, 2002; Schaller et al., 2004). However, which metabolites are active or inactive is not clear. For example, MeJA is highly active in bioassays for JA activity and it has been assumed that exogenously applied or airborne MeJA is de-esterified to JA as the primary intracellular signal transducer in plant tissues. The characterization of a JA carboxyl methyltransferase (JMT), however, indicates that the opposite may also be true: formation of MeJA may actually be necessary to activate JA responses (Seo et al., 2001). Transgenic *Arabidopsis* plants constitutively expressing the JMT gene had elevated levels of MeJA while the JA content remained unchanged. JA-responsive genes were expressed constitutively and enhanced resistance to *Botrytis cinerea* was observed in the transgenic plants, suggesting MeJA is a signal in defense response. Another example is from cis-jasmone (cisJ), which was found to be inactive in induction of volatile emissions in several plant species. Therefore, the formation of cisJ was suggested to be an efficient way for the inactivation of JA and the disposal into the gas phase as an infinite sink (Koch et al., 1997). In *N. attenuata*, however, only a small fraction of the herbivore-elicited JA burst was channeled into MeJA and cisJ, and neither one was recovered in the headspace, suggesting that *Nicotiana* plants do not dispose of JA in the form of its volatile derivatives (von Dahl and Baldwin, 2004). Furthermore, Birkett and colleagues reported that cisJ functioned as a semiochemical in plant defense (Birkett et al., 2000), showing it to be active on three trophic levels by inducing volatile emission in bean plants (1st level), repelling aphids (2nd level), and attracting aphid antagonists (3rd level). Which JA metabolites are active or inactive cannot be generalized: JA may be metabolized for inactivation in one species, while the very same compound in other species may serve a specialized signaling function.To study whether other JA-amino acid conjugates like JA-Ile act as

signal molecules, it is helpful to determine which genes are regulated by exogenous applications of synthetic JA-amino acid conjugates, and for this a microarray is useful. Measurement of direct defenses such as TPI and nicotine will also clarify the inducibility of JA-amino acid conjugates. More work, however, will be required to uncover both the variations of the JA structure and how jasmonate signaling regulates jasmonate-responsive genes.

The strong herbivore-induced expression of TD may indeed reflect the high demand of the enzyme for the synthesis of defense compound precursors. A possible role for TD can also be inferred from α-aminobutyric acid, a derivative of α-keto butyric acid (Zhao et al., 1998). Although there is no evidence for the involvement of α-aminobutyric acid in herbivore defense, there is some for the involvement of γ-aminobutyric acid (GABA) in defense against insects and pathogens. Because GABA is a neurotransmitter in vertebrates and invertebrates, it has been speculated that plants produce GABA to deter feeding insects. GABA levels are elevated by mechanical stimulation or damage (Ramputh and Bown, 1996), and even by insects crawling on leaves (Bown et al., 2002), and it is possible that the ingested GABA interferes with the normal development of insects (Shelp et al., 1999; Bouche and Fromm, 2004). For instance, transgenic tobacco plants containing elevated GABA levels were shown to be resistant to the tobacco budworm larvae (MacGregor et al., 2003). Moreover, GABA may function as a signaling molecule given that GABA is a neurotransmitter in animals with a clearly defined role in signaling.

A possible role for TD can also be inferred from a recent report that analyzes plant proteins in the midgut of *M. sexta*. Chen *et al.* used MS-based approach to identify tomato JA-inducible proteins (JIPs) that accumulate in and interfere with digestive processes in the midgut of *M. sexta* larvae. They found that several JIPs significantly alter the protein content of the *M. sexta* midgut, and that two such proteins, arginase and TD, act in the gut to deplete amino acids that are required for insect growth (Chen et al., 2005). These findings imply that host plant enzymes such as TD that metabolize essential nutrients in the insect digestive tract play a role in plant resistance to herbivore attack.

To summarize, I propose the following model to account for TD's role in development and defense signaling: During development, JA is produced throughout seedling and flowering stages. Ile is synthesized from Thr by TD in particular tissues at particular times. When attacked by herbivores, plants produce JA and activate TD

in the attacked tissues. Ile synthesized from Thr by TD is conjugated with JA by JAR1. The resulting JA-Ile regulates plant development and elicits the accumulation of the direct defenses, TPI and nicotine (Fig. 5). The fundamental roles of amino acids

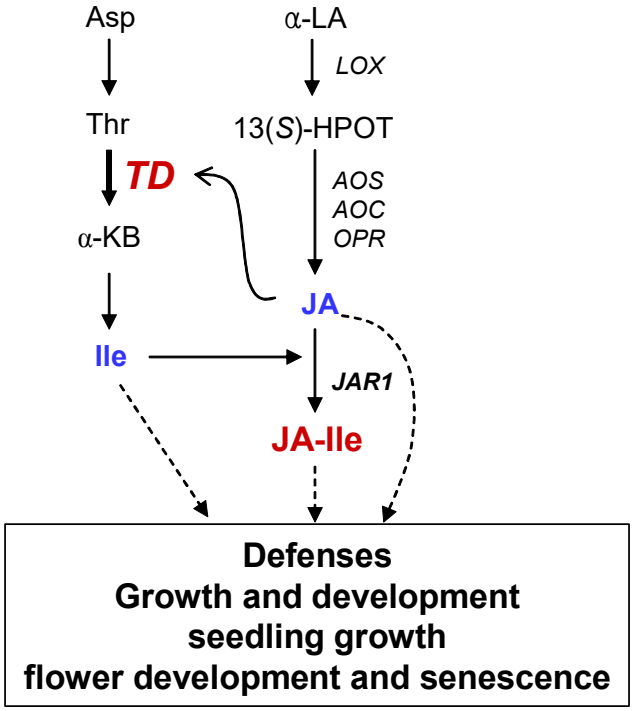

Figure 5. The Role of TD in plant defense and development.
JA biosynthetic enzymes and JA-amino synthetase are represented in boxes. Dashed arrows represent signal transduction pathways. *TD*, threonine deaminase; *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, 12-oxo-phytodienoic acid reductase; *JAR1*, JA-amino synthetase; Asp, aspartic acid; Thr, threonine; α-KB, α-keto butyric acid; Ile, isoleucine; α-LA, α-linolenic acid; 13-HPOT, 13(*S*)-hydroperoxy linolenic acid; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine conjugate.

in protein synthesis and growth notwithstanding, our research demonstrates that much is still to be learned from studying amino acid biosynthesis and highlights the value of working with subtle phenotypes in plants whose ecological performance is well understood.

TD promoter and its use

To understand whether the expression of TD is tissue-specific and developmentally regulated, a genomic clone containing 5' sequences of the TD gene was isolated and characterized (Manuscripts I and II). The analysis of TD promoter:GUS fusions demonstrated that TD promoter directed GUS expression to cotyledons, stigmas, and anthers. The tissue-specific expression was consistent with asTDS plants that have morphological changes in cotyledons and flowers. In addition to its tissue-specific expression, TD promoter:GUS fusions were also elicited by mechanical wounding and MeJA treatment (Manuscript I).

Deletion analysis of TD promoter identified two regions important for expression in cotyledons, stigmas, and anthers as well as a region involved in wound and MeJA elicitation (Manuscript II). These regions contain putative cis-acting regulatory elements; GTGA and QELEMENTZMZM13 motif for regulating pollen expression; W box for wound expression; WRKYs for various biological functions, including senescence and trichome dvelopment as well as defense against pathogen infection; and GT and CATG box for wound and MeJA elicitation. To examine which cis-acting regulatory elements are responsible for tissue-specific expression in addition to wound- and MeJA-inducible expression, more detailed research is needed. For example, where the cotyledon-, wound-, and MeJA-responsive regions of the TD promoter are located within 110 bp, there are still many putative cis-acting regulatory elements. Gain of function study and mutation analysis could be useful to confirm which putative cis-elements are required for the tissue-specific expression and the elicitation.

The efficiency of the formation of transcription initiation complex is largely influenced by the regulatory transcription factors that bind to short-sequence elements that activate or repress genes in a manner that is specific for the tissue, the developmental stage, or the stress conditions. Once cis-elements are found, a one-hybrid screening system could be used to isolate cDNA for cis-element binding protein(s). For example, a promoter element involved in jasmonate and elicitor-responsive gene expression (JERE) was identified in the terpenoid indole alkaloid's (TIA) biosynthetic gene Strictosidine synthase (Str) (Menke et al., 1999). Yeast one-hybrid screening with this element as a bait has identified two transcription factors of the AP2/ERF-domain family that were called ORCA1 and ORCA2 (octadecanoid-

responsive catharanthus AP2; Menke et al., 1999). ORCA2 expression was induced by MeJA and an elicitor. Furthermore, ORCA2 showed sequence-specific binding and trans-activated gene expression via the JERE, demonstrating the mechanisms whereby jasmonate signaling triggers gene expression.

Plant promoters contain multiple cis-acting regulatory elements, only some of which may contribute to their gene inducibility and tissue-specific expression. Recently, synthetic promoters in response to pathogens have provided direct evidence that a range of pathogen-inducible cis-acting elements could mediate pathogen-inducible expression in plants (Rushton et al., 2002). When taken out of their native promoter contexts, these elements retained pathogen inducibility as a component (Rushton et al., 2002). Similarly, if herbivore-specific cis-acting elements are found in TD or other herbivore-responsive promoters, synthetic promoters containing these cis-acting elements will be useful for many applications, for example, as molecular markers, to engineer crops with increased disease resistance. Researchers have identified numerous plant and insect genes that can be used to study plant-insect interaction and to increase crop resistance to invading insects. These strategies involve expression of gene products toxic to certain insects and enhancement of the plant's own natural resistance mechanisms. Such introduced genes are usually placed under the control of strong promoters, yielding constitutive expression of the gene product in all tissues of the plant and detrimentally effect plant growth, development, and crop yield. Use of the synthetic promoters may overcome those detrimental effects. Insect-responsive promoters will allow researchers not only to engineer insect-resistance, but also to analyze the contribution of individual transgenes to the Darwinian fitness of natural plant populations exposed to herbivory.

4. Conclusion

Jasmonic acid (JA) is a naturally occurring signaling compound that regulates plant growth and development, and is involved in plant responses to several abiotic and biotic stresses. Not only JA but also related compounds derived from fatty acids may contribute to signaling, which shows the importance of a comprehensive analysis of different oxylipins, termed the ‘‘oxylipin signature’’ (Weber et al., 1997). The absolute amounts, as well as the ratio of these different compounds, may determine development and stress responses (Hause et al., 2000).

In this study, the roles of threonine deaminase (TD) in plant development and defense were demonstrated by connecting TD expression to jasmonic acid-isoleucine conjugate (JA-Ile) production with antisense TD transgenic plants and TD promoter: GUS fusion transgenic plants. Antisense TD transgenic lines were characterized as having one of two growth phenotypes: 1) plants with severely reduced TD expression and activity, and retarded growth (asTDS plants), and 2) plants with mildly reduced TD expression and activity but otherwise wild type (WT) growth and development patterns (asTDM plants). asTDS plants have morphological differences in young seedlings and flowers compared to WT plants and impaired direct defenses in floral buds, which is associated with reduced levels of α-keto butyrate (α-KB) and an imbalance of JA and JA-Ile. The seedling phenotypes of asTDS plants were partially recovered by supplementing the germination media with α-KB, Ile, or JA-Ile. These results suggest that TD supplies Ile for JA-Ile synthesis and JA-Ile acts as a signal molecule for plant development.

asTDM plants are more susceptible to *M. sexta* attack than are WT plants, probably owing to reduced levels of JA-Ile and direct defenses (nicotine, trypsin protease inhibitors [TPI]). Adding labeled Ile to oral secretions and treating wounds with these revealed that Ile is rapidly conjugated to JA. The defense phenotypes were restored by applying JA-Ile to asTDM plants, implying that TD is involved in herbivore defense by regulating Ile formation at the wound site and that Ile is involved in the conversion of JA to JA-Ile. These data indicate that JA-Ile rather than JA itself is active as signal molecules at least for direct defenses, suggesting the importance of JA-amino acid conjugates as signal molecules.

Analysis of TD promoter: GUS fusion transgenic plants demonstrated that TD is specifically expressed in seedlings and floral organs, as well as strongly elicited by

wounding and MeJA in leaves. Alalysis of TD promoter deletion revealed promoter regions capable of directing minimal expression in cotyledons, stigmas, and anthers as well as regions important for elicitation by wounding and methyl jasmonic acid (MeJA) in leaves. However, more research is needed to delineate the cis-elements responsible for cotyledon-, stigma-, and anther-specific expression in addition to wound- and MeJA-inducible expression. The discoveries of MeJA- or herbivore-responsive cis-acting elements in future work will help clarify their use in development of insect-specific resistance. Moreover, it will help analyze the contribution of individual transgenes to the Darwinian fitness of natural plant populations exposed to herbivory.

5. Zusammenfassung

Jasmonsäure (JS) ist ein Signalhormon, das Pflanzenwachstum und Entwicklung reguliert, sowie Stressreaktionen der Pflanze auf Veränderungen der biotischen und abiotischen Umwelt. Nicht nur Jasmonsäure, sondern auch ihr verwandte Fettsäurederivate (Oxilipine) sind mögliche Vermittler solcher Reaktionen. Deshalb ist es notwendig, die ganze ‘‘Oxilipin-Signatur’’ (Weber et al., 1997) zu untersuchen, da sowohl verschiedene Konzentratioenen als auch veränderte Verhältnisse der einzelnen Substanzen zueinander die Reaktion der Pflanze bestimmen (Hauser et al., 2000).

In dieser Arbeit wurden die Funktionen von Threonindeaminase (TD) in der Pflanzenentwicklung und Herbivorenabwehr anhand von transgenen *Nicotiana attenuata* Pflanzen untersucht, zum einen in bezug auf die Produktion von Jasmonsäure-Isoleuzin-Konjugaten (JS-Ile), zum anderen durch exprimierte TD Promotor:GUS Fusionen. Transgene Pflanzen zeigten zwei verschiedenen Geno- und Phänotypen: 1) sehr stark reduzierte TD Expression und Aktivität verbunden mit beeinträchtigtem Wachstum (stark veränderter Phänotyp, asTDS) und 2) Leicht reduzierte TD Expression und Aktivität gekoppelt mit Wildtyp (WT) ähnlichem Wachstum und Entwicklung (mild beeinflusster Phänotyp, asTDM). asTDS Pflanzen zeigten morphologische Unterschiede im Sämlingsstadium und in der Blütenbildung im Vergleich zum Wildtyp, sowie eine reduzierte direkte Abwehr in Blütenknospen, die mit reduziertem α-Ketobutyrat (α-KB) und einem Ungleichgewicht zwischen JS und JS-Ile verbunden ist. Die Unterschiede im Sämlingswachstum konnten teilweise durch Zugabe von α-KB, JS oder JS-Ile aufgehoben werden, was die Vermutung nahe legt, dass TD Isoleuzin zur JS-Ile-Bildung bereit stellt und dass JS-Ile als Signalsustanz in der Entwicklung der Pflanzen eine Rolle spielt.

asTDM Pflanzen sind anfälliger als der Wildtyp gegenüber den Raupen des Tabakschwärmers *Manduca sexta*, einem natürlichen Frassfeind von *Nicotiana attenauta*. Die reduzierte Resistenz konnte auf eine geschwächte direkte Abwehr durch reduzierten Nikotingehalt und reduzierte Mengen an Trypsin Proteinaseinhibitoren aufgrund des geringen JS-Ile Vorkommen zurückgeführt werden. Die normale Abwehr der asTDM Pflanzen konnte durch Zugabe von JS-Ile wieder hergestellt werden. Desweiteren zeigte die Applikation von radioaktiv

markiertem Ile zu Raupenregurgitat behandelten Blattwunden, dass dieses direkt mit JS konjugiert wurde. Diese Ergebnisse demonstrieren, dass TD Ile zur Konjugatbidlung von JS-Ile bereit stellt und das JS-Ile eine wichtige Rolle in der dirketen Abwehr von *Nicotiana attenuata* spielt. Dies zeigt, dass nicht nur JS, sondern auch ihre Aminosäurekonjugate als Signalmoleküle fungieren.

Untersuchungen des TD Expressionsmusters anhand von Promotor:GUS Fusionen zeigten die stärkste konstitutive TD Expression in Sämlingen und Blütenorganen und eine Induktion von TD durch Verwundung und Applikation von Metyhljasmonsäure (MeJS) in Blättern. TD Promotor „Deletionen“ zeigten, dass es sowohl spezifische Regionen für die örtliche Expression in Kotyledonen, Stigma und Antheren gibt, als auch für die Induktion bei Verwundung und MeJS in Blättern. Weitere Untersuchungen sind nötig, um die cis-Elemente zu spezifizieren. Die Entdeckung von MJ- und herbivorenspezifischen cis-Elementen wird in der Entwicklung von insektenspezifischen Resistenzen hilfreich sein und auch in der Erforschung der Funktion einzelner Gene in Bezug auf die Darwin´sche Fitness in natürlichen Populationen.

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7. Acknowledgments

Many thanks to

- **Prof. Ian Baldwin for his supervision, ongoing support, and many inspirational ideas and discussions at every stage of the project.**
- **Dieter & Ursula for isolation and characterization of threonine deaminase gene, stimulating an interest in the function of threonine deaminase.**
- **Tamara, Michelle, & the gardeners for the huge amount of transformation and growing enormous amounts of plants.**
- **Bernd for the synthesis of JA-Ile and set-up for the measurement of JA-related compounds.**
- **Klaus, Susi, Wibke, & Thomas for their huge help with the chip business and sequencing.**
- **Lei for the VIGS experiment.**
- **Jianqiang, Dominik, Rayko, Anke, Wioletta, & Christstian for data analysis and helping hands.**
- **Evelyn for invaluable help with administrative matters.**
- **Emily for editorial assistance.**
- **My office mates - Claudia, Ursula, Yonggen, Oz, & Sirsha – and my group members for making the MPI years happy and unforgettable.**
- **The Max-Planck-Society for funding this work.**
- **All people at the Friedrich-Schiller-University directly or indirectly involved in the dissertation procedure.**
- **My family – Eun-Ju, Kyoung-Jin, Hye-Jin– for immense patience, support, and understanding as well as for their endless love.**

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9. Selbständigkeitserklärung

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Personen, die an der Durchführung und Auswertung des Materials und bei der Herstellung der Manuskripte beteiligt waren sind am Beginn der Arbeit („Manuscript Overview“) und jedes Manuskriptes angegeben.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen.

Die vorgelegte Arbeit wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

Jin-Ho Kang