AN ABSTRACT OF THE DISSERTATION OF

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Title: Molecular Cloning and Characterization of the *Diageotropica* Gene in Tomato (*Lycopersicon esculentum* Mill.)

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Terri L. Lomax

The auxin-resistant *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum* Mill.) has a pleiotropic phenotype including a lack of lateral roots and reduced gravitropic response, apical dominance, vascular development, and fruit growth. The *dgt* mutation reduces the auxin sensitivity of only a subset of auxin responses while levels, metabolism, and transport of auxin appear normal, suggesting that the *Dgt* gene encodes a component in an auxin-signaling pathway.

This dissertation reports isolation and characterization of the *Dgt* gene. Delineation of three microsyntenic regions in the *Arabidopsis* genome containing genes homeologous to genetic markers near the *Dgt* gene allowed isolation of additional ESTs from the corresponding tomato region, significantly reducing the mapping distance to the *dgt* locus. Further analysis determined that the *Dgt* gene encodes a cyclophilin (LeCYP1), a previously unidentified component of auxin signaling. Each known *dgt* allele contains a unique mutation in the coding sequence of *LeCyp1*. In addition, the wild-type *Dgt* gene can complement *dgt*

mutant plants. Cyclophilins characteristically have peptidylprolyl *cis-trans* isomerase (PPIase) activity, but it is unclear whether that activity is necessary for all of their biological functions. Each allelic *dgt* mutation reduces or nullifies PPIase activity of LeCYP1 fusion proteins *in vitro*. Immunoblot analysis indicates that all three *dgt* mutations are null mutations. Phylogenetic comparisons of tomato and *Arabidopsis* cytosolic-type cyclophilins could not identify any single *Arabidopsis* member as orthologous to LeCYP1/DGT. Five T-DNA insertion mutants were analyzed to determine if mutations in *Arabidopsis* cytosolic-type cyclophilins phenocopy the pleiotropic *dgt* phenotype. Overall seedling growth and morphology appear normal in the mutants, however, their gravitropic response is slow. The lack of exact phenocopy may be due to the redundant nature of *Cyp* genes in *Arabidopsis,* which has over twice as many *Cyp* genes as tomato. In tomato, the cyclophilin inhibitor cyclosporin A (CsA) inhibits auxin-induced adventitious root initiation and expression of two early auxin response genes, *LeIAA10* and *11*, that are also affected by the *dgt* mutation. Taken together, these results suggest that the cyclophilin encoded by the *Dgt* gene plays an important role in auxin signal transduction.

Molecular Cloning and Characterization of the *Diageotropica* Gene in Tomato (*Lycopersicon esculentum* Mill.)

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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KwangChul Oh, Author

CONTRIBUTION OF AUTHORS

All experiments in this dissertation were planned, conducted, and evaluated by the primary author. Kristine Hardeman, Mary Ellard-Ivey, and Andreas Nebenführ were involved in the initial work of high-resolution mapping in Chapter 2. Maria G. Ivanchenko and TJ White assisted in some of the experiments of Chapter 2 and 3.

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DEDICATION

This thesis is dedicated to the memory of my mother, Taekyung Lee, who never failed to encourage me, even on the night before she passed away.

Molecular cloning and characterization of the *Diageotropica* gene in tomato (*Lycopersicon esculentum* Mill.)

1. INTRODUCTION

"A prime function of hormones in plants, which lack a nervous system, is to convey information from one part of the plant to another."

– Peter J. Davies, 1995

Plant growth and development are controlled by the orchestrated actions of genes, environmental stimuli, and hormones. Genes encode numerous endogenous developmental programs and the environmental stimuli trigger or halt the developmental programs. Plant hormones play a major role in harmonizing this internal and external developmental information by mediating, amplifying, and integrating signals between cells, as well as intracellular signaling networks. Plant hormones are a group of naturally occurring organic substances that influence physiological processes at low concentrations (Davies, 1995a). Due to the nature of plant compared to animal hormones, the concept of plant hormones has been debated for many years. However, hormone concentration, sensitivity, transport, and redistribution upon environmental signals are critical factors for the function of plant hormones (reviewed in Davies, 1995b). A specific signaling pathway, also called hormone signal transduction, is necessary for a cell to translate an external signal into a specific cellular response, which can be either

perception, amplification of the signal, or gene regulation.

1.1 Role of auxin in plant growth and development

"If an auxin was discovered today, would we call it a hormone? Probably not, because it has all the characteristics of a morphogen, not a hormone"

- Alan M. Jones, 1994

Among plant hormones, auxin is often considered a "master" hormone or a "morphogen" because it regulates a multitude of events throughout the plant life cycle via an auxin gradient along the axis of the plant. These events include cell division and enlargement, vascular tissue differentiation, root initiation, tropic responses, apical dominance, senescence, abscission, assimilate partitioning, fruit ripening, flowering and floral development (for review Jones, 1994; Davies, 1995a;Trewavas, 2000). Auxin levels, transport, and perception, as well as auxin-induced gene regulation, are all important factors that control growth and development. While auxins regulate many different processes, other plant hormones influence or affect the auxin-regulated processes at the same time within a plant. For example, auxins interact closely with cytokinins in regulating apical dominance, leaf senescence, adventitious root initiation, cell elongation, and organ differentiation in tissue culture. Interactions between auxin and cytokinins can be synergistic, additive, or antagonistic depending on the development response (reviewed in Coenen and Lomax, 1997). In this dissertation, I will emphasize recent molecular genetic analyses of polar auxin

Figure 1.1. A current model of auxin action mechanism. Polar auxin transport is mediated by asymmetric localization of auxin-influx (AUX1) and -efflux (PIN1) carriers. Vesicle-dependent transport of the PIN1 protein to the membrane appears to be along actin filament tracks, interacting with a 1-*N*-naphthylphthalamic acid-binding protein (NBP), and directed by regulator proteins such as GNOM and BIG. Perception of auxin remains unclear although an auxin-binding protein (ABP1) plays a role in regulating some aspects of auxin response and is a candidate auxin receptor. A mitogen-activated protein kinase (MAPK) cascade, or yet unrevealed signaling pathway, may transduce the auxin signal to the nucleus and activate the transcription of auxin response genes. Auxin-regulated gene expression involves auxin-response factors (ARFs) and AUX/IAAs as well as potentially additional unknown transcription factors. Increased levels of the auxin promote the ubiquitin (Ub)-dependent degradation of the AUX/IAA proteins following their phosphorylation by various candidate protein kinases. Abbreviations: AuxRE, auxin-response *cis* element ; E1, ATP-dependent Ub-activating enzyme; E2, Ub-conjugating enzyme; ECR1-AXR1, RUB-activating enzyme (E1); PINOID, Pinoid protein kinase; PK, protein kinase; RCN, protein phosphatase 2A; RNA Pol II, RNA polymerase II; Rub, Ub-related protein; Skp1-CUL-F-box/TIR1, E3 Ub-ligase complex; TF, unknown transcription factor.

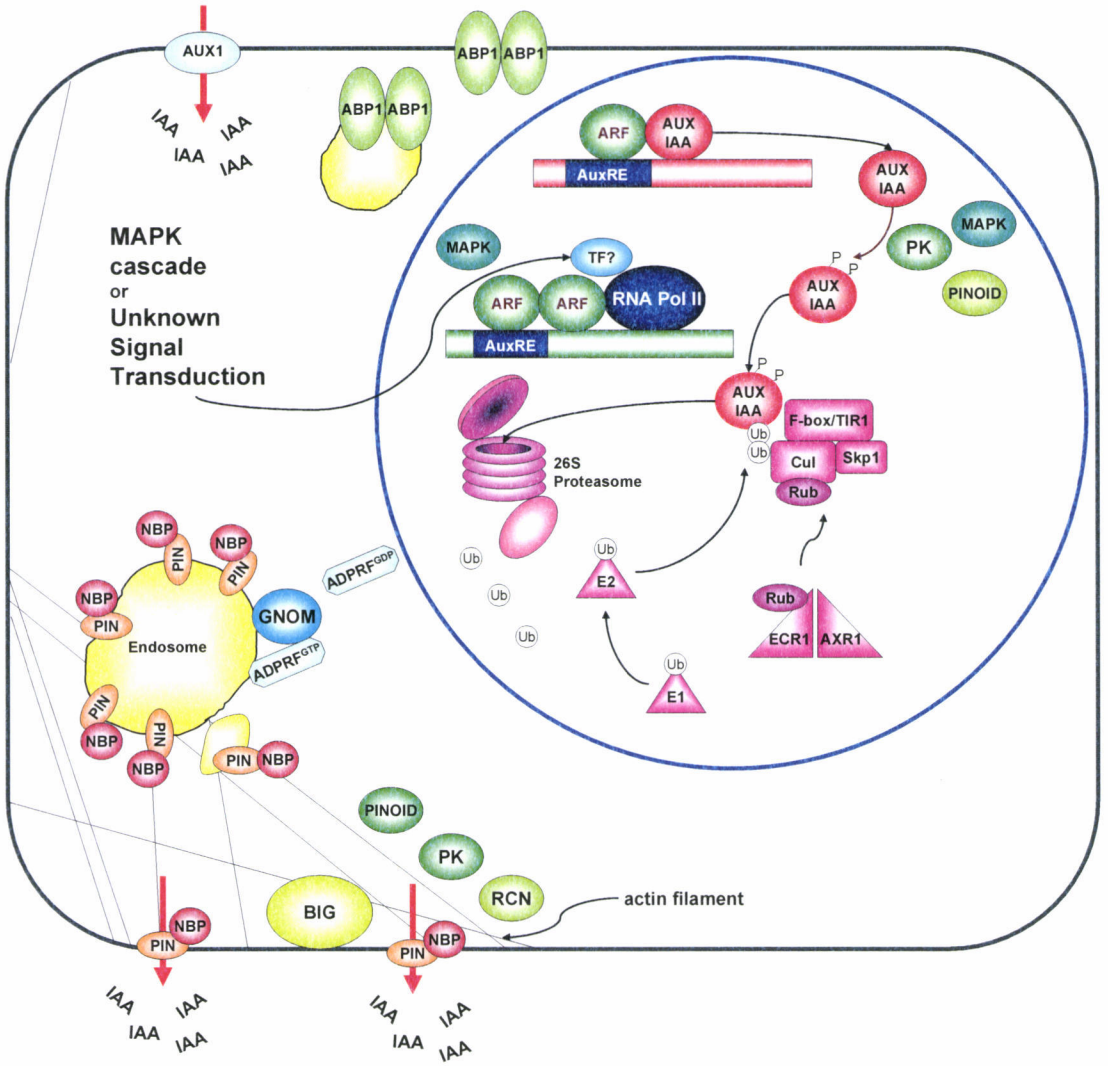
AUX1
IAA
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Figure 1.1

transport and auxin-induced gene expression to understand current models of auxin action (Figure 1.1).

1.1.1 Auxin Level

The most abundant and naturally occurring auxin is indole-3-acetic acid (IAA). The cellular concentration of IAA is tightly controlled and maintained, because endogenous IAA-homeostasis is critical during the plant life cycle to ensure appropriate developmental signals. The *rooty* (*rty*) mutant of *Arabidopsis*, which is defective in IAA-homeostasis, exhibits increased numbers of lateral and adventitious roots, absence of an apical hook when grown in the dark, epinastic cotyledons and leaves, and lack of flower development (King et al., 1995).

IAA-homeostasis depends not only on *de novo* synthesis of IAA thorough tryptophan-dependent and –independent pathways but also on metabolic transformations between various IAA conjugates (for review Bandurski et al., 1995). However, in spite of its importance, the mechanism of IAA-homeostasis is still elusive, because no auxin auxotrophic mutant has yet been isolated. This may be due to the many complicated metabolic transformations of IAA-precursors and/or –conjugates that could compensate for the lack of a particular metabolic pathway, or it may be that auxotrophic mutants are lethal.

1.1.2 Polar Auxin Transport

"*When seedlings are freely exposed to a lateral light some influence is transmitted*

from the upper to the lower part, causing the latter to bend."

– Charles and Francis Darwin, 1880

In animal developmental biology, a morphogen is defined as a secreted signaling molecule that organizes a field of surrounding cells into patterns, forming a gradient of concentration emanating from a localized source, that determines the arrangement and fate of responding cells according to the different concentrations of the morphogen perceived by the cells (Gilbert, 1991). Auxin is normally synthesized in young apical shoot tissues and by the embryo of developing seeds. The auxin gradient along the primary axis of a plant is established by distinct polar transport characteristics, and it significantly contributes to auxin's role as a plant morphogen. During polar auxin transport, auxin is transported basipetally via starch-sheath cells surrounding the vascular bundles in the shoot, moving acropetally to the root tip through the central cylinder, and then basipetally to the root elongation zone through the root epidermal layers (Lomax et al., 1995; Jones, 1998). Important features of plant development, such as hypocotyl elongation (Jensen et al., 1998), apical dominance (Shimizu-Sato and Mori, 2001), leaf and lateral root formation (Casimiro et al., 2001, Reinhardt et al., 2000), tropic responses (Lomax et al., 1995), vascular differentiation (reviewed in Aloni, 1995), and shade avoidance (reviewed in Morelli and Ruberti, 2000), depend upon proper auxin gradients established through cell-to-cell transport, both along the primary axis of the plant and the lateral flux of auxin initiated upon stimulation, e.g. light, touch, wounding, or a change in the gravity vector. Polar auxin transport involves two families of

proteins, auxin-influx and -efflux carriers. The polarity of auxin transport is thought to be controlled by the asymmetric localization of auxin-influx and -efflux carriers (Gälweiler et al., 1998; Müller et al., 1998; Swarup et al., 2001).

The auxin-resistant *aux1* mutant of *Arabidopsis* has reduced numbers of lateral roots (Hobbie and Estelle, 1995), a modified root architecture (Marchant et al., 2002), and impaired auxin-influx carrier activity in seedlings (Marchant et al., 1999), suggesting a putative role of the AUX1 protein as an auxin-influx carrier. The *AUX1* gene encodes a highly hydrophobic polypeptide containing 10 to 12 transmembrane spanning domains and the primary sequence is similar to a family of plant amino acid permeases (Bennett et al., 1996; Parry et al., 2001). The AUX1 protein regulates root gravitropism (Bennett et al., 1996; Marchant et al., 1999), as well as lateral root initiation (Casimiro et al., 2001) with the cooperation of an auxin-efflux carrier. Although sequence and phylogentic analyses of amino acid permeases demonstrate that at least three other amino acid permeases, like-AUX1s (LAXs), are included in the same clade in the *Arabidopsis* genome (Parry et al., 2001), characterization of the auxin-influx carrier has been limited to only AUX1 to date.

Compared to the limited information on the auxin-influx carrier, auxin-efflux carriers are relatively well characterized. The best-studied auxin efflux carriers, the PIN proteins, were first identified in mutants with shoot (*pin1*) and root (*agr1/eir1/pin2/wav6*) phenotypes consistent with reduced IAA transport (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Asymmetric cellular localization of PIN proteins is consistent with a role in controlling the polar efflux of IAA from cells (Gälweiler et

al., 1998; Müller et al., 1998) and in redistribution upon gravitropic stimulation (Friml et al., 2002).

The subcellular localization of PIN1 is tightly regulated by vesicle trafficking involving an ADP-ribosylation factor (ADPRF)-guanine nucleotide exchange factor (GEF), a calossin-like protein (BiG), and 1-*N*-naphthylphthalamic acid (NPA, auxin-efflux inhibitor)-binding proteins. A mutatnt in ADPRF-GEF function, *gnom*, displays severe abnormality in embryonic development including loss of the embryo axis and fusion or deletion of hypocotyls and cotyledons (Shelvell et al., 1994; Busch et al., 1996). The *gnom* phenotype can be phenocopied by high-dose application of auxin or polar auxin transport inhibitors in the embryo cultures of *Brassica juncea* (Hadfi et al., 1998). Brefeldin A (BFA), a vesicle movement blocking agent, abolishes the normal asymmetric distribution of PIN1 and results in its accumulation in internal cellular compartments (Steinmann et al., 1999). A recent study using engineered BFA-resistant ADPRF-GEF demonstrated that GNOM proteins regulate specific endosome trafficking that recycles PIN1s between transport vesicles and cell surface. (Geldner et al., 2001 and 2003). A calossin-like protein, named BIG due to its huge molecular weight, is an integral membrane protein encoded by the *TIR3/DOC1* gene, and is believed to be essential for secretory vesicle formation (Gil et al., 2001). Application of NPA to *tir3/doc1* mutants demonstrated that BIG is likely to regulate the distribution of PIN1, however, whether the NPA-dependent effect is directly involved in PIN distribution is questionable (Ruegger et al., 1997; Gil et al., 2001). NPA-binding proteins also interact with the actin cytoskeleton (Butler et al., 1998; Hu et al., 2000), suggesting that this NPA-binding protein may provide a bridge between

efflux carriers and the actin cytoskeleton used to transport and/or localize these complexes. When the polarity of auxin transport is altered by changes in the direction of the gravity vector, auxin-efflux carriers are redistributed and positioned to the appropriate side of the plasma membrane by rapid vesicle cycling.

In addition to the aforementioned regulator proteins, protein phosphorylation switches are likely to modulate polar auxin transport. Mutations in protein kinases or phosphatases result in phenocopies of auxin transport mutants. A mutant *pinoid* (*pid*) displays reduced auxin transport and *pin*-like phenotypes and encodes a protein kinase C–like protein, (Bennett et al., 1995; Christensen et al., 2000). Overexpression of the PID protein leads to growth effects consistent with increased auxin transport (Christensen et al., 2000; Benjamins et al., 2001). In contrast, the *rcn1* mutation in a protein phosphatase 2A regulatory subunit gene results in reduced phosphatase 2A activity (Deruère et al., 1999), increased root basipetal IAA transport, and gravitropic defects. Attempts to identify the target of RCN1 action finds no evidence that this regulation is at the level of the putative auxin carriers (Rashotte et al., 2001) and is consistent with an upstream action.

1.1.3 Auxin Perception and Signaling Cascades

"Receptors are proteins which specifically and reversibly bind chemical signals but, unlike enzymes, do not convert them chemically. Upon binding, the receptor molecules are, through a conformational change, transformed into an activated

state."

Kees R. Libbenga and Albert M. Mennes, 1995

To understand how auxin is perceived by target cells, the identification of auxin receptors has been pursued for more than 40 years. No protein has been definitely proven to be an auxin receptor in the sense of fulfilling definition of plant hormone receptor as described above and proving the hypothesis that plant hormones are detected by specific receptor proteins (Libbenga and Mennes, 1995; Leyser, 2002). However, the best candidate for an auxin receptor is auxin-binding protein 1 (ABP1), first characterized in maize membrane fractions and subsequently isolated, purified (Hertel et al., 1972; reviewed in Jones, 1994), and identified as a soluble 22 kDa glycoprotein. ABP1 is found predominantly within the endoplasmic reticulum (ER) where it is retained by KDEL, a C terminal ER retention signal (Lys-Asp-Glu-Leu) sequence. The presence of smaller quantities of ABP1 at the cell surface (Diekmann et al., 1995) caused a great deal of debate about whether ABP1 functions as a hormone receptor on the plasma membrane. ABP1 is dimeric in solution (Shimomura et al., 1986) and has submicromolar affinity for auxins, consistent with its ability to bind free auxin at physiological concentrations (Löbler and Klämbt, 1985). There is increasing evidence that ABP1 functions as a putative auxin receptor: Ectopic and inducible expression of ABP1 confers auxin-dependent cell expansion in tobacco cells normally lacking auxin responsiveness (Jones et al., 1998); antisense suppression of ABP1 eliminates auxin-induced cell elongation and reduces cell division; and a homozygous null mutation in ABP1 confers embryo lethality in *Arabidopsis* (Chen et al., 2001).

How the auxin signal is delivered to the nucleus, where gene expression occurs in response to auxin (see the next section), should be addressed. Protein phosphorylation switches are a tempting pathway to explain the connection between auxin and auxin-regulated gene expression. In the *Arabidopsis* genome, there are more than 2500 proteins containing protein kinase or phosphatase domains (The *Arabidopsis* Genome Initiative, 2000) that can serve as major signal mediators in cells. Mitogen-activated protein kinase (MAPK) cascades, consisting of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK), are known to be major downstream components of receptors or sensors that transduce extracellular or endogenous signals in yeast and animal cells. Plant MAPKs are believed to transduce stress stimuli such as H_2O_2, ozone, osmotic and salt stress, and pathogen attacks, as well as plant hormone signals such as ethylene, abscisic acid, and jasmonic acid (reviewed in Zhang and Klessing, 2001). There is evidence that MAPK cascades also transduce auxin signals. A tobacco MAPKKK, NPK1, suppresses the expression of a *GH3* promoter-driven *GUS* reporter gene in response to auxin treatment of protoplasts (Kovtun et al., 1998), and an auxin-stimulated MAPK has been identified in *Arabidopsis* root tissue (Mockaitis and Howell, 2000). In addition to the MAPK cascade, a small GTPase, NtRac1, is proposed to activate auxin-induced gene expression as a potential upstream signaling component of the MAPK cascade (Tao et al., 2002). However, we can not rule out the possibility that 1) ABP1 may directly deliver auxin signals to the nucleus, similar to the nuclear receptors for steroids, retinoids, and thyroids in vertebrates (Mangelsdorf and Evans, 1995) or 2) other as yet unrevealed signaling pathways transduce auxin signals.

1.1.4 Gene Expression and Ubiquitin-Dependent Protein Degradation

"Whatever the auxin receptor and the signal transduction pathways, it is clear the exogenously applied auxin can rapidly and specifically alter the expression of selected genes in different tissue and organs."

- Tom J. Guilfolye, 1998

Ever since Silberger and Skoog (1953) found that IAA induced changes in nucleic acid content and growth in tobacco tissue, the identification and characterization of primary auxin-response genes, genes rapidly and specifically regulated by auxin application but independent of *de novo* protein synthesis, have been pursued for 50 years. Three families of primary auxin-responsive genes have been identified: *GH3*-like genes (*GH3*; Hagen et al., 1991; Hagen, 1995), *small auxin up-regulated RNAs* (*SAURs*; McClure and Guilfoyle, 1987; McClure and Guilfoyle, 1989), and *AUX/IAA* genes (Ainley et al., 1988; Conner et al., 1990; Abel et al., 1995; reviewed in Liscum and Reed, 2002). Although *SAUR* and *GH3* appear to be expressed in tissue- or developmental-stage specific manners (McClure and Guilfoyle, 1987; McClure and Guilfoyle, 1989), the exact role of these genes in auxin-regulated gene expression still remains unclear. On the other hand, Aux/IAA family members encoding short-lived nuclear proteins have been extensively studied because they are putative repressors of auxin-regulated transcription (Tiwari et al., 2001, reviewed in Liscum and Estelle, 2002).

Auxin-regulated transcription involves another family of proteins, auxin-response factors (ARFs), that bind to an auxin-response *cis* element (AuxRE),

along with AUX/IAA proteins. ARFs contain a DNA-binding domain (DBD) at the N-terminus, a variable glutamine (Q)- or proline/serine/threonine (P/S/T)-rich domain in the middle region (MR), and domains III and IV at the C-terminus. The DBD is present in all ARFs and binds to the AuxRE. ARFs with Q-rich middle domains activate transcription in transient expression assays, whereas those with P/S/T-rich middle domains repress transcription (Ulmasov et al., 1999b). Domain III and IV are shared with the AUX/IAA proteins and are critical protein-protein interaction domains that mediate both homo- and heterodimerization between AUX/IAA and ARF families (Kim et al., 1997; Ulmasov et al., 1997; Ulmasov et al., 1999a). While the function of domain I in the AUX/IAA proteins is not known, domain II appears to be critical for stability and rapid turnover of the protein. When auxin concentrations are low or below a threshold, early auxin response genes containing TGTCTC AuxREs are actively repressed by heterodimers of AUX/IAA repressors and ARF transcriptional activators (Figure 1.1). When auxin concentrations are increased, AUX/IAA proteins are degraded, which effectively relieves the repression of early auxin response genes and results in gene activation. Gene activation might be enhanced further by the homodimerization of ARF transcriptional activators to ARFs that are bound to AuxRE target sites. Currently, at least 23 *ARF* and 29 *AUX/IAA* genes have been identified in *Arabidopsis* (Liscum and Reed, 2002). The staggering number of possible combinations between ARF and AUX/IAA proteins suggests that each dimer activates or represses the transcription of specific downstream auxin-response genes, including the primary auxin-response genes themselves, by a feedback mechanism. The involvement of additional transcription factors in auxin-response

gene expression remains an open question.

The fast response and efficient adaptation to endogenous and exogenous stimuli suggests that selective and rapid proteolysis is involved in auxin signal transduction. Auxin stimulates not only the induction of AUX/IAA proteins, but also their rapid degradation (Zenser et al., 2001; Kepinski and Leyser, 2002; Hellmann and Estelle, 2002). Ubiquitination is a highly conserved pathway that covalently attaches ubiquitin (Ub) to protein substrates through the sequential action of three enzymes: ATP-dependent Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub-protein ligase (E3). The resulting ubiquitinated proteins are recognized and degraded by the 26S proteasome and the Ub moieties are released for reuse. The 26S proteasomes are found in both the eukaryotic cytoplasm and nucleus and can be deployed to different locations in the cytosol or nucleus, wherever its action is required (reviewed in Voges et al., 1999). Degradation of AUX/IAA proteins by the 26S proteasome might occur in the nucleus since NtPSA1, a homolog to the yeast and human α-type proteasome subunit, was found in the nucleus, and its antisense expression resulted in reduced sensitivity to auxin treatment (Bahrami et al., 2002).

The first crucial evidence that the Ub-proteasome pathway is involved in auxin signal transduction was provided by characterization of two auxin-related mutants, *auxin-resistant1-12* (*axr1-12*) and *transport inhibitor response* (*tir1-1*) of *Arabidopsis* (Lincoln et al., 1990; Ruegger et al., 1997). The *AXR1* gene encodes a subunit of E1 Ub-related protein (RUB)-activating enzyme that forms a dimer with ECR1, ubiquitin-activating enzyme, that activates RUB1 (del Pozo et al., 1998 and 2002). The *TIR1* gene encodes an F-box protein subuit of E3 Ub-ligase

(Ruegger et al., 1997). The F-box protein interacts with either of the *Arabidopsis* Skp1-like proteins, ASK1 or ASK2, and the cullin AtCUL1 protein to form the functional Skp1/Cullin/F-box (SCF) complex^{TIR1} (Gray et al., 1999 and 2001). The *ask1-1* mutant displays reduced auxin-response (Gray et al., 1999). In AUX/IAA, a core GWPPV motif in domain II has been suggested to confer the destabilization signal and ubquitination site (Ramos et al., 2001). SCF-mediated ubiquitination is usually preceded by substrate modification. These changes turn stable proteins into susceptible targets for SCF. The most common modification is phosphorylation (Deshaies, 1999). Kepinski and Leyser (2002) suggest that auxin might increase the affinity of SCF^{TIR1} for AUX/IAAs by stimulating phosphorylation of AUX/IAAs in a manner similar to ubiquitination of phosphorylated NF-κB activation upon stress stimuli (reviewed in, Karin and Ben-Neriah, 2000). In fact, the N-termini of AUX/IAA proteins can be phosphorylated by phytochrome A *in vitro* (Colon-Carmona et al., 2000), suggesting that phosphorylation of AUX/IAA may target them to the proteasome.

1.2 The *diageotropica* Mutant of Tomato

The *diageotropica* (*dgt*) mutant of tomato was first identified by its horizontal growth characteristics in the field (Zobel, 1972). Many reports have characterized the highly pleiotropic phenotype of *dgt* plants that includes a slow gravitropic response (Rice and Lomax, 2000), lack of lateral roots (Zobel, 1974; Muday et al., 1995), hyponastic leaves (Ursin and Bradford, 1989), reduced apical dominance (Coenen and Lomax, 1998), and altered vascular development (Zobel,

1974; Madlung and Lomax, unpublished results). Stems of the *dgt* mutant seedling have fewer tracheary elements (TEs) than wild type, however, *dgt* plants produce more TEs than wild-type plants in tissue culture (Madlung et al., unpublished results). Although the auxin-resistant *dgt* mutant was reported to exhibit normal reproductive behavior (Fujino et al., 1988; Ludford, 1995), Balbi and Lomax (2003) demonstrated an effect on overall fruit development, including fruit size, fruit weight, number of locules, number of seeds, and fruit set, regardless of the background of each mutant allele.

The *dgt* mutant, formerly described as having altered ethylene synthesis (Zobel, 1973; Zobel, 1974), is actually auxin-resistant. Ethylene is produced normally in a variety of stress responses but *dgt* shoots do not produce ethylene or elongate in response to exogenously-applied auxin (Kelly and Brandford, 1986). The auxin insensitivity of *dgt* is unlikely to result from altered auxin levels, metabolism, or polar transport. Endogenous IAA levels are normal in *dgt* shoot apices and roots (Fujino et al., 1988; Lomax, unpublished), and rates of basipetal IAA polar transport are identical, or nearly identical in hypocotyl sections of *dgt* and wild-type (Daniel et al., 1989). Auxin uptake and efflux also appear to be normal in *dgt* seedlings (Rice and Lomax, 2000).

The *dgt* mutation affects the expression of many auxin-regulated genes, including primary auxin-response genes such as *LeAUX/IAA* and *LeSAUR,* in a tissue- and developmental-stage specific manner (Table 1.1). Differential expression of the auxin-regulated genes affected by *dgt* could partially explain the *dgt* phenotype. The auxin-induced expansin (*LeEXP2*) and xyloglucan endotransglycosylase (*LeXET*) genes, proposed to be involved in cell elongation,

are down- and up-regulated by the mutation, respectively (Caderas et al., 2000), while the *LeSAUR* and *LeACS* genes involved in gravitropic response and ethylene synthesis, respectively, are down-regulated (Coenen et al., 2003). It is noteworthy that the primary auxin-response genes, such as *LeIAA2, 8, 10,* and *11,* are regulated by the *DGT* protein and are believed to be repressors proteins required for regulating the transcription of auxin-response genes (Nebenführ et al., 2000). These data suggest that the DGT gene product may function upstream of auxin-regulated gene expression.

The *Dgt* gene also appears to be involved in cell cycle regulation. The lack of optima for both auxin and cytokinin in dgt callus cultures demonstrates that both auxin and cytokinin stimulate callus growth via a DGT-dependent process (Coenen and Lomax, 1998). Flow-cytometric analyses reveal that mitotic activity is different in wild-type and *dgt* ovaries during fruit development, suggesting that the reduction in fruit size by the *dgt* mutation might be determined via cell cycle regulation during early flower development (Balbi and Lomax, 2003).
In addition, the *dgt* mutant can be considered as a useful tool to dissect interactions between auxin and other hormone signaling mechanisms. Ethylene partially restores the wild-type response in *dgt* plants. While *dgt* plants do not exhibit upward shoot growth (Jackson, 1979) or petiole epinasty in response to exogenous auxin, epinasty is restored by ethylene treatment without additional auxin (Ursin and Bradford, 1989). The reduced gravitropic response of the *dgt* mutant is also restored to wild-type levels by ethylene treatment (Madlung et al., 1999). Epibrassinolide, a brassinosteroid, also restores auxin-stimulated hypocotyl elongation but not the altered gravitropic response in *dgt* seedlings (Park, 1998).

Exogenous application of cytokinin to wild-type plants results in a phenocopy of the pleiotropic *dgt* phenotype in wild-type plants including shortened internodes, stunted roots, increased pigmentation, retarded senescence of cotyledons, and overall slowing of development (Coenen et al., 2003). Cytokinin treatment of wild-type plants not only phenocopied the *dgt* morphology but also inhibited the expression of two DGT-dependent auxin-response genes, *LeSAUR* and *LeACS3*, in hypocotyls (Coenen et al., 2003). These data suggest that the *DGT* gene may selectively interact with the signaling mechanism of other hormones.

1.3 Objectives of This Work

Molecular and genetic analyses of allelic forms of genes encoding signaling components have made great advances in understanding the mechanism of auxin actions. Nevertheless, the connections between auxin signaling networks remain unclear. The single gene, auxin-insensitive *dgt* mutant could provide a clue to connect the spotting information on auxin signaling. Physiological and molecular characteristics of the *dgt* mutant have led to the hypothesis that the *Dgt* gene encodes an integral component in the auxin signal transduction pathway. However, despite the potential significance of the *Dgt* gene in the molecular mechanism of auxin action, the nature of the *Dgt* gene has not been determined. The *Arabidopsis nonphototropic hypocotyls4/massugu1* (*nph4/msg1*) mutant also has a pleiotropic phenotype in which hypocotyls fail to elongate in response to exogenous auxin application and are non-gravitropic. Also, the respective positive and negative phototropic responses of hypocotyls and roots are altered (Liscum

Table 1.1. Differential gene expression regulated by the *DGT* gene

	Expression level $_{tissue}$		Reference
LeACS3	WT > *dgt* $_{hypocotyls}$	ethylene synthesis	Coenen et al. (2003)
LeACS5	WT > *dgt* $_{hypocotyls}$	ethylene synthesis	Coenen et al. (2003)
LeACS6	WT > *dgt* $_{fruit}$	ethylene synthesis	Balbi and Lomax (2003)
LeACS7	WT > *dgt* $_{fruit}$	ethylene synthesis	Balbi and Lomax (2003)
LeAUX	WT > *dgt* $_{hypocotyls}$		Mito and Bennett (1995)
LeIAA2	WT > *dgt* $_{fruit}$	transcription control	Balbi and Lomax (2003)
LeIAA8	WT > *dgt* $_{fruit, hypocotyl}$	transcription control	Balbi and Lomax (2003), Nebenführ et al., (2000)
LeIAA10	WT > *dgt* $_{hypocotyls}$	transcription control	Nebenführ et al. (2000)
LeIAA11	WT > *dgt* $_{fruit, hypocotyls}$	transcription control	Nebenführ et al. (2000); Balbi and Lomax (2002)
LeSAUR	WT > *dgt* $_{hypocotyl}$		Mito and Bennett (1995)
LeCDKA	WT > *dgt* $_{hypocotyl}$	cell cycle control	Balbi et al., (2003)
LeEXP2	WT > *dgt* $_{hypocotyl}$	cell elongation	Caderas et al., (2000)
LeXET2	WT < *dgt* $_{hypocotyl}$	cell elongation	Catalá et al., (2001)

and Briggs, 1995; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998). The *NPH4* gene encodes ARF7, as identified by map-based cloning (Harper et al., 2000). Interestingly, addition of ethylene suppresses the aphototropic response of *nph4* while restoring the gravitropic response of *dgt* (Madlung et al., 1999; Harper et al., 2000). However, no mutations were found in the tomato *ARF7* homolog (Oh and Lomax, unpublished result). Therefore, our laboratory used the knowledge that the *dgt* locus is located in the long arm of chromosome 1 (Tanksley et al., 1992), to apply a positional cloning approach to isolate the *Dgt* gene. The goals of the work presented in this dissertation were to isolate and characterize the *Dgt* gene. Also, *Arabidopsis* homologs of the *Dgt* gene were analyzed since hormone signaling has been extensively studied in *Arabidopsis*. To achieve these goals, the following specific aims were pursued:

1) Comparative mapping, using microsynteny analysis, of the tomato and *Arabidopsis* genomes to facilitate the positional cloning of the *Dgt* gene.
2) Identification of the *Dgt* gene by DNA sequence analysis of the BAC clone containing the two closest flanking genomic markers and verification of the candidate gene by complementation of the *dgt* phenotype.
3) Characterization of the *Dgt* gene at the RNA and protein levels in both mutant and wild-type tissues to determine how *Dgt* expression is regulated.
4) Characterization of putative functional homologs of the *Dgt* gene in *Arabidopsis* to provide insight into the function of DGT in auxin signaling and its interactions with other hormones and stimuli.

2. FINE MAPPING IN TOMATO USING MICROSYNTENY WITH THE *ARABIDOPSIS* GENOME: THE *DIAGEOTROPICA* LOCUS

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2.1 Abstract

The *Arabidopsis thaliana* genome sequence provides a catalog of reference genes applicable to comparative microsynteny analysis of other species, facilitating map-based cloning in economically important crops. We have applied such an analysis to the tomato expressed sequence tag (EST) database to expedite high-resolution mapping of the *Diageotropica* (*Dgt*) gene within the distal end of chromosome 1 in tomato (*Lycopersicon esculentum*). A BLAST search of the *Arabidopsis* database with nucleotide sequences of markers that flank the tomato *dgt* locus revealed regions of microsynteny between the distal end of chromosome 1 in tomato, two regions of *Arabidopsis* chromosome 4, and one on chromosome 2. Tomato ESTs homeologous to *Arabidopsis* gene sequences within those regions were converted into co-dominant molecular markers via cleaved amplified polymorphic sequence (CAPS) analysis and scored against an informative backcross mapping population. Six new microsyntenic EST (MEST) markers were rapidly identified in the *dgt* region, two of which further defined the placement of the *Dgt* gene and permitted the selection of a candidate tomato bacterial artificial chromosome clone for sequence analysis. Microsynteny-based comparative mapping combined with CAPS analysis of recombinant plants rapidly and economically narrowed the *dgt* mapping region from 0.8 to 0.15 cM. This approach should contribute to developing high-density maps of molecular markers to target-specific regions for positional cloning and marker-assisted selection in a variety of plants.

2.2 Introduction

High-resolution mapping and chromosome walking, critical steps in the positional cloning of a mutant gene, may become problematic and tedious without high-density molecular markers. Although a number of molecular-marker maps are available for various species, further resolution of the target region is often required, as markers may be irregularly spaced along the chromosome owing to uneven rates of recombination. In addition, focusing the genetic interval reduces the time and resources necessary for chromosome walking. Comparative mapping is based on regions of microsynteny between two organisms and provides a powerful technique for enriching molecular markers in the region surrounding a gene of interest. A number of researchers have suggested that map-based cloning in economically important crop species can be expedited by utilizing chromosomal microsynteny between the target and a model species (Bennetzen and Freeling , 1993; Moore et al., 1995; Schmidt, 2000). The recently completed sequence of the entire genome of *Arabidopsis thaliana* (The *Arabidopsis* Genome Initiative, 2000) now provides a catalog of ordered reference genes immediately applicable to other higher plant species (Mysore et al., 2001). It is well documented that synteny is conserved in closely related species within the same family: e.g. *Arabidopsis* vs. *Brassica oleracea* (Kowalski et al., 1994); rice vs. barley (Kilian et al., 1997); tomato, potato, and pepper (Tanksley et al., 1992; Livingstone et al., 1999). Recent comparative sequence analyses and mapping studies have indicated that microsynteny and macrosynteny are also well conserved between *Arabidopsis* and evolutionarily divergent species such as tomato (Ku et al., 2000;

Rossberg et al., 2001) or soybean (Grant et al., 2000). This indicates that comparative mapping can potentially provide a rapid method to identify additional molecular markers in a region of interest in those species.

The single gene *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum*) demonstrates a pleiotropic phenotype including reduced auxin sensitivity (Kelly and Bradford, 1986). A number of physiological and molecular studies suggest that a *Dgt*-dependent auxin signal transduction pathway regulates a subset of early auxin-response genes (Coenen and Lomax, 1998; Mito and Bennett, 1995; Nebenführ et al., 2000; Rice and Lomax, 2000); however, the nature of the *Dgt* gene is still unknown. We have been using a map-based cloning strategy to isolate the *Dgt* gene, previously mapped to the long arm of chromosome 1 (Tanksley et al., 1992). Based on recent comparative sequence analyses that have demonstrated well-conserved microsynteny between the tomato and *Arabidopsis* genomes within relatively small regions (Ku et al., 2000; Rossberg et al., 2001), we applied microsynteny-based comparative mapping to facilitate the positional cloning of the *Dgt* gene and successfully reduced the genetic interval with new molecular markers.

2.3 Materials and Methods

2.3.1 Mapping Population

A mapping population consisting of 1308 backcross (BC_1) plants derived from a backcross between *L. esculentum* cv. Alisa Craig (*dgt/dgt*) X F_1 [*L.*

esculentum cv. Alisa Craig (*dgt/dgt*) X *L. pennellii* (*Dgt/Dgt*)] were initially screened with RFLP markers localized to the distal end of chromosome 1 (Tanksley et al., 1992). RFLP analyses were performed to identify markers closely linked to the *Dgt* gene (Figure 2.1). Two RFLP markers, TG269 and CT190, were converted to CAPS markers (Konieczny and Ausubel, 1993) and established as flanking markers to *dgt*, approximately 0.8 cM apart. Ten plants were identified as recombinant within this interval and were designated as the informative recombinant population in this study. Ten non-recombinant plants were randomly selected for use as the non-informative population.

2.3.2 Comparative Sequence Analysis

Arabidopsis genome database searches were performed with BLASTN software in The *Arabidopsis* Information Resource (TAIR; http://www.*Arabidopsis*.org/blast). Tomato EST database searches were performed with TBLASTN software in the Tomato Gene Index at The Institute for Genome Research (Davis et al., 1999). The resulting tentative consensus (TC) sequences or high scoring singleton EST sequences were analyzed and used for designing PCR primers. Further sequence analysis was carried out with the Genetics Computer Group 10 (GCG; Madison, WI) program. Alignments of conserved regions within a multigene family were made using PILEUP and adjusted manually to design gene-specific primers. All PCR primers used in this research were designed using Primer3 (Rozen and Skaletsky, 2000) software and are listed in Table 2.1.

Figure 2.1. Three microsyntenic regions identified by BLASTN on *Arabidopsis* chromosomes. Arrows indicate *Arabidopsis* BAC clones with BLASTN matches for the tomato RFLP marker. MSR = microsyntenic region. Solid segments indicate the MSRs defined here. The default values for TAIR BLAST and the Blosum 62 scoring matrix were used for the BLASTN parameter options. Hatched segments on *Arabidopsis* chromosomes (AtCHR) 2 and 4 represent duplicated chromosomal segments reported previously (Blanc et al., 2000).

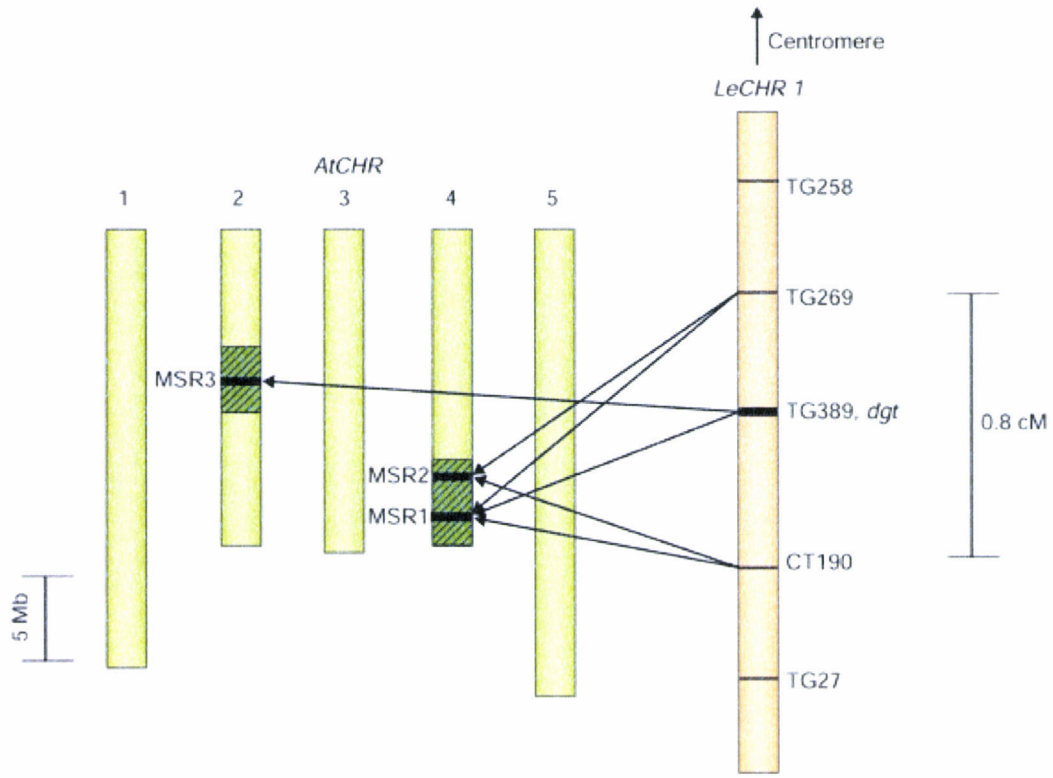
Centromere
LeCHR 1
AtCHR
1
2
3
4
5
TG258
TG269
TG389, dgt
CT190
TG27
MSR3
MSR2
MSR1
0.8 cM
5 Mb

Figure 2.1

Table2.1. CAPS markers converted from RFLP markers and microsyntenic ESTs[a]

Arabidopsis gene[b]	C/P/H[c]	Molecular markers mapped in the *dgt* region[d]	Type	Expect value[e]	Primer Sequences[f]	Restriction enzyme
AT4g38580	-	CT190[g]	RFLP	-	5′-TTCTCGTCGCTAAAGGCAGT-3′ 5′-TCACACAAAACAATGGGTGTTCTT-3′	*Hin*fI
AT4g38620	2 / 2 / 40					
AT4g38630	1 / 1 / 1					
AT4g38660	1 / 2 / 16					
AT4g38670	1 / 2 / 15					
AT4g38680	1 / 1 / 1	TC98260 (GRP)	EST	$1.7e^{-40}$	5′-GTGCCTCACAATCAAAGGGTTTTA-3′ 5′-CTCCATAACCACGATTTCCTCCTC-3′	*Rsa*I
AT4g38690	4 / 4 / 5	TC92082 (PPLC)	EST	$1.4e^{-84}$	5′-TGGTTGAGCTGATTTTCTTGGTTT-3′ 5′-CCTGGTTCTGATTATCGCTCAGAT-3′	*Hin*fI
AT4g38700	1 / 2 / 5	AW624844 (DRRP)	EST	$1.2e^{-28}$	5′-AAACGTCATGGGCTAAGAGAGTTG-3′ 5′-TCTAGATGCAATGGCTTGTTTTCC-3′	*Apo*I
AT4g38730	-	TG389	RFLP	-	5′-TCACTAGCTCAAGGGAGTCATCTG-3′ 5′-ACCACTTTGACCATCATCGCAAGC-3′	*Hin*fI
AT4g38740	1 / 3 / 10	TC89380 (PPI)	EST	$5.2e^{-76}$	5′-CAAATCCAAAGGTTTTCTTTGACC-3′ 5′-CTGGTAGAAGCAACACAACAACCA-3′	*Hae*III
AT4g38790	1 / 2 / 4					

Table 2.1. (continued)

AT4g38810	1 / 2 / 2	TC85079 (EFP)	EST	$4.0e^{-129}$	5′-CGAAACTGGCTTCCCTTCTA-3′ 5′-AGTCAGGTGATGGACGGTTC-3′	*Ban*I
AT4g38830	10 / 12 /146					
AT4g38840	1 / 1 / 6					
AT4g38850	-	TG269	RFLP	-	5′-CAAATTCTTCCTCAGCTTGACT-3′ 5′-TGATCTCACATCTTGCTTGCG-3′	*Dde*I
AT4g38880	1 / 1 / 1	TC87150 (APRT)	EST	$3.8e^{-90}$	5′-CAGAAAAATGACTTGGAGGGAGAG-3′ 5′-CCAAGATTGTGAGGCTGTTAAAGG-3′	*Rsa*I
AT4g38900	1 / 1 / 3	TC47447 (bZIP)	EST	$1.3e^{-98}$	5′-AACTTGGAAGCGTCTGCACT-3′ 5′-GGACGACCTGTTTTCTGCAT-3′	*Rsa*I
AT4g34830	1 / 1 / 1	BG643476	EST	$5.3e^{-86}$	5′-GGTTGATGGACTGCATAAAAATCC –3′ 5′-TGCAAATTCCCAATTTACCATTTT -3′	*Hha*I
AT4g35050	2 / 3 / 4					

[a]The conversion rate of the amplicons, generated from ESTs in the target region, to CAPS was 75% (30/40) and 20% of CAPS markers (6/30) were successfully mapped in the *dgt* region. [b]Genes in MSR1 with exceptions of AT4g34830 and AT4g35050 in MSR2. [c]C/P/H, the number of CAPS markers developed/the number of PCR products investigated/the number of TBLASTN matches of interest ($< e^{-20}$). [d]Abbreviations in parentheses: GRP, glycine-rich protein; PPLC, phosphatidylinositol-specific phospholipase C precursor; DRRP, disease-resistance response protein; PPI, cyclophilin-type peptidyl-prolyl isomerase; EFP, EF-hand containing protein-like protein; APRT, amidophosphoribosyl transferase; bZIP; basic leucine zipper transcription factor. [e]Each value represents the *E*-value of a TBLASTN search using the *Arabidopsis* microsyntenic gene against the Tomato Gene Index using the Blosum 62 scoring matrix and default parameters. The *E*-value of a BLASTN search using tomato RFLP markers is not described in this table (see text). [#]Oligonucleotide sequences are only indicated for each MEST marker. [f] Amplified by modified PCR conditions: 3 min 30 sec for elongation and 2.5 mM $MgCl_2$.

2.3.3 Cleaved Amplified Polymorphic Sequence (CAPS) Analysis of Molecular Markers

Tomato genomic DNA was extracted from leaf tissue as described by Dellaporta et al. (1983). RFLP markers and ESTs were converted to co-dominant PCR-based molecular markers (CAPS) as described by Konieczny and Ausubel (1993). Amplification reactions consisted of a 25 μl reaction containing 100 ng genomic DNA or 20 ng BAC DNA, 200 μM dNTPs, 200 nM each forward and reverse primer, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, and 1 U of Maxi Taq polymerase (Pluthero, 1993). Standard temperature parameters for amplifying ESTs from genomic DNA in this study were as follows: initial denaturation at 94°C for 3 min; 40 cycles of 94°C for 45 sec, 58°C for 1 min, and 72°C for 3 min; final elongation at 72°C for 5 min. All PCR experiments were performed in a RoboCycler 96 Gradient with Hot Top Assembly (Stratagene, La Jolla, CA). Following PCR, products were digested with the indicated restriction enzyme (Table 2.1) to yield co-dominant markers. The digested PCR products were resolved in either 2% or 2.5% agarose electrophoretic gels in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized by ethidium bromide staining.

2.3.4 BAC Library Screening and Analysis

TG389, an RFLP marker tightly linked to dgt and situated between markers TG269 and CT190, was used to screen a tomato (*L. esculentum* cv. Heinz

1706) bacterial artificial chromosome (BAC) library (Budiman et al., 2000) as described by the Clemson University Genome Institute (http://www.genome.clemson.edu). Two tomato BAC clones, 52M1 and 93O2, isolated by hybridization to the TG389 probe were used in this study. BAC DNA was isolated by alkaline lysis (Sambrook et al., 1989) modified for a 200 mL LB broth culture volume containing 17 mg/L chloramphenicol. BAC DNA samples were digested with NotI to liberate tomato genomic insert DNA then digested with HindIII and separated by electrophoresis in 1% agarose gels. Fractionated DNAs were transferred to Hybond-XL membrane (Amersham Pharmacia Biotech, Piscataway, NJ) as described by Sambrook et al. (1989). Membranes were prehybridized for 1 to 2 h in Church buffer (Church and Gilbert, 1984) prior to adding denatured probe in fresh Church buffer. Labeled probe was synthesized using a High Prime random priming kit, according to the manufacturer's instructions (Roche Molecular Biochemicals,Indianapolis, IN), from 100 ng of genomic PCR product and 50 μCi [^{32}P]-α-dCTP. After a 30 min incubation at 37°C, labeled probes were purified on Sephadex G50 spin columns. Membranes were hybridized for 16 h at 65°C and briefly rinsed in low stringency buffer containing 40 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA (pH 8.0), and 5% SDS at room temperature, followed by washing in low stringency buffer at 65°C for 30 min. Membranes were washed twice at 65°C for 30 min in high stringency buffer containing 40 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA (pH 8.0), and 1% SDS, before exposure to autoradiographic film.

2.3.5 Direct BAC End Sequencing

For sequencing, BAC DNA was isolated from a 100 ml LB culture containing 17 mg/l chloramphenicol using a QIAGEN Plasmid Midi Kit (Valencia, CA), following the manufacturer's instruction. The DNA sample was then subjected to digestion of co-eluted residual genomic DNA with Plasmid-Safe™ ATP-dependent DNase (Epicentre, Madison, WI). BAC insert ends were sequenced on an ABI377 automated sequencer (Applied Biosystems, Foster City, CA) using the ABI PRISM® BigDye™ terminator cycle sequencing kit with T7 and SP6 sequencing primers. Sequencing was performed by the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University.

2.4 Results

2.4.1 Identification of Microsyntenic Regions in *Arabidopsis* Chromosomes

Using RFLP and RFLP-derived CAPS markers (Figure 2.1) based on previously published tomato genetic and RFLP maps (Tanksley et al., 1992), initial studies mapped the *dgt* locus to a region of ca. 0.8 cM near the bottom of the long arm of tomato chromosome 1. Of 1308 backcross (BC_1) individuals screened, 10 plants were determined to be recombinant between markers TG269 and CT190, whereas no plants were found to be recombinant between TG389 and *dgt* (Figure 2.1). In order to identify additional genes within that region by finding

Figure 2.2. Three MSRs have conserved content and order of genes. Each MSR is aligned by the orientation of tomato RFLP markers. The *Arabidopsis* homeologs of tomato RFLP markers closely linked to the *Dgt* gene are indicated by arrows. Syntenic genes are represented by the same symbol and abbreviated name. EFP (AT4g38810) and PP (AT4g34830) are unique genes in MSR1 and MSR2, respectively, that were used for genotyping (see text). Abbreviations: APRT, amidophosphoribosyl transferase; bZIP, basic leucine zipper transcription factor; COSAP, cluster of SAUR and auxin-induced proteins; DRRP, disease-resistance response protein; EFP, EF-hand containing protein-like protein; ERLP, endoplasmic reticulum lumen protein-retaining receptor; ExLP, extensin-like protein; FP, farnesylated protein; GRP, glycine-rich protein; HP, hypothetical protein; Myb, Myb transcription factor; pEARLI4, phospholipase-like protein; PPLC, phosphatidylinositol-specific phospholipase C precursor; PP, putative protein; PPI, cyclophilin-type petidyl-prolyl isomerase; β-gal, β-galactosidase-like protein. Bars to the right of the MSRs represent the corresponding annotated *Arabidopsis* BAC clones.

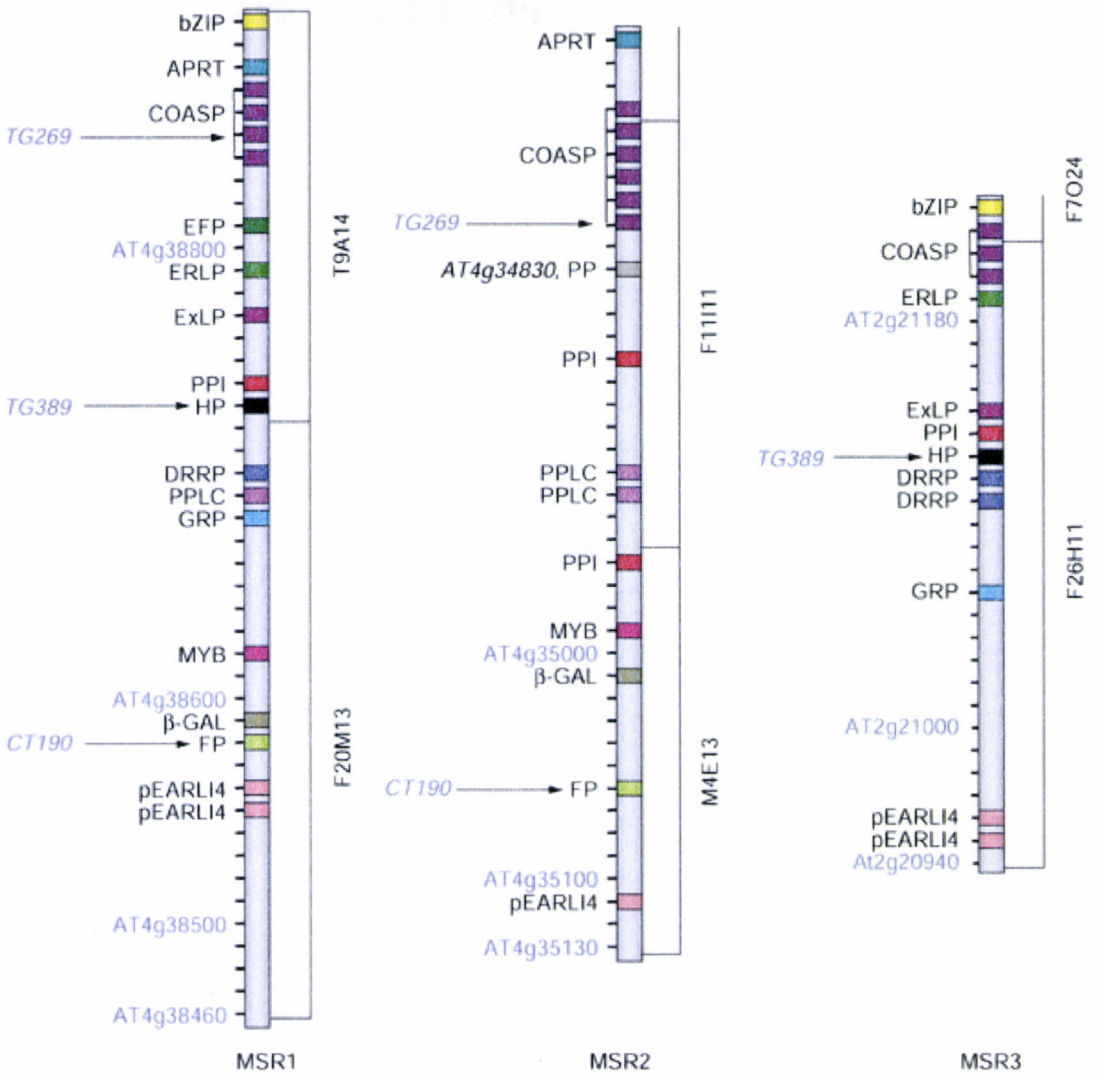
bZIP
APRT
COASP
TG269
EFP
AT4g38800
ERLP
ExLP
PPI
TG389
HP
DRRP
PPLC
GRP
MYB
AT4g38600
β-GAL
CT190
FP
pEARLI4
pEARLI4
AT4g38500
AT4g38460
T9A14
F20M13
MSR1
APRT
COASP
TG269
AT4g34830, PP
PPI
PPLC
PPLC
PPI
MYB
AT4g35000
β-GAL
CT190
FP
AT4g35100
pEARLI4
AT4g35130
F11I11
M4E13
MSR2
bZIP
COASP
ERLP
AT2g21180
ExLP
PPI
TG389
HP
DRRP
DRRP
GRP
AT2g21000
pEARLI4
pEARLI4
At2g20940
F7O24
F26H11
MSR3

Figure 2.2

microsyntenous regions in *Arabidopsis*, nucleotide sequences of the three RFLP markers most closely linked to the *dgt* locus (TG269, TG389, and CT190) were used to identify homeologous sequences in the *Arabidopsis* genome database (http://www.*Arabidopsis*.org). BLASTN matches with an arbitrary threshold expect value (E-value) of less than 0.01 were investigated as significant matches. Three putative microsyntenic regions (MSRs) were identified in the *Arabidopsis* genome (Figures 2.1 and 2.2). In MSR1, homeologs of all three tomato RFLP markers were found in the same linear order on two adjacent *Arabidopsis* BAC clones from chromosome 4 (*Arabidopsis* accession numbers are in parentheses): F20M13 (AL035540) and T9A14 (AL035656). The *Arabidopsis* homeologs of TG269, TG389, and CT190 in MSR1 had corresponding E-values of $4e^{-3}$, $2e^{-7}$, and $3e^{-5}$, respectively. The *Arabidopsis* homeologs of markers TG269 (AT4g38850), TG389 (AT4g38730), and CT190 (AT4g38580) encode a small auxin up-regulated protein (SAUR), a hypothetical protein of unknown function (HP), and a farnesylated protein (FP), respectively. The second microsyntenic region (MSR2) spanned two *Arabidopsis* BAC clones, F11I11 (AL079347) and M4E13 (AL022023), and was also located on chromosome 4, but only yielded homeologs for tomato markers TG269 and CT190 (E-values of $1e^{-3}$ and $8e^{-3}$, respectively). The third microsyntenic region (MSR3) was located on *Arabidopsis* chromosome 2 and spanned *Arabidopsis* BACs F26H11 (AC006263) and F7O24 (AC007142). This region only contained a homeolog to TG389 (AT2g21120, E-value of $9e^{-4}$). However, additional auxin-regulated genes were identified in this region (AT2g21200 to AT2g21220), which made continued analysis potentially beneficial. Additionally, this region had previously been reported to be syntenic to MSR1

(Blanc et al., 2000). Therefore, we also included MSR3 in the comparative analysis of genes in MSRs between tomato and *Arabidopsis*.

Close inspection of the microsyntenic regions detected by BLASTN analysis suggested that the genomic microstructure was highly conserved in all three MSRs. Eighteen of 45 genes (40%) identified in MSR1 also have homologs in MSR2 and/or MSR3 (Figure 2.2). The order of the microsyntenic genes was highly conserved in the three regions with the colinear pattern of three genes, phospholipase-like protein (pEARLI 4), cyclophilin-type petidyl-prolyl cis/trans isomerase (PPI), and a cluster of SAUR and auxin-induced proteins (COSAP), serving as a common footprint for these MSRs. At the same time, variation in syntenic genes by gene duplication and/or translocation was also evident in all three MSRs. For example, the PPI (AT4g38740), phosphatidylinositol-specific phospholipase C precursor (PPLC, AT4g38690) and disease-resistance response protein (DRRP, AT4g38700) sequences of MSR1 are duplicated only in either MSR2 or MSR3, respectively. PPLCs (AT4g34920/AT4g34930) and DRRPs (AT2g21110/AT2g21120) remain tandemly duplicated in MSR2 and MSR3, respectively, whereas PPI (AT4g34960) appears to have been translocated after gene duplication in MSR2.

2.4.2 High-Resolution Mapping of the *dgt* Locus by MEST Markers

Because *Arabidopsis* MSR1 contained homeologs in common with three RFLP markers in the *dgt* region of tomato, we used it as our main target region to search for corresponding tomato homeologs. MSR1 contained 25 intervening

genes between the homeologs of TG269 and CT190. TBLASTN analysis of each intervening *Arabidopsis* gene against the Tomato Gene Index (http://www.tigr.org/tdb/lgi) generated 260 TBLASTN hits that were chosen for further investigation based on the original annotation of the *Arabidopsis* gene and a low expect value ($<e^{-20}$). generated 260 TBLASTN hits that were chosen for further investigation on the basis of the original annotation of the *Arabidopsis* gene and a low expect value ($< e^{-20}$). From those, 40 robust PCR products were obtained and selected for development into new CAPS markers. The original 40 products provided 30 new CAPS markers (Table 2.1) that were used for PCR-based genotyping against the informative mapping population (Figure 2.3). The 10 previously identified informative recombinant plants, as well as an equal number of non-informative plants, were used to analyze the new microsyntenic EST (MEST) markers. Of the 30 CAPS markers tested, six MEST markers mapped in the *dgt* region. Two MESTs, BG643476 (putative protein, PP) and TC85079 (EF-hand containing protein-like protein, EFP), identified a crossover between TG269 and TG389/*dgt*, and one MEST, TC98260 (glycine-rich protein, GRP), revealed a crossover between TG389/*dgt* and CT190. These three new markers, which flank *dgt*, narrowed the target region from 0.8 cM to 0.15 cM in our small mapping population. Three MEST markers, TC89380 (PPI), TC92082 (PPLC), and AW624844 (DRRP), co-segregated with TG389 and *dgt*. Most of the MEST markers derived from MSR1 have paralogs in other MSRs, but BG643476 (PP, AT4g34830) and TC85079 (EFP, AT4g38810) are unique to MSR1 and MSR2, respectively (Figure 2.2).

We screened a BAC library with TG389 and obtained two BAC clones, 52M1

Figure 2.3. MEST markers substantially narrow the *dgt* mapping region. Each bar shows the putative breakpoint of recombination in an informative BC_1 individual. Closed and open bars represent the chromosomal fraction of *L. pennellii* (wild-type phenotype) and *L. esculentum* (mutant phenotype), respectively. Mutant (M) or wild-type (W) phenotype is indicated for each individual plant and identifying number. Based on the CAPS analysis, three MEST markers, BG643476, TC85079, and TC98260, were identified as new intervening markers between TG389 and the flanking RFLP markers, TG269 and CT190, with new recombination events identified in BC_1 individuals M710 and W505, respectively. TC89380, AW624844, and TC92082 cosegregated with TG389 and, thus, were tightly linked to the *Dgt* gene. Genetic distances between molecular markers are indicated at the top.

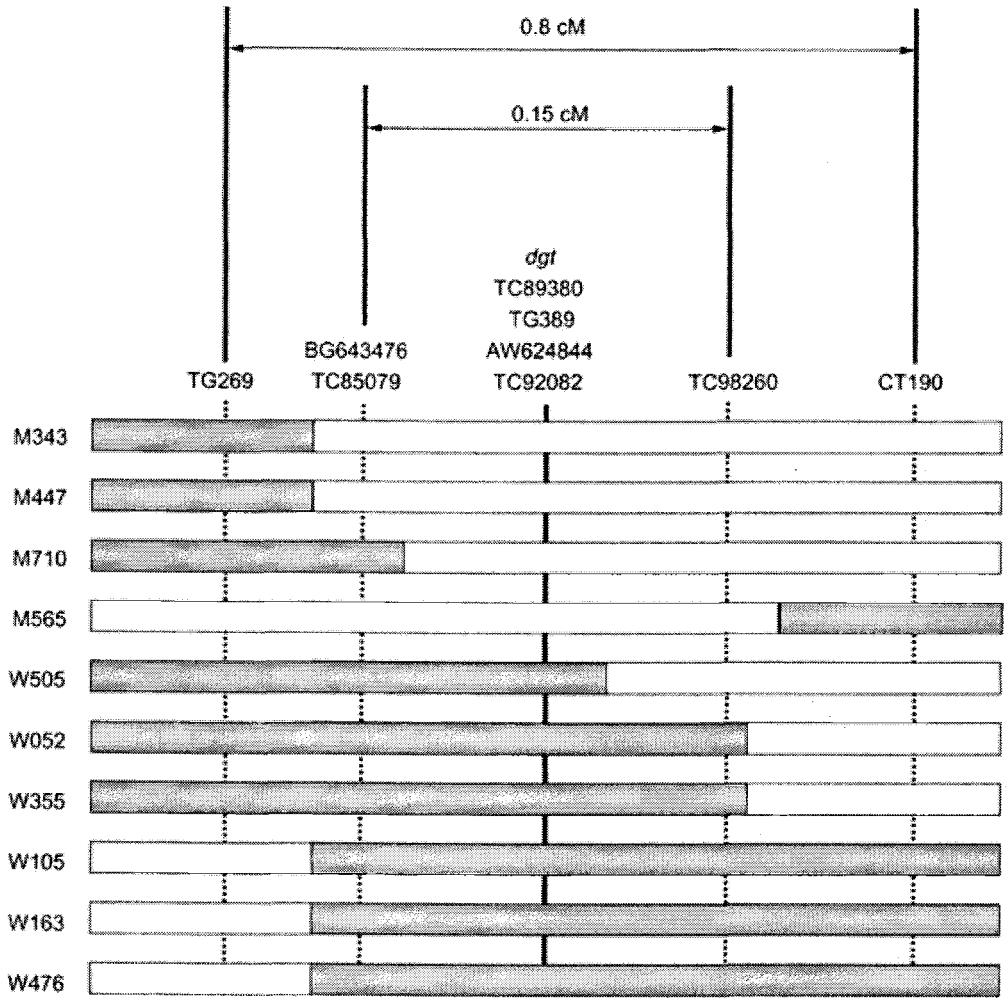
0.8 cM
0.15 cM
dgt
TC89380
TG389
AW624844
BG643476
TC85079
TC92082
TG269
TC98260
CT190
M343
M447
M710
M565
W505
W052
W355
W105
W163
W476

Figure 2.3

and 9302. Each tomato BAC clone was estimated to be approximately 120 kb in length by pulsed-field gel electrophoresis (data not shown), but neither contained marker TG269 or CT190 sequences (data not shown) to allow confirmation that the *dgt* locus was located on either BAC clone. When both BAC clones were probed with the newly identified MEST molecular markers, TC98260 (the intervening marker between TG389 and CT190), was detected only on BAC 52M1, whereas BG643476 and TC85079 (the intervening markers between TG269 and TG389), were present only on BAC 93O2 (Figure 2.4). The three MEST markers that co-segregated with TG389 were detected on both BAC clones. These results permitted partial ordering of the newly identified MEST markers and demonstrated that the *dgt* locus was present within the two BAC clones.

Subsequent BAC end-sequencing and BLAST searches of the Tomato Gene Index identified BAC 9302 and 52M1 insert DNA ends nearest the telomere as containing genomic nucleotide sequence for two previously identified MESTs - TC92082 and TC98260, respectively. This information placed TC92082 between TC98260 and the MEST markers that co-segregated with TG389 (Figures 2.3 and 2.5). The BAC end-sequences nearest to the centromere for BACs 9302 and 52M1 were identified as BG643476 (PP) and TC87127 (chalcone synthase-like protein, CHSP), respectively. BG643476 was confirmed as a MEST when the sequence was later posted to the tomato EST database. The BAC end-location placed BG643476 between markers TG269 and TC85079. Taken together, these results strongly suggest that TC85079 and TC98260 are the closest flanking molecular markers to *dgt* present in the two overlapping BAC clones containing TG389. BLAST searches of the BAC end-sequences against the *Arabidopsis* Information Resource (TAIR)

Figure 2.4. Southern hybridization of TG389-containing BAC clones identifies MEST gene order. DNA samples of BAC clones were digested with *Hin*dIII. The separated fragments were blotted and probed with MEST markers indicated at the top of each panel. The left and right lanes of each panel are 93O2 (1) and 52M1 (2), respectively.

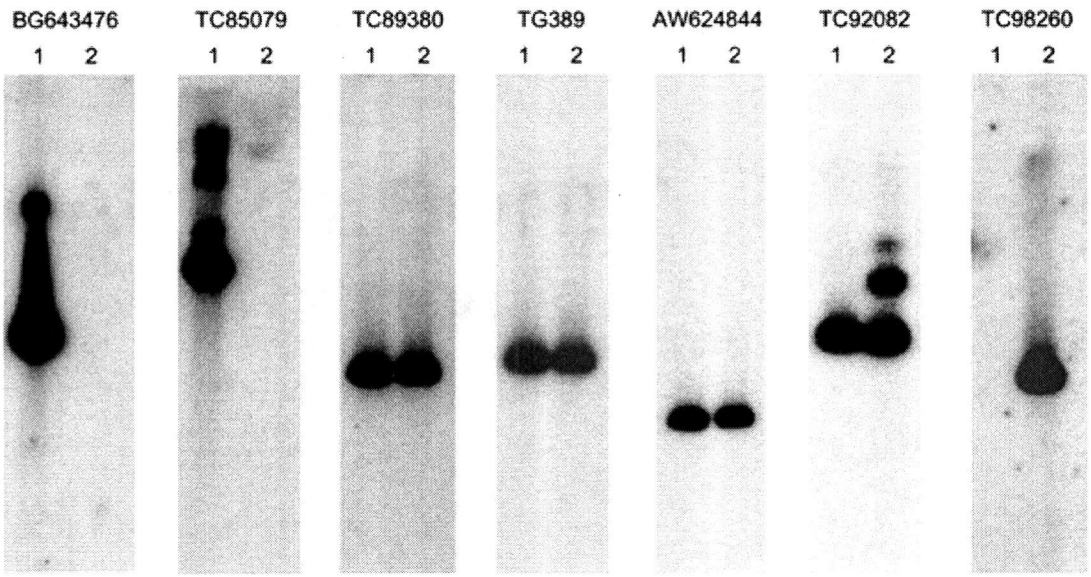
BG643476
1 2
TC85079
1 2
TC89380
1 2
TG389
1 2
AW624844
1 2
TC92082
1 2
TC98260
1 2

Figure 2.4

database further confirmed microsynteny between the dgt region of tomato chromosome 1 and the three MSRs in *Arabidopsis* (Figure 2.5).

As would be expected if microsynteny were maintained, two additional markers, TC87150 (amidophosphoribosyi transferase, APRT) and TC47447 (basic leucine zipper transcription factor, bZIP), were determined to be non-informative MEST markers with respect to our positional cloning strategy because they fell outside TG269. However, their sequences maintained microsyntenic alignment and co-segregated with tomato RFLP marker TG258 (data not shown), which is proximal to TG269 on chromosome 1 (see Figure 2.1). Given no rearrangement of tomato chromosome 1 and that microsynteny between tomato and *Arabidopsis* genomes remains firm, the predicted order of the new MEST and BAC end-generated markers in tomato would be as follows: TC47447 → TC87150 → TG269 → BG643476 → TC85079 → TC87127 → TC89380 → TG389 → AW624844 → TC92082 → TC98260 → CT190 (centromere to telomere, Figure 2.5). The exact order of these markers and the position of dgt will be determined by complete sequencing of tomato BACs 93O2 and 52M1.

2.5 Discussion

With the sequencing of the entire *Arabidopsis* genome, comparative mapping and homeology-based gene cloning is now available in other species via microsyntenic alignment of molecular markers and genes against the sequenced reference genome (Bancroft, 2001). Although exceptions to the blanket application of this approach have been noted (Bennetzen, 2000), our study

successfully applied microsynteny analysis between the tomato and *Arabidopsis* genomes to facilitate positional cloning of the *Dgt* gene in tomato. Using three sequential tomato RFLP markers from our target region, we searched the *Arabidopsis* genome database and found three MSRs in the *Arabidopsis* genome that enabled us to construct a detailed molecular map of the target area. Comparison of the three MSRs from the *Arabidopsis* genome shows that the content and order of the genes are well conserved within these regions. MSR1 and MSR3 exhibit reverse polarity compared to orientation of tomato RFLP markers, whereas the chromosomal segment MSR2 has the same polarity as tomato. Using information from the three *Arabidopsis* MSRs, we identified eight new molecular markers on tomato chromosome 1, two of which narrowed the genetic distance to the *dgt* locus from 0.8 cM to less than 0.2 cM and provided the necessary data to confirm the location of the dgt locus on two overlapping BAC clones, thus avoiding the need for a chromosomal walk.

Our strategy for microsynteny-based comparative mapping was straightforward and simple. First, the BLASTN program at TAIR was used to identify microsyntenic regions in *Arabidopsis* containing homeologs of tester markers (tomato RFLPs) in similar order within a relatively small physical interval (1-2 cM). Several published reports supported our strategy of using simple computer-based comparisons between tester and reference nucleotide sequences. Two recent comparative sequence analyses clearly presented microsynteny of a 105 kb BAC DNA insert (Ku et al., 2000) and a 57 kb cosmid DNA insert (Rossberg et al., 2001) from tomato chromosomes 2 and 7, respectively, to their homeologous counterparts in the *Arabidopsis* genome. Paterson et al. (1996)

Figure 2.5. The *dgt* locus is contained within two overlapping BAC clones. The comparative map shows the position of each MEST in tomato chromosome 1 (LeCHR I) based on microsynteny. The orientation of LeCHR I and MSR2 is the same but that of MSR1 and MSR3 is reversed (note arrowheads on each chromosome). The arrows from LeCHR I to the *Arabidopsis* MSRs represent the BLASTN matches of tomato RFLP markers and BAC insert ends, whereas the arrows from MSR1 to LeCHR I indicate MESTs confirmed by CAPS analysis. See the legends for Table 2.1 and Figure 2.2 for the abbreviation for each MEST. The order of MESTs is predicted by the order of *Arabidopsis* genes in the MSRs, DNA blot analysis, and sequence analysis (Figure 2.4). Based on this comparative map, the *Dgt* gene would be located between TC85079 and TC98260 on BAC 93O2 and/or 52M1. Note that the scale bar represents physical length for the *Arabidopsis* chromosome.

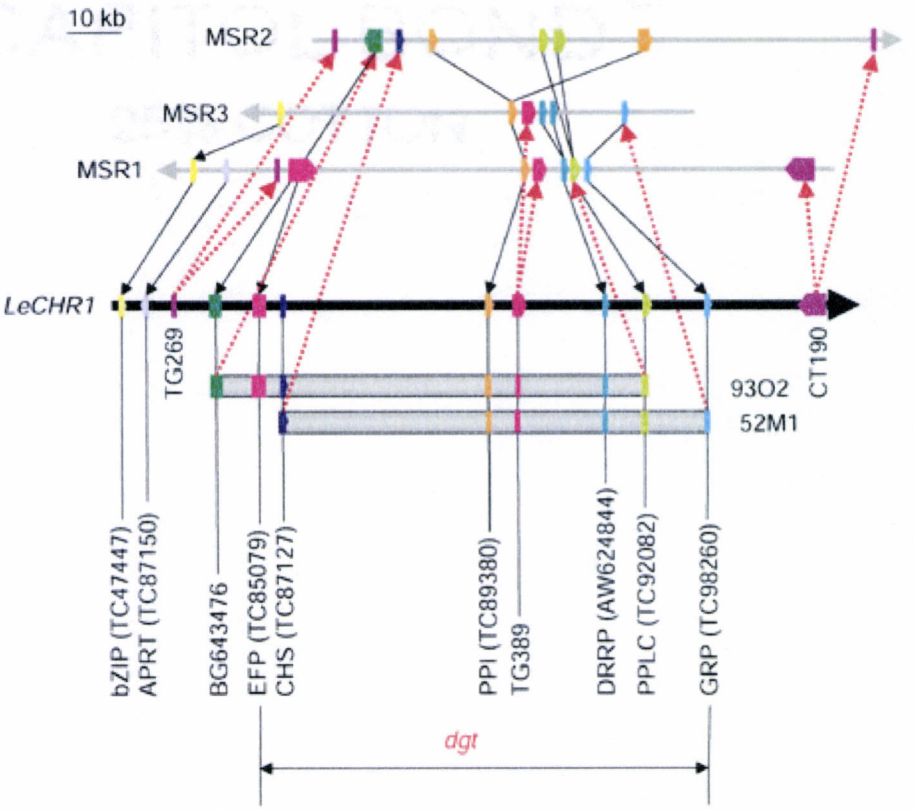
10 kb
MSR2
MSR3
MSR1
LeCHR1
TG269
93O2
52M1
CT190
bZIP (TC47447)
APRT (TC87150)
BG643476
EFP (TC85079)
CHS (TC87127)
PPI (TC89380)
TG389
DRRP (AW624844)
PPLC (TC92082)
GRP (TC98260)
dgt

Figure 2.5

suggested that 43% to 58% of chromosomal segments of less than 3 cM remain colinear in distantly related species. Direct nucleotide sequence comparisons (BLASTN) were used for each search as this provides a more stringent test of homology between tomato and *Arabidopsis* sequences than do the conceptual translations of DNA sequences (TBLASTX) that can be used for less stringent comparisons between evolutionarily divergent species (Grant et al., 2000). In our study, BLASTN searches using default parameters enabled us to find a high level of microsynteny without encountering any major differences between the highest-scoring matches in either TBLASTX or BLASTN searches (data not shown). However, the success of EST-derived CAPS markers depends on a well-established EST database. The Tomato Gene Index of The Institute for Genomic Research (TIGR) is the third largest sequence database for plants and provided 131,988 EST sequences at the time of this study (Quackenbush et al., 2001). The tentative consensus (TC) sequences of assembled ESTs can be used for integration of complex mapping data and identification of orthologous genes between divergent species (Quackenbush et al., 2001). Expressed gene sequences were used in this study as they can be converted to molecular markers more consistently than non-coding regions when comparing distantly related species (Grant et al., 2000). Moreover, two recent comparative sequence analyses using monocots versus *Arabidopsis* (Dubcovsky et al., 2001) and dicots versus *Arabidopsis* (Rossberg et al., 2001) clearly showed that exon sizes are well conserved in homeologs even between monocots and dicots, whereas intron length varied in rice versus barley and tomato versus *Arabidopsis*. In BLAST searches using either the processed nucleotide sequence or the predicted protein sequence of an *Arabidopsis* gene, the

score of each BLAST match was clearly higher and the E-value was significantly lower when compared to the use of either non-processed nucleotide sequence or intergenic nucleotide sequence. Presumably, use of the predicted protein sequence of an *Arabidopsis* gene increases the probability of identifying the microsyntenic EST in comparative mapping of distantly related species, considering the average substitution rate, 6 × 10-9/nucleotide site/year, of nucleotides in plants (Wolfe et al., 1987) and the separation, 112 million years ago, of tomato and *Arabidopsis* (Ku et al., 2000).

The MEST-derived comparative map indicating that three regions of the *Arabidopsis* genome are related to each other (Figures 2.2 and 2.5) supports the hypothesis that at least two rounds of duplication occurred in the *Arabidopsis* genome followed by selective deletion of genes and/or minor rearrangements (Ku et al., 2000). Minor rearrangements of microsyntenic genes could potentially impede high-resolution mapping if placement of the syntenic markers within a relatively large segment indicated a missing sequence fragment, as reported for the comparative mapping of maize and sorghum (Tarchini et al., 2000). Given that several genes exist between the homeologs of the two closest flanking MESTs, TC85079 (EFP) and TC98260 (GRP), and neither a deletion nor local rearrangement of the Dgt counterpart has occurred in the MSRs, the Dgt counterpart could be one of the annotated genes between the homeologs of the two closest flanking MESTs. It remains to be seen, however, whether additional tomato genes are present in the region.

Our results indicate that comparative microsynteny-based mapping can facilitate positional cloning of a target gene when information on genomic location

is limited. Ku et al. (2001) recently utilized microsynteny-based comparative mapping to add new molecular markers in a 0.067 cM region defined by a previously determined 100 kb BAC clone containing the ovate locus in tomato. Here, microsynteny-based comparative mapping was used to define the position of the Dgt gene within a much larger region (0.8 cM) of the tomato genome by contributing six intervening MEST markers, initially derived from *Arabidopsis* gene sequences, between TG269 and CT190. The approach proved to be less technically complicated than other fingerprinting methods and points to several co-segregating genes for further investigation as potential Dgt candidates. We anticipate that this general approach will contribute significantly to the development of dense molecular marker maps for a variety of higher plant species to expedite map-based cloning.

2.6 Acknowledgements

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3. THE *DIAGEOTROPICA* GENE OF TOMATO ENCODES A CYCLOPHILIN, A NOVEL PLAYER IN AUXIN SIGNALING

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3.1 Abstract

The single gene, auxin-resistant *diageotropica* (*dgt*) mutant of tomato displays a pleiotropic phenotype that includes a slow gravitropic response, lack of lateral roots, reduced apical dominance, altered vascular development, and reduced fruit growth. We hypothesize that the *Dgt* gene encodes a component in an auxin signaling pathway since *dgt* affects only a subset of auxin responses and levels, metabolism, and transport of auxin appear normal. By combining map-based cloning with comparative microsynteny, we determined that the *Dgt* gene encodes a cyclophilin (LeCYP1), a previously unidentified component of auxin signaling. Each known *dgt* allele contains a unique mutation in the coding sequence of *LeCyp1*. Single nucleotide point mutations generate an amino acid change ($G_{137}R$) and stop codon (W_{128}stop) in $dgt^{1\text{-}1}$ and $dgt^{1\text{-}2}$, respectively, and dgt^{dp} has an amino acid change ($W_{128}C\Delta 129\text{-}133$) preceding a 15 bp deletion. Complementation of *dgt* mutant plants with *LeCyp1* restored the wild-type phenotype. Each allelic mutation reduced or nullified PPIase activity of glutathione-S-transferase (GST)–LeCYP1 fusion proteins *in vitro*. RT-PCR and immunoblot analyses indicate that the *dgt* mutations do not affect the expression of *LeCyp1* mRNA but the accumulation of LeCYP1 protein is greatly reduced for all three alleles. The cyclophilin inhibitor, cyclosporin A, inhibits auxin-induced adventitious root initiation in tomato hypocotyl sections and reduces the auxin-induced expression of two early auxin response genes, *LeIAA10* and *11*, that are also affected by the *dgt* mutation. This provides further support that the tomato cyclophilin encoded by the *Dgt* gene plays a important role in auxin signal

transduction.

3.2 Introduction

Auxins play an important role in many aspects of plant growth and development including cell division, elongation, and differentiation as well as apical dominance and tropic responses. Analyses of various auxin-insensitive mutants have deciphered the mechanism of auxin action for regulating various cellular and developmental responses. Polar auxin transport contributes to changes in the developmental plasticity of the primary axis of plants (for review, Friml, 2003). Auxin influx (AUX1) and efflux carrier (PINs) proteins have been implicated in the primary mechanism of auxin action and are believed to mediate directional cell-to-cell movement of auxin by their asymmetric localization (Swarup et al., 2001). Understanding the regulation of downstream auxin-mediated gene expression is also integral to elucidating the mechanism of auxin action. A current model of the signaling mechanism for auxin-induced gene expression involves two protein families, auxin-responsive element-binding auxin response factors (ARF) and AUX/IAA proteins (for review, Reed 2001; Liscum and Reed, 2002). In this model, an AUX/IAA protein heterodimerizes with an activator-class ARF. At basal levels of endogenous auxin, the ARF:AUX/IAA complex acts to repress early auxin-induced gene expression at basal levels of endogenous auxin (Tiwari et al., 2003). Once auxin levels increase in the cell, the AUX/IAA protein dissociates from the ARF:AUX/IAA heterodimer. The remaining ARF then activates transcription of early auxin-response genes either by itself or

by dimerizing with another ARF-transcriptional activator. In this model, the ubiquitin-dependent degradation of AUX/IAA is crucial to the regulation of cellular AUX/IAA protein levels. Although knowledge about the molecular mechanism of auxin action has expanded through analyses of many auxin-insensitive mutants, the identity of additional participants and their relationship to each other in auxin signaling networks remains to be elucidated.

The single gene, auxin-resistant *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum*) demonstrates a pleiotropic phenotype that includes a slow gravitropic response, lack of lateral roots, reduced apical dominance, altered vascular development, and reduced fruit growth (Zobel, 1973; Zobel 1974, Coenen and Lomax 1998; Rice and Lomax, 2000; Balbi and Lomax, 2003). While displaying reduced auxin sensitivity with respect to elongation growth and ethylene production (Kelly and Bradford, 1986), the *dgt* mutant does not exhibit altered levels (Fujino et al., 1988) or transport of auxin (Rice and Lomax, 2000). Interestingly, the *dgt* mutation appears to affect the expression of only a subset of auxin-regulated genes in a tissue- and developmental stage-specific manner (Balbi and Lomax, 2003; Mito and Bennett, 1995; Nebenführ et al., 2000). The nature of the *Dgt* gene has not been revealed in spite of its potential significance in the molecular mechanism of auxin action. We hypothesize that the *Dgt* gene encodes an integral component in an auxin signal transduction pathway. We have recently mapped the *Dgt* gene to a 0.15 cM interval by microsyteny-based comparative mapping in conjunction with conventional map-based cloning (Oh et al., 2002). Here, we report that the *Dgt* gene encodes a cyclophilin, LeCYP1, a novel component involved in auxin signaling.

3.3 Materials and Methods

3.3.1 Plant Material

Three *dgt* alleles of tomato (*Lycopersicon esculentum* Mill.), as well as their corresponding wild-type varieties, VFN8, VF36, and Chatham, were used in this study. The three *dgt* alleles were each generated by a different mechanism (dgt^{1-1}, spontaneous; dgt^{1-2}, EMS; and dgt^{dp}; x-ray, Jones and Jones, 1996). Seeds of each genotype were surface sterilized for 20 min in 20% Chlorox, sown onto moist Whatman 3MM paper, and germinated in constant darkness at 28°C. All chemicals were, unless otherwise stated, from Sigma.

3.3.2 Identification of the *Dgt* Gene

High-resolution mapping and bacterial artificial chromosome (BAC) clones in the *dgt* region were previously described (Oh et al., 2002). BAC 52M1 was chosen for complete sequencing via the shotgun-sequencing method (University of Washington Genome Center) and assembled with the PHRED/PHRAP software package (http://www.phrap.org/). The final sequence of BAC 52M1 was analyzed to identify putative genes, as described by Van der Hoeven et al. (2002), using the *Arabidopsis* genome database (http://www.*Arabidopsis*.org) and the tomato gene index (TGI, http://www.tigr.org/tdb/tgi/lgi/). Individual predicted genes with at least 95% identity to ESTs in the tomato gene index were subject to cleaved amplified polymorphic sequence (CAPS) analysis (Konieczny and Ausubel, 1993)

of the informative mapping population (Oh et al., 2002) to narrow the target region. Each predicted gene was amplified by *Pfx* polymerase (Invitrogen) from genomic DNA (Dellaporta et al., 1983) isolated from the three mutant alleles and their corresponding wild-type backgrounds, then sequenced to identify mutations.

3.3.3 Complementation of the *dgt* Phenotype with Wild-Type *LeCyp1*

A 4.8 Kb- *Hin*dIII fragment of BAC 52M1containing *LeCyp1* was subcloned into the *Hin*dIII site of a binary plasmid pCB302-3 (Xiang et al., 1999) for complementiation of *dgt* plants. *Agrobacterium tumefaciens* strain EHA105 carrying the *LeCyp1* gene construct was introduced into 2-week-old light-grown hypocotyl sections of dgt^{1-1} as described (Knapp et al., 1994). Transformed shoots were selected on Murashige and Skoog (MS) medium supplemented with Gamborg vitamin B5 mix (Gamborg et al., 1968), 5 μM 6-benzyladenine, 0.5 μM 1-NAA, and 0.025% to 0.1% (w/v) glufosinate ammonium (BASTA, Fluka). Regenerated shoots were rooted on phytohormone-free selection medium then transplanted to soil.

3.3.4 Sequence and Phylogenetic Analyses

Cyclophilin (CYP) and CYP-like protein sequences of tomato, potato, *Arabidopsis*, rice, and soybean were downloaded from the *Arabidopsis* genome database (ftp://ftpmips.gsf.de/cress/) and the Plant Gene Index (http://www.tigr.org/tdb/tgi/plant.shtml) and used for phylogenetic analyses.

Prior to performing distance analysis, the petidyl-prolyl *cis-trans* isomerase (PPIase, Pfam number: PF00160) domain of each CYP was identified by Pfam 8.0 search (http://pfam.wustl.edu/hmmsearch.shtml). An alignment of selected PPIase domain sequences was generated with the ClustalX program (Thompson et al., 1997). The protein sequences used for alignment are listed in Table 3.1. The matrix of distances was subjected to a cluster analysis by using the Neighbor-Joining program from the ClustalX program. For statistical analysis, 1000 bootstrap replications were performed. The consensus tree was established by using the TreeView program (Page, 1996; version 1.6.6, available at http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

3.3.5 Protein Expression in *Escherichia coli* and PPIase assay

The DNA sequence encoding each open reading frame was amplified by PCR from genomic DNA of wild-type and mutant plants in order to construct recombinant plasmids containing fusion proteins GST::LeCYP1, GST::dgt$^{1-1}$, GST::dgt$^{1-2}$, and GST::dgt^{dp}. The *Arabidopsis* cyclophilin, AtRoc1 (Grebe et al., 2000) in expression vector pGEX-4X-1 (Amersham Bioscience), was used as a positive control. For constructing the recombinant plasmids, PCR products were amplified by *Pfx* polymerase (Invitrogen) using primers that contained *Bam*HI and *Xho*I sites and cloned into pGEMTeasy (Promega). The *Bam*HI-*Xho*I fragments were subcloned into the *Bam*HI and *Xho*I sites of pGEX-5X-1 (Amersham Bioscience). Each fusion construct in the recombinant plasmid was sequenced to confirm the in-frame open reading frame. The fusion proteins were purified as

Table 3.1 Protein sequences used for alignment and phylogenetic analysis

Annotation	Accession number EST number AGI number	Annotation	Accession number EST number AGI number
At1/ROC1	At4g38740	LeTC6	TC106667
At2/ROC2	At3g56070	LeTC7	TC102792
At3/ROC3	At2g16600	LeTC8	TC108129
At4/ROC4	At3g62030	LeTC9	TC103462
At5/ROC5	At4g34870	Gm1	AAL50187
At6/ROC6/CYP2	At2g21130	GmTC1	TC144425
At7/ROC7	At5g58710	GmTC2	TC144427
At8/ROC8	At5g13120	GmTC3	TC158696
At9/ROC9	At1g01940	GmTC4	TC168006
At10/ROC10	At2g36130	GmTC5	TC149256
At11/ROC11/CYP5	At2g29960	GmTC6	TC161297
At12/ROC12	At2g38730	GmTC7	TC148829
At13/ROC13	At4g34960	GmTC8	TC145744
At14/ROC14	At3g55920	GmTC9	TC151936
At15/ROC15	At3g22920	GmTC10	TC164167
At16/ROC16	At2g47320	St1	T50771
At17/ROC17	At3g66654	StTC1	TC46429
At18/ROC18/SQN	At2g15790	StTC2	TC55401
At19/ROC19	At3g01480	StTC3	TC41998
At20/ROC20	At4g33060	StTC4	TC53587
At21/ROC21	At1g53720	StTC5	TC56849
At22/ROC22	At3g63400	StTC6	TC53494
At23/ROC23	At5g67530	StTC7	TC50526
At24/ROC24	At3g44600	StTC8	TC50451
At25/ROC25	At4g32420	StTC9	TC42174
	At1g26940	Os1	S48018
	At5g35100	Os2	S48017
	At1g74070	OsTC1	TC108589
LeCYP1	P21568	OsTC2	TC100389
LeTC1	TC102127	OsTC3	TC105213
LeTC2	TC99522	OsTC4	TC105227
LeTC3	TC113347	OsTC5	TC99499
LeTC4	TC100355	OsTC6	TC106657
LeTC5	TC103768	OsTC7	TC105683

described by Hammarberg et al. (2000) with slight modifications. PPIase activities of the fusion proteins were assayed by a protease-coupled method (Liu et al., 1990). A 5 ml overnight culture of *E. coli* strain BL21 Gold (Stratagene) carrying each fusion protein construct was inoculated into 500 ml of 2X YT medium containing ampicillin (100 µg/ml) and incubated at 37°C with vigorous shaking. At optical density A_{600} = 1.5, cells were induced for protein overexpression by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 100 µM. Cultures were incubated for 3 h and cells harvested by centrifugation at 4000 × *g* for 15 min. Prior to homogenization, pellets were incubated in 10 ml of lysis solution (50 mM triethanolamine HCl, pH 8.0; 5 mM EDTA; 2 mM dithiothreitol, 50 mM phenylmethylsulfonyl fluoride; 1 mg/ml lysozyme) at room temperature for 5 min, followed by 25 min on ice. Lysed cells were sonicated (2 × 35 s) using Sonifier® cell disruptor (Branson Sonic Power, model W185) at level 4 then centrifuged at 20,000 × *g* for 15 min. Following filtration (0.22 µm), the supernatant was applied onto a GSTrap™ FF affinity column (Amersham Bioscience) and the GST fusion proteins were purified according to the manufacturer's instruction. Protein concentrations were determined spectrophotometrically against bovine serum albumin standards (0.5 to 10 mg/ml) using a protein assay dye reagent (Bio-Rad) according to manufacturer's instruction. The purified GST-LeCYP1, each GST-mutant CYP1, or the GST control was pre-equilibrated with 35 mM HEPES buffer (pH 8.0) containing 25 µM N-succinyl-Ala-Ala-Pro-Phe-*p*-nitranilide at room temperature for 10 min. Reactions were initiated by adding chymotrypsin and then monitored spectrophotometrically at 390 nm and 15°C in 0.5 s intervals (Hewlett-Packard

model 8453).

3.3.6 Protein Extraction and Immunoblot Analysis

Whole protein extracts were prepared from hypocotyls of 7-day-old dark-grown wild-type (VFN8) tomato seedlings and the three mutant alleles. Approximately one gram of 1 to 2 cm long sections was excised from the upper hypocotyls and ground to a fine powder in a mortar and pestle with liquid nitrogen. Extraction buffer (20 mM MES, pH 6.1; 4 mM $MgCl_2$; 0.3 M sucrose; 0.1 mM EGTA; 1 mM dithiothreitol; 0.1 mM phenylmethylsulfonyl fluoride; 20 μM leupeptin; 1 μM pepstatin) was added to the powder in a ratio of 10 ml/g fresh weight. Homogenates were filtered through Miracloth (Calbiochem) and clarified by differential centrifugation at 5000 x *g*, 12000 x *g*, and 50000 x *g* for 30 min each. Proteins from the clear supernatants were precipitated with 5 volumes of 100% ethanol, air-dried, and dissolved in 2X SDS-PAGE sample buffer (Ausubel et al., 1997). Protein samples from the rosette leaves of wild-type *Arabidopsis thaliana,* as well as *Nicotiana benthamiana* leaves overexpressing the FLAG-epitope-tagged LeCYP1, were prepared as described above for use as controls in immunoblot analysis. Proteins were separated by 10% SDS-PAGE and transferred electrophoretically to nitrocellulose membrane (Ausubel et al., 1997). Blots were hybridized with primary and secondary antibodies using standard techniques (Ausubel et al., 1997). Anti-AtROC1 antiserum (Lippuner et al., 1994) and M2 anti-FLAG monoclonal antibody (Sigma) were used at 1:8000 and 1:1000 dilution, respectively. Anti-rabbit and anti-mouse horseradish peroxidase-conjugated goat

IgGs (Bio-Rad) were used for the secondary antibody conjugation then detected by chemiluminescence. Chemiluminescence detection was performed by incubating the membranes with 100 mM Tris-HCl (pH 8.8), 0.28% H_2O_2, 0.43 mM luminol, and 0.07 mM *p*-coumaric acid for 1 min followed by exposure to autoradiographic film.

3.3.7 Adventitious Root Initiation Assay

Mutant and wild-type seedlings were grown in 0.5X MS medium for 7 to 8 d. One-cm hypocotyl sections were placed on MS medium containing 2% sucrose and supplemented with vitamin B5 mix (pH 6.0) containing 1-NAA and/or cyclosporin A (CsA) at indicated concentrations. The rooting rate and the number of adventitious roots per section were measured after 6 d incubation.

3.3.8 Total RNA Isolation and RT-PCR Analysis

The hypocotyls of 7-d-old etiolated seedlings (*dgt*$^{1-1}$, *dgt*$^{1-2}$, *dgt*^{dp} and their corresponding wild-type cultivars) were harvested to extract total RNA for RT-PCR analysis of *LeCyp1* expression. For analysis of auxin-induced gene expression, the hypocotyls of 7-d-old etiolated seedlings (VFN8 and *dgt*$^{1-1}$) were harvested and cut into 1 cm sections. After depleting endogenous auxins in 1% sucrose, 10 mM MES (pH6.0) for 2 hrs in the dark, the hypocotyl sections were further incubated in buffer containing 10 μM IAA and/or 10 μM CsA for 2 hr. Following incubation, the hypocotyl sections were briefly blotted on filter paper and frozen in liquid

nitrogen. Total RNA was extracted from the frozen ground tissue using TriZol reagent (Invitrogen). First strand of cDNA was synthesized from 5 μg of total RNA using oligo(dT) primers and SuperScript II (Invitrogen) according to manufacturer's instruction. Expression of *LeIAA10, 11, LeCyp1,* and *RPL2* was analyzed by RT-PCR in the presence of specific primers (*LeIAA10*: sense 5'-CAT CAA GAA TTC AAC ACA CAA AAC C-3' and antisense 5'-CGT TTG CAA GAA TCA ACA AAC ATC-3'; *LeIAA11*: sense 5'- GTG TGT GCT TAC CGG AAA AAG AAC-3' and antisense 5'-GAC TTA TCT GCA TCC TCC AAT GCT-3'; *LeCyp1*: sense 5' 5'-CAA ATC CAA AGG TTT TCT TTG ACC-3' and antisense 5'-CTG GTA GAA GCA ACA CAA CAA CCA-3'; *RPL2*: sense 5'-ATT CCG TCA TCC TTT CAG GTA CAA-3' and antisense 5'-ACT GTA CTG GCA TGA CCA ATG TGT-3').

3.4 RESULTS

3.4.1 The *Dgt* Gene Encodes a Cyclophilin

Map-based cloning and comparative microsynteny analysis between the tomato and *Arabidopsis* genomes isolated the *Dgt* gene to two tomato BAC clones, 52M1 and 93O2 (Oh et al., 2002). Nucleotide sequence analysis of BAC clone 52M1 was assembled from 1,536 reads with a 4.5-fold average depth of coverage into five contigs that span about 120 Kb. Sequence information from microsyntenic EST markers and BAC-end sequences (Oh et al., 2002) determined the orientation and order of the contigs. CAPS analysis using genes predicted to lie within BAC 52M1 pinpointed the *Dgt* locus between two EST markers,

Figure 3.1. The *Dgt* gene encodes a cyclophilin. (A) Sequence information and CAPS analyses of ESTs for putative genes in BAC 52M1 determine that *Dgt* is located between two EST markers, TC102622 (primer sets: 5′-TCA CCG CTA TTT GGT TCA AAT CT-3′ and 5′-AAA CCT CAC GTC TAG GAG ATC ACG-3′; restriction: *Hae*III) and BI208338 (primer sets: 5′-CAT GGG CTC ATA CGA TTA CGA CTT-3′ and 5′-CCA AAG GGG TTT TAG GAT TTC TGT-3′; restriction: *Alu*I). Sequencing of putative genes using genomic DNA from three mutant alleles and their backgrounds indicate that the *Dgt* gene encodes LeCYP1. Mutations in the three alleles are $G_{137}R$, $dgt^{1\text{-}1}$; W_{128}stop, $dgt^{1\text{-}2}$; and $W_{128}C\Delta(129\text{-}133)$, dgt^{dp}. (B) Lateral roots are initiated from complemented mutant plant ($dgt^{1\text{-}1}$) grown in phytohormone-free selection medium (Left). 8 week-old complemented plant with restored wild-type growth (Right).

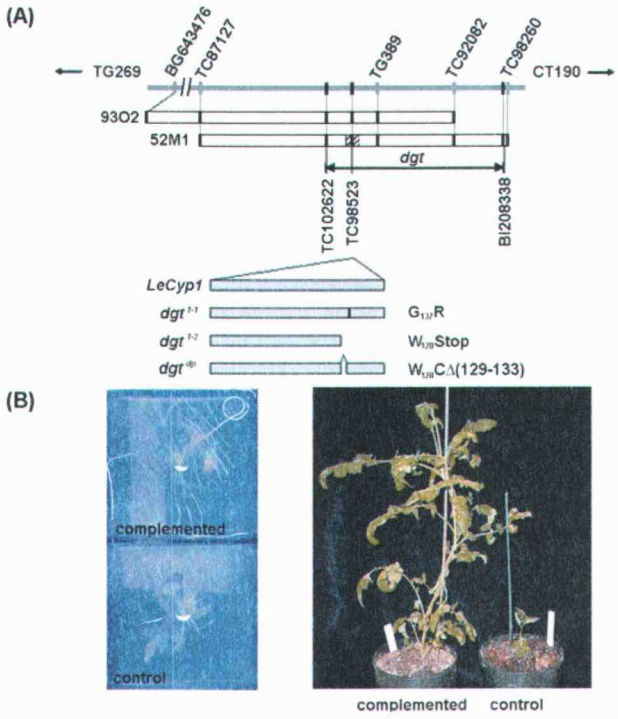
(A)
TG269
BG643476
TC87127
TG389
TC92082
TC98260
CT190
93O2
52M1
dgt
TC102622
TC98523
BI208338
LeCyp1
dgt 1-1
G137R
dgt 1-2
W178Stop
dgt 4b
W108CΔ(129-133)
(B)
complemented
control
complemented
control

Figure 3.1

TC102622 and BI208338, encoding a translocon-associated protein α-subunit and a putative glycine-rich protein, respectively (Figure 3.1*A*). This region spans about 66 Kb region containing only 14 to 16 putative genes. Sequencing of PCR products from the intervening genes revealed only one gene (*LeCyp1,* Gasser et al., 1990), encoding a cyclophilin (CYP), that contained a unique mutation in each *dgt* allele. Alleles $dgt^{1\text{-}1}$and $dgt^{1\text{-}2}$ contain single nucleotide point mutations that generate an amino acid change ($G_{137}R$) and a stop codon (W_{128}stop), respectively, while dgt^{dp} has an amino acid change ($W_{128}C\Delta 129\text{-}133$) preceding a 15 bp deletion (Figure 3.1*A*). Complementation of the mutant phenotype verified that the *Dgt* gene encodes *LeCyp1.* A 4.8 Kb subclone of *Hin*dIII-digested BAC 52M1 containing only the predicted promoter, coding region, and terminator of *LeCyp1* gene, as well as intergenic sequence and partial sequences of flanking genes, was introduced into $dgt^{1\text{-}1}$-containing mutant tissue (Figure 3.1*B*). Complemented plants initiated lateral root formation on the selection medium containing 25 ppm BASTA. Transgene insertion was confirmed in the complemented plant by normal growth in the presence of 100 ppm BASTA applied to the leaves after rooted plantlets were transferred to soil.

To gain insight into the structural relationship between LeCYP1 and other plant CYPs, we performed phylogenetic analysis on plant CYP proteins from tomato, *Arabidopsis*, rice, soybean, and potato (Figure 3.2). Since few plant CYPs other than *Arabidopsis* CYPs are currently annotated in GenBank, putative CYPs and CYP-like proteins (CYPLs) identified by TBLASTN searching the plant gene index using LeCYP1 were used for constructing a phylogenetic tree (Table 3.1). Database searches revealed 25 annotated CYPs, as known as ROCs, and three

Figure 3.2. Phylogenetic relationships among plant CYPs and CYPLs. Bootstrap values of 1000 bootstrap iterations are shown (> 70%). Clades with different colored-branches represent their subcellular localization. The scale represents a distance of 0.1 units. At, *Arabidopsis*; Gm, soybean; Le, tomato; Os, rice; St, potato, ER, endoplasmic reticulumn; TC, deduced protein from EST.

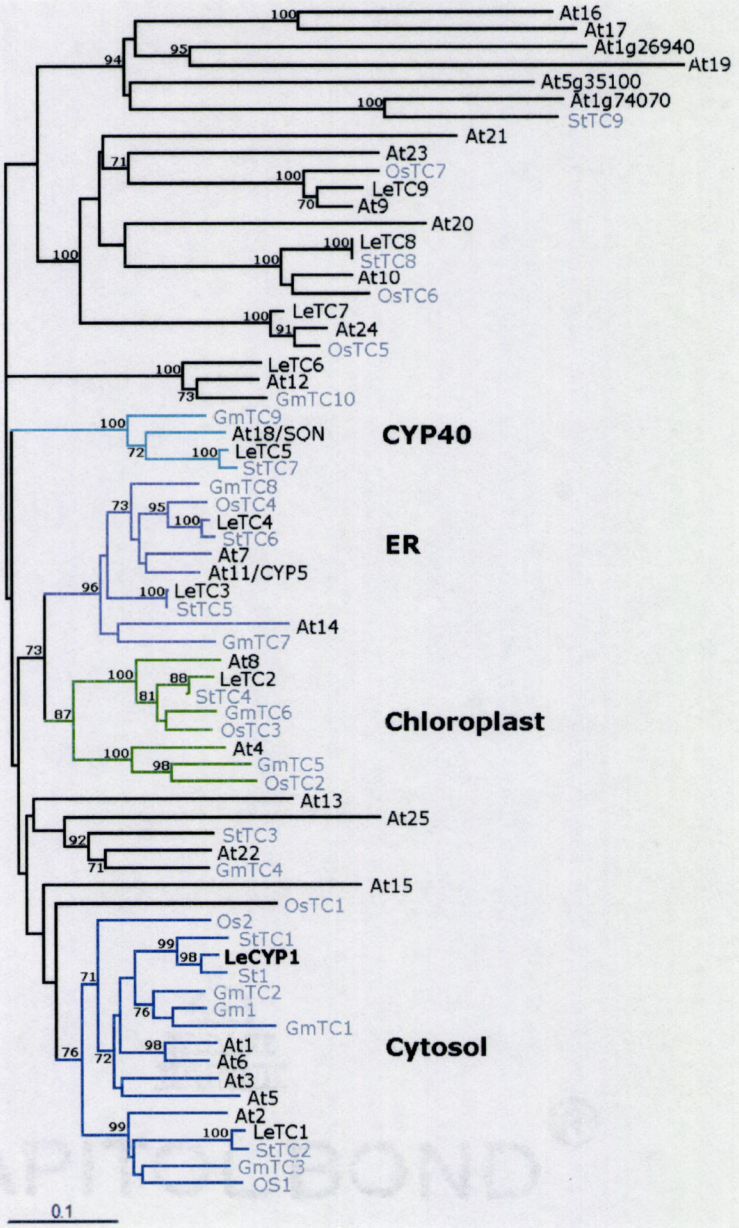
At16
At17
At1g26940
At19
At5g35100
At1g74070
StTC9
At21
At23
OsTC7
LeTC9
At9
At20
LeTC8
StTC8
At10
OsTC6
LeTC7
At24
OsTC5
LeTC6
At12
GmTC10
GmTC9
At18/SON
LeTC5
StTC7
CYP40
GmTC8
OsTC4
LeTC4
StTC6
At7
At11/CYP5
LeTC3
StTC5
ER
At14
GmTC7
At8
LeTC2
StTC4
GmTC6
OsTC3
Chloroplast
At4
GmTC5
OsTC2
At13
At25
StTC3
At22
GmTC4
At15
OsTC1
Os2
StTC1
LeCYP1
St1
GmTC2
Gm1
GmTC1
At1
At6
At3
At5
At2
LeTC1
StTC2
GmTC3
OS1
Cytosol
0.1

Figure 3.2

CYPLs in the *Arabidopsis* genome. A neighbor joining phylogenetic tree was generated from the aligning the PPIase domain of 68 plant CYP and CYPLs (Figure 3.2). A nearly identical tree was obtained when we used the full-length amino acid sequences of the plant CYPs were used (data not shown). The phylogenetic tree clearly delimits clades corresponding to subcellular localization of CYPs. Interestingly it exhibits two different subgroups in the cytosolic CYP clade. Two LeCYPs, LeCYP1 and the other putative LeCYP encoded by EST TC102127, are included in each group of the cytosolic CYP clade. Five *Arabidopsis* ROCs (ROC 1-3, 5, and 6) are included in the same clade and either AtROC1 or 6 appear to be the ortholog of LeCYP1.

3.4.2 The *dgt* Mutations Nullify or Reduce PPIase Activities *In Vitro*

PPIase activities of GST-fusion proteins of LeCYP1 and the three mutant proteins were determined *in vitro*. Some minor contaminant proteins were identified by SDS-PAGE analysis but AtROC1 antiserum detected only single bands at the correct size for the GST-fusion proteins (Figure 3.3*A*). The purified GST-tagged LeCYP1 is functional protein with higher intrinsic PPIase activity than GST-tagged AtROC1 (Figure 3.3*B*). The $G_{137}R$ mutant protein, GST::dgt$^{1\text{-}1}$, retained 20% catalytic activity, compared to wild-type fusion protein levels. The opal mutant fusion protein, GST::dgt$^{1\text{-}2}$, and the $W_{129}C\Delta$(129-133) deletion mutant fusion protein, GST::dgt^{dp}, did not exhibit any PPIase activity, over the negative control, when tested at 50 nM protein concentrations (Figure 3.3*C*). Reaction mixtures containing 50 nM of each fusion protein was used to test the sensitivity

Figure 3.3. PPIase activity is reduced or abolished by the *dgt* mutations *in vitro*. (A) The molecular weight of each fusion protein overexpressed in *E. coli* was verified (top panel; lane 1, GST::LeCYP1; lane 2, GST::dgt$^{1-1}$; lane 3, GST::dgt$^{1-2}$; lane 4, GST::dgt^{dp}; lane 5, GST::AtROC1; lane 6, GST control). Immunoblot analysis (bottom panel) indicates that all fusion proteins contain PPIase proteins. (B) GST-tagged LeCYP1 appears to retain higher intrinsic PPIase activity than GST-tagged AtROC1. Nearly identical curves are generated by 50 nM GST and the blank chymotrypsin control. (C) The $G_{137}R$ mutation, GST::dgt$^{1-1}$, appears to retain 20% catalytic efficiency of the wild-type fusion protein. The opal mutation, GST::dgt$^{1-2}$, and the $W_{129}C\Delta$(129-133) mutation, GST::dgt^{dp}, do not demonstrate any PPIase activity. (D) CsA (1 μM) substantially diminishes the PPIase activity of GST::LeCYP1 and GST::dgt$^{1-1}$ whereas GST::dgt$^{1-2}$ and GST::dgt^{dp} activity is unchanged in response to CsA treatment. Each curve represents the average values of triplicates in three independent experiments.

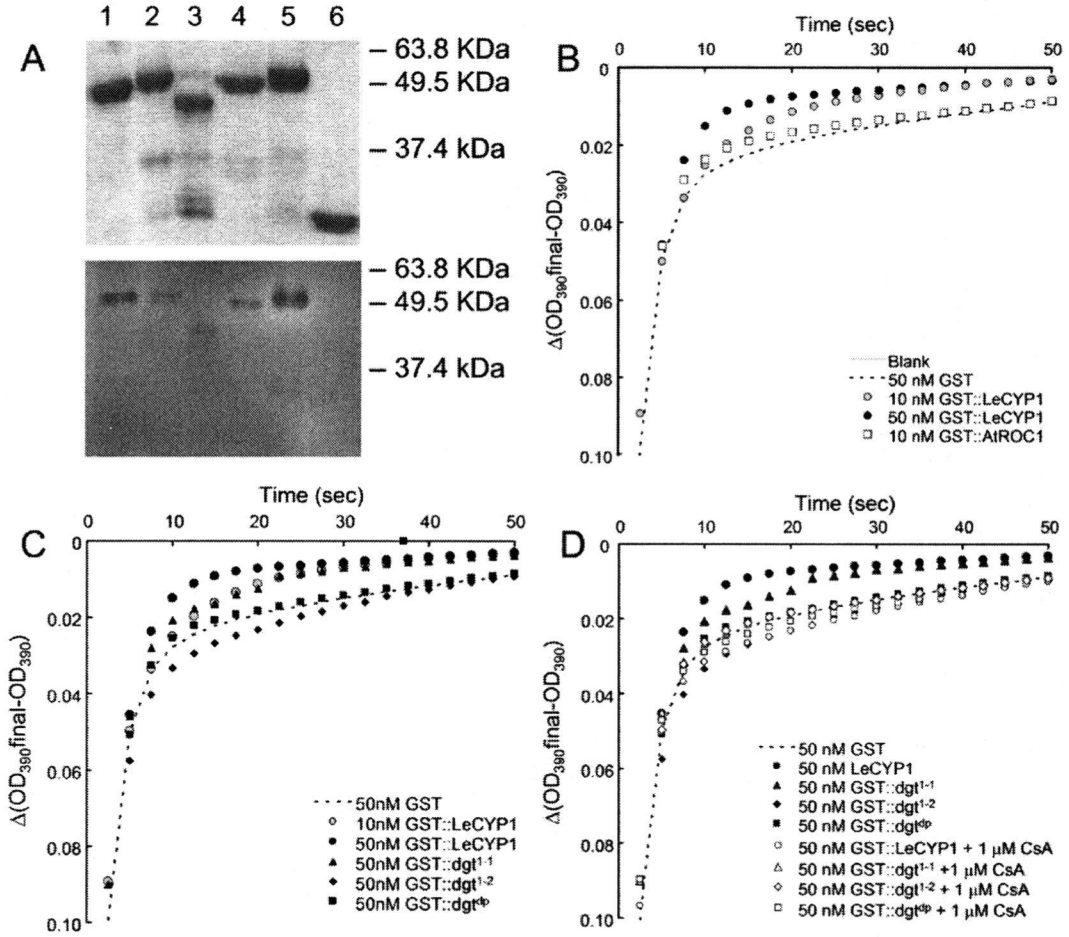
A
1 2 3 4 5 6
– 63.8 KDa
– 49.5 KDa
– 37.4 kDa
– 63.8 KDa
– 49.5 KDa
– 37.4 kDa
B
Time (sec)
Δ(OD390final-OD390)
Blank
50 nM GST
10 nM GST::LeCYP1
50 nM GST::LeCYP1
10 nM GST::AtROC1
C
Time (sec)
Δ(OD390final-OD390)
50nM GST
10nM GST::LeCYP1
50nM GST::LeCYP1
50nM GST::dgt1-1
50nM GST::dgt1-2
50nM GST::dgtdp
D
Time (sec)
Δ(OD390final-OD390)
50 nM GST
50 nM LeCYP1
50 nM GST::dgt1-1
50 nM GST::dgt1-2
50 nM GST::dgtdp
50 nM GST::LeCYP1 + 1 μM CsA
50 nM GST::dgt1-1 +1 μM CsA
50 nM GST::dgt1-2 + 1 μM CsA
50 nM GST::dgtdp + 1 μM CsA

Figure 3.3

of PPIase activity to CsA. At 1μM, CsA substantially diminished the catalytic efficiency of GST::LeCYP1. Inhibition of the GST::dgt$^{1-1}$ protein by 1 μM CsA was similar, suggesting the $G_{137}R$ mutation may not interfere with protein's ability to bind CsA. Addition of CsA did not alter GST::dgt$^{1-2}$ or GST::dgt^{dp} PPIase activity (Figure 3.3*D*).

3.4.3 The *dgt* Alleles Are Null-Mutations

RT-PCR analysis showed that the level of *LeCyp1* transcripts was unaffected by the mutations (Figure 3.4*A*). Thus, immunoblot analysis using proteins extracted from etiolated hypocotyls was performed to see how the mutations affect the accumulation of proteins *in planta.* Anti-AtROC1 antiserum detected wild-type LeCYP1, as well as endogenous tobacco CYP, but was not able to detect LeCYP1 in protein extracts from all three alleles (Figure 3.4*B* and *C*).

3.4.4 CsA Inhibits Auxin-Responses

Application of CsA to wild-type and *dgt* hypocotyl sections demonstrated the inhibition of auxin responsiveness by disruption of CYP action. Hypocotyl sections of *dgt* plants exhibit reduced sensitivity to exogenously-applied 1-NAA (Figure 3.5*A*). The reduced sensitivity of *dgt* can be partially mimicked in wild-type shoots by application of CsA (Figure 3.5*B*). Inhibition of 1-NAA-induced adventitious rooting by CsA was dosage-dependent (data not shown), and did not appear to result from general toxicity of CsA, as shoots remained green and

formed callus (Figure 3.5*C*). The reduced rooting response of *dgt* shoots is inhibited even further by CsA treatment, which may reflect inhibition of other CYP isomers that could partially compensate for the mutated *LeCyp1 in planta*. Treatment of wild-type hypocotyls segments with CsA inhibited not only 1-NAA-induced rooting, but also auxin-induced expansion. Semi-quantitative RT-PCR analysis indicated that IAA-induced expression of two DGT-dependent members of the *AUX/IAA* gene family, *LeIAA10* and *11* (Nebenführ et al., 2000), was partially inhibited in etiolated hypocotyl sections by the treatment with 10 μM CsA for 2hr (Figure 3.6). Incomplete inhibition may be due in part to inefficient uptake of CsA through hypocotyls segments, or to the poor solubility of CsA at concentrations above 10 μM. Expression of *LeCyp1* was not affected by either exogenous IAA or CsA in other wild-type or mutant hypocotyls as compared to the *RPL2* controls, previously shown to reflect the overall distribution of mRNA and used as a general marker for small cells with high metabolic activity (Fleming et al., 1993; Reinhardt et al., 1998).

3.5 DISCUSSION

The identification of three distinct allelic mutations in the coding sequence of *LeCyp1* and the restoration of lateral root initiation in dgt^{1-1}plants by complementation with *LeCyp1* provide compelling evidence that the *Dgt* gene encodes the tomato gene *LeCyp1*. LeCYP1 is a cytosolic CYP, also reported as CYP A in mammalian cells (reviewed in Göthel and Marahiel, 1999) and previously identified as a cellular receptor for the immunosuppressive drug, CsA. CYPs have

Figure 3.4. The *dgt* alleles are null-mutations. (A) Expression of *LeCyp1* was unaffected by the *dgt* mutations. First strand of cDNAs synthesized from 100 ng of total RNA were amplified by intial denaturation at 94°C for 2 min and 40 cycles of amplification (94°C for 15 sec, 58 °C for 30 sec, 72 °C for 60 sec), followed by final extension (72 °C for 5 min). The genomic DNA from VFN8 was used as a negative control (gDNA) for genomic DNA contamination in total RNA preparations (B) Protein samples (10 μg each) from hypocotyl sections of 7-d old etiolated seedlings, *Arabidopsis* rosette leaves, and tobacco leaves overexpressing a FLAG-epitope-tagged LeCYP1 along with molecular standards (M), were separated by SDS-PAGE and stained with Commasie Blue. (C) The same protein samples (10 μg) were transferred to a nitrocellulose membrane and probed with AtROC1 antiserum. The bands of LeCYP1 (17.9 KDa), AtROC1 (18.4 KDa), and a doublet of endogenous tobacco CYP and FLAG::LeCYP1 (19.3 KDa) were detected only from protein samples of wild-type (VFN8) hypocotyls (W), *Arabidopsis* (At), and tobacco leaves (N), respectively, whereas no band was detected in the three mutant alleles (lane $dgt^{1\text{-}1}$, $dgt^{1\text{-}2}$ and dgt^{dp}). To confirm the detection of FLAG::LeCYP1 in the doublet bands by anti-AtROC1 antiserum (N), M2 anti-FLAG monoclonal antibody was used detect FLAG::LeCYP1 in the protein extracts of tobacco leaves overexpressing FLAG::LeCYP1 (N-Fl).

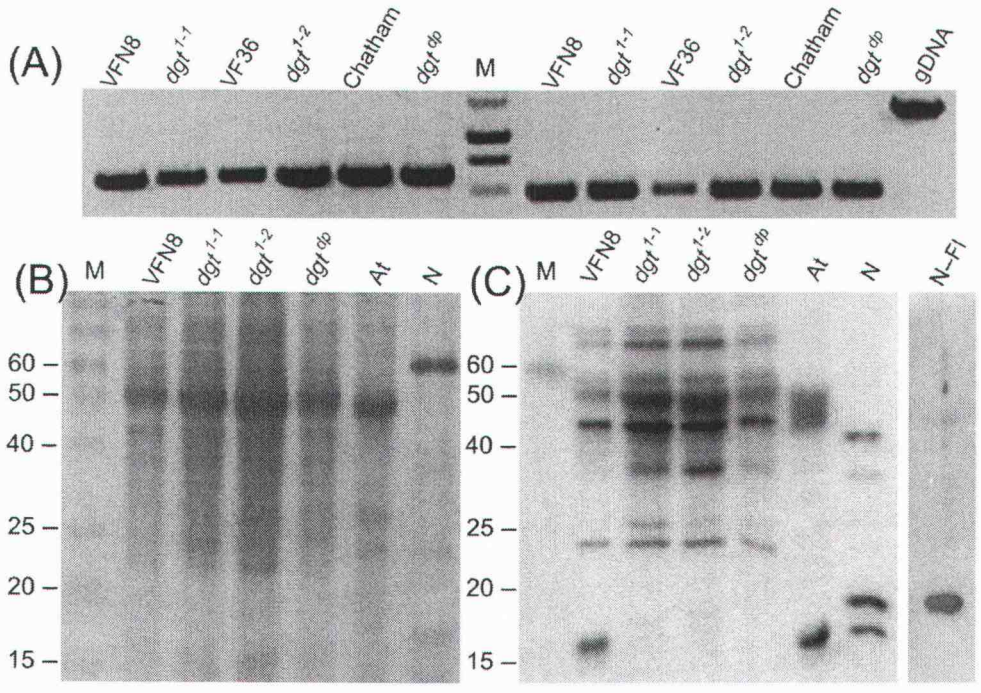
(A)
VFN8
dgt 1-1
VF36
dgt 1-2
Chatham
dgt dp
M
VFN8
dgt 1-1
VF36
dgt 1-2
Chatham
dgt dp
gDNA
(B)
M
VFN8
dgt 1-1
dgt 1-2
dgt dp
At
N
60
50
40
25
20
15
(C)
M
VFN8
dgt 1-1
dgt 1-2
dgt dp
At
N
N-FI
60
50
40
25
20
15

Figure 3.4

Figure 3.5. Cyclosporin A inhibits auxin responses in wild-type hypocotyl segments. (A) Hypocotyl segments (~1 cm long) from 8-9 d old wild-type and *dgt* seedlings were grown for 6 d on MS medium containing the indicated auxin (1-NAA) concentration. (B) Hypocotyl segments of *dgt* and wild-type were grown for 6 d on MS medium containing 1 μM 1-NAA in the absence or presence of 10 μM CsA. (C) The effect of 10 μM CsA on 1-NAA-induced adventitious rooting of wild-type and *dgt* hypocotyl segments. The photograph was taken after 7 d incubation. All data were average of triplicates in three independent experiments (n= 9 to 12).

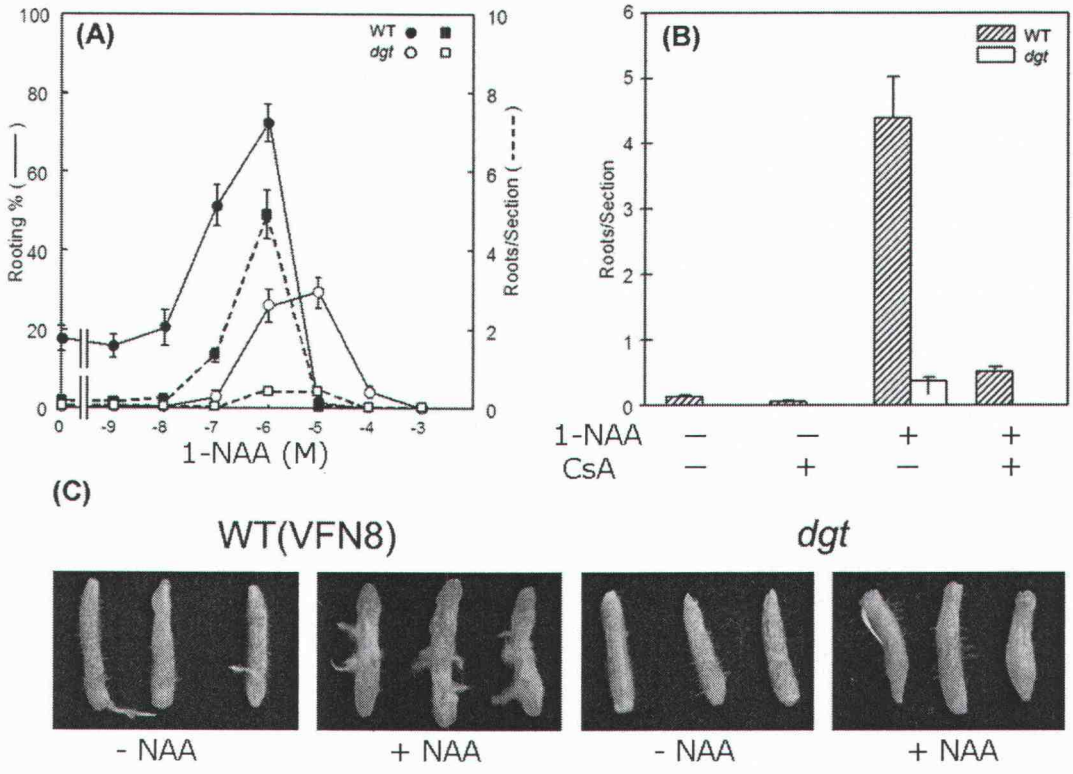
(A)
WT
dgt
Rooting % (——)
Roots/Section (- - - -)
1-NAA (M)
(B)
WT
dgt
Roots/Section
1-NAA
CsA
(C)
WT(VFN8)
dgt
- NAA
+ NAA
- NAA
+ NAA

Figure 3.5

Figure 3.6. Cyclosporin A inhibits auxin-induced expression of *LeIAA* genes. Expression of *LeIAA10, 11,* and *LeCyp1* as well as the *RPL2* control was analyzed by semiquantitative RT-PCR in the presence or absence of 10 μM IAA and/or 10 μM CsA. First strand cDNAs synthesized from 250 ng of total RNA were amplified by initial denaturation at 94°C for 2 min and 25 cycles of amplification (94°C for 15 sec, 58°C for 30 sec, 72°C for 60 sec), followed by final extension (72°C for 5 min). IAA-induced expression of *LeIAA10* and *11* from etiolated wild-type (WT) hypocotyl sections treated with CsA partially mimics *dgt* phenotype.

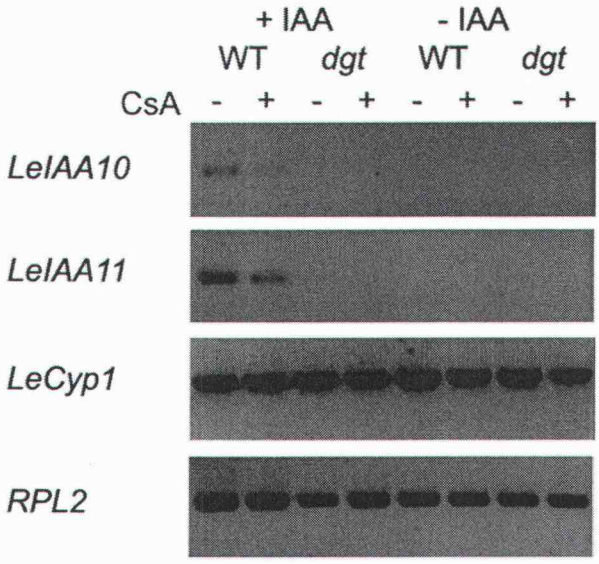

Figure 3.6

PPIase activity that catalyzes the *cis-trans* isomerization of proline residues in polypeptides (Liu et al., 1990; Fischer et al., 1989; Takahashi et al., 1989). Plant CYPs were first reported in tomato, maize, and rape (Gasser et al., 1990) and many *Cyp* genes have since been isolated and characterized in a variety of plant species (Gasser et al., 1990; Buchholz et al., 1994; Marivet et al., 1994; Marivet et al., 1995; Küllertz et al., 1999, Saito et al., 1999; Nuc et al., 2001). The two candidates, AtROC1 and 6, of the ortholog for LeCYP1 (Figure 3.2) are located in the microsyntenic regions to *DGT* (Oh et al., 2002), suggesting that these microsyntenic regions containing AtROC1 and 6 are duplicated after the speciation.

Identification of the *Dgt* gene led us to address whether cyclophilin action is independent of or dependent on the intrinsic PPIase activity or if it results from it. PPIase assays demonstrated that the $G_{137}R$ mutant protein (GST::dgt$^{1\text{-}1}$) severely reduced PPIase activity *in vitro*, compared to wild-type LeCYP1, but retained binding ability to CsA. As might be expected, the other two mutant fusion proteins, GST::dgt$^{1\text{-}2}$ and GST::dgt^{dp}, due to early stop codon and the five amino acid deletion, respectively, in the CsA binding site (Mikol et al., 1994) exhibited no PPIase activity and did not respond to addition of CsA. Levels of *LeCyp1* transcripts isolated from hypocotyl sections of the three alleles were unaffected by the allelic mutations in the coding region. However, phenotypic characteristics are similar in all three *dgt* alleles, suggesting that all are likely to be null mutations. The null mutant assumption is supported by the finding that anti-AtROC1 antiserum did not detect LeCYP1 in protein extracts from any of the three mutant alleles (Figure 3.4). The lack of detection was not due to changes in antigenicity of the mutant proteins since overexpressed GST-fusion mutant proteins were

identified by the antiserum (Figure 3.3). Since many higher molecular weight bands were detected by anti-AtROC1 antiserum in wild-type and *dgt* protein extracts due to the presence of CYP-domain-containing RNA-binding proteins and higher molecular weight CYPs such as CYP40, the specificity of anti-AtROC1 for LeCYP1 was tested using FLAG::LeCYP1 overexpressed in tobacco (Figure 3.4*C*, lane 6). A slightly higher molecular-weight band (19.3 kDa) was recognized by both the anti-AtROC1 antiserum and the M2 anti-FLAG antibody, indicating that the anti-AtROC1 antiserum can recognize LeCYP1 in protein extracts from wild-type hypocotyls. Although the overexpressed $dgt^{1\text{-}1}$ fusion protein retained minimal PPIase activity *in vitro*, the $dgt^{1\text{-}1}$ protein was not detected *in planta*, suggesting that the G_{137} residue may be critical for protein accumulation and enzymatic function *in planta*.

The *dgt* mutation reduces the expression of only a subset of *Aux/IAA* genes (Balbi and Lomax, 2003; Nebenführ et al., 2000). Therefore, we investigated the effect of CsA, a specific CYP inhibitor, on auxin-regulation of development and the expression of known DGT-dependent *LeIAAs* in tomato hypocotyl sections (Figure 3.5). Absence of LeCYP1 protein indicates that mutant proteins do not accumulate and suggests that the lack of LeCYP1 reduces auxin-induced responses such as adventitious root initiation and expression of DGT-dependent *AUX/IAA* genes. A DGT-independent pathway for some adventitious root initiation may exist and/or other tomato CYPs may compensate for the absence of DGT. Addition of CsA to wild-type seedlings partially phenocopies the *dgt* phenotype. Although auxin-induced expression of *LeIAA10* and *11* was not completely inhibited by CsA (Figure 3.6), this may be due to the low solubility of

CsA or reduced uptake by tomato hypocotyl sections. CsA inhibition of auxin-stimulated adventitious root formation indicates that LeCYP1 affects auxin signaling in a CsA-dependent manner and that the intrinsic PPIase activity of LeCYP1 may be a necessary component of auxin action.

The exact role of CYPs in plant growth and development still remains unclear. The AtCYP5 protein has an N-terminal endoplasmic reticulum transport signal and interacts with the N-terminal region of the GNOM protein, a guanine nucleotide exchange factor (GEF) that is required for the proper polar localization of PIN1(Gelnder et al., 2003). The *Arabidopsis gnom* mutant exhibits defective embryo development and since *AtCyp5* is also expressed in developing embryos, CYP5 may regulate the GEF function of the GNOM protein during embryogenesis (Grebe et al., 2000). The *Arabidopsis Squint* (*Sqn*) gene encodes AtCYP40 and *sqn* mutants have reduced numbers of juvenile leaves (Berardini et al., 2001), providing the first direct evidence of a role for CYPs in regulating vegetative development. CYP40, complexed with HSP90, functions as a chaperone required for proper folding of steroid receptors (Pratt et al., 2001). However, it remains to be determined whether AtCYP40 actually functions as a chaperone and with what other proteins. Another CYP proposed to affect auxin action is AtROC7, a CYP containing a 30 amino acid N-terminal extension (Jackson and Söil, 1999). In yeast two-hybrid studies, AtROC7 interacts with the regulatory subunit of protein phosphatase A (PP2A) (Garbers et al., 1996). Mutation of the PP2A regulatory subunit, (*rcn1*, Garbers et al., 1996) is known to alter polar auxin transport and the root growth response in *Arabidopsis* (Rashotte et al., 2001). However, antisense expression of *AtRoc7* alters only the root elongation response, but not

auxin transport (Jackson and Söil, 1999). None of the discussed CYPs are cytosolic forms of CYP, as is LeCYP1. To our best knowledge, LeCYP1 is the first demonstration of the involvement of a cytosolic-type *Cyp* gene in the early steps of auxin-induced gene expression.

Our results suggest that LeCYP1 affects auxin signaling in a CsA-dependent manner, indicating that PPIase activity is involved. The best-known mechanism of CYP A action is requires the formation of CYP-CsA complexes that suppress immune responses by binding to the regulatory B subunit of Ca^{2+}-dependent protein phosphatase 2B, also known as calcienurin (Cyert et al., 1991; Liu et al., 1991). Complexes between a *Vicia faba* CYP A and CsA inhibits both animal calcineurin and plant calcineurin-like protein phosphatase activity (Luan et al., 1994; Kinoshita et al., 1999). Additionally, there is growing evidence that calcineurin-B subunit-like proteins (CBLs) of *Arabidopsis* mediate plant signal transduction pathways in response to light (Nozawa et al., 2001) and salt stress (Lippuner et al., 1996; Kudla et al., 1999) by serving as potential Ca^{2+} sensors. To date, we have found no evidence in the literature to implicate calcineurin or CBL involvement in auxin-regulated growth and development. Whether calcineurin or CBL is involved in DGT-dependent auxin signaling remains to be determined.

Since LeCYP1 affects the expression of a subset of *LeIAA*s, we postulate that LeCYP1 is localized in the nucleus where it may interact with other proteins to regulate the expression of early auxin-response genes. Also, immunofluorescence microscopy with anti-CYP A monoclonal antibodies demonstrates localization of CYP A in both the nucleus and cytosol of mouse macrophages (Krummrei et al., 1995). Using a yeast two-hybrid system, human CYP A interacts with the

mammalian YY1 zinc finger transcription factor. This interaction was also identified in HeLa cell culture (Yang et al., 1995). However, we do not rule out the possibility of CYP functions that regulate additional components of auxin signal transduction. Information as to the subcellular localization of LeCYP1 protein is crucial to understanding its role in auxin signal transduction. We have demonstrated that the *Dgt* gene encodes LeCYP1 and that LeCYP1 acts on specific auxin signaling pathways. These findings will provide a new insight toward understanding the mechanism of auxin signal transduction.

3.6 ACKNOWLDEGEMENTS

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4. SUBCELLULAR LOCALIZATION OF THE LECYP1/DGT PROTEIN AND CHARACTERIZATION OF CYCLOPHILIN ACTION IN *ARABIDOPSIS THALIANA*

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4.1 Abstract

The tomato gene *Diageotropica* (*Dgt*) encodes a cytosolic cylophilin, LeCYP1, that is a novel component in auxin signaling and confers selective regulation of transcriptional repressor AUX/IAA proteins. Sequence and phylogenetic analyses between tomato and *Arabidopsis* cytosolic cyclophilins demonstrate two subgroups of cytosolic cyclophilins that appear to diverge before speciation, suggesting that any *Arabidopsis* cyclophilin member in Group 1 might be orthologous to LeCYP1/DGT. The phenotypes of five T-DNA-insertion *Atroc* mutant alleles of cyclophilin were analyzed to determine if mutations in *Arabidopsis* Group 1 cyclophilins could phenocopy the pleiotropic *dgt* phenotype. Overall seedling growth of the *Atroc* mutant alleles appeared normal with slight changes in the gravitropic response. Cytosolic cyclophilins interact with many nuclear proteins in mammalian and yeast cells and LeCYP1/DGT appears to regulate the expression of transcription factors for auxin-induced gene expression. Green fluorescent protein fusions to LeCYP1/DGT were predominantly expressed in the nucleus of maize epidermal cells and we hypothesize that the LeCYP1/DGT protein may be involved in nuclear signal transduction. AUX/IAA gene expression was differentially affected by exogenous application of the cyclophilin inhibitors cyclosporin A and Sanglifehrin A in *Arabidopsis* plants harboring *AtIAA3/SHY2* and *AtIAA28* promoter*::GUS* reporter gene constructs. Cyclosporin A repressed auxin-induced *AtIAA3/SHY2* expression, but had no effect on auxin-induced expression of *AtIAA28*. Sanglifehrin A partially affected *AtIAA3/SHY2* expression, suggesting that a Ca^{2+}-dependent protein phosphatase 2B may be involved in

auxin signaling.

4.2 Introduction

Cyclophilins (CYPs) were discovered as receptors for the immunosuppressant drug cyclosporin A (CsA), but have subsequently been shown to have many other cellular functions in a variety of organisms. CYPs have an intrinsic petidyl-prolyl *cis-trans* isomerase (PPIase) activity, also known as rotamase (ROC), that accelerates *cis-trans* isomerization of prolyl residues in nascent polypeptides, a process significant to proper protein folding. The PPIase activity of CYPs is shared with two other families, FK506-binding proteins (FKBPs) and parvulins, that are not structurally related. CYP/ROCs, encoded by multigene families, are ubiquitously present in all classes of organisms and found in either small, single-domain proteins or large multi-domain proteins. Depending on their subcellular localization signals, additional domains, and molecular weights, mammalian CYP/ROCs are classified into at least six groups, CYPA to E and CYP40 (reviewed in Göthel and Marahiel, 1999). The 18 kDa CYPA consists only of the CYP core domain, is localized primarily in the cytosol, and is highly conserved from yeast to humans with 65% sequence identity. CYPB and CYPC, 21 kDa and 33 kDa proteins, respectively, are mainly localized in the endoplasmic reticulum (ER) and secretory pathways. CYPD is a 20 kDa mitochondrial protein and CYPE (33 kDa) contains an RNA recognition motif (Mi et al., 1996). CYP40 consists of the core CYP domain plus a tetratricopeptide repeat (TPR) domain and forms a chaperone-like complex with heat shock protein 90 (HSP90) that is required for

proper folding of steroid receptors (Duina et al., 1996; Owens-Grillo et al., 1996a and b). Cytosolic CYPA was the first CYP/ROC to be isolated and the target of its PPIase action is Ca^{2+}/calmodulin-dependent protein phosphatase 2B, also known as calcineurin (Klee et al., 1998). Calcineurin is involved in the nuclear targeting of nuclear-factor activated transcription factors upon increased cytoplasmic Ca^{2+}, a crucial step for immune responses in mammalian cells (Liu et al., 1991; Swanson et al., 1992).

Plant *Cyp/Roc* genes were first reported in tomato, maize, and rape (Gasser et al., 1990) and many *Cyp/Roc* genes have been isolated and characterized in a variety of plant species (Buchholz et al., 1994; Marivet et al., 1994; Chou and Gasser, 1997; Küllertz et al., 1999; Saito et al., 1999; Nuc et al., 2001). Expression patterns of plant *Cyp/Roc* genes suggested their involvement in signaling processes responsive to environmental stresses (Marivet et al., 1994) and hormones (Marivet et al., 1995); however, the cellular functions of plant CYP/ROCs are unknown, perhaps due to the deficiency of information on plant *cyp/roc* mutants and their phenotypes. The first described mutation of plant *Cyp/Roc* genes was the *squint* (*sqn*) mutant of *Arabidopsis,* which causes reduced numbers of juvenile leaves and subtle changes in inflorescence morphology (Berardini et al., 2001). The *Sqn* gene encodes CYP40, a protein that contains a PPIase domain and three tetratricopeptide repeat (TPR) units, resembling the domain organization of another type of PPIase, PAS1/FKBP52, involved in cell proliferation and differentiation in *Arabidopsis* seedlings (Vittorioso et al., 1998). The TPR units of both PPIases are required for interaction with HSP90, suggesting that both PPIases may function as protein chaperones.

The AtCYP5/ROC11 protein of *Arabidopsis* has an N-terminal ER transport signal and interacts *in vitro* with the N-terminal region of the GNOM protein, a ADP-ribosylation factor guanine nucleotide exchange factor (ARF-GEF), involved in the redistribution of auxin efflux-carrier protein PIN1 (Geldner et al., 2003). The *gnom* mutant exhibits defective embryo development. *AtCyp5/Roc11* gene expression in developing embryos suggests that AtCYP5/ROC11 may regulate the GEF function of the GNOM protein during embryogenesis (Grebe et al., 2000). Another ER-type CYP, AtROC7, was identified in yeast two-hybrid studies as interacting with the regulatory subunit of protein phosphatase 2A (Jackson and Söll, 1999), a protein implicated in polar auxin transport (Garbers et al., 1996; Deruère et al., 1999). Further characterization of the putative role of ER-type CYP/ROCs in polar auxin transport has not been reported.

Mutational and protein interaction analyses in *Arabidopsis* indicate that several CYP/ROCs containing localization signals or multiple domains are involved in regulating plant growth and development. Recently, we provided the first evidence of CYP/ROC involvement in auxin signaling by demonstrating that the auxin-resistant *diageotropica* (*dgt*) mutant results from a lesion in *LeCyp1*, which encodes a 18 KDa, single domain cytosolic CYP/ROC (Chapter 3). Although many plant *CYPA* genes are differentially regulated in response to various environmental stimuli and stresses (Marivet et al., 1994 and 1995; Saito et al., 1999), northern and RT-PCR analyses of the *LeCyp1/Dgt* gene exhibit that the *LeCyp1/Dgt* gene appears to be expressed constitutively (Gasser et al., 1990; Balbi and Lomax, unpublished results; Chapter 3). Mutations in members of the *AUX/IAA* gene family (Rouse et al., 1998; Tian and Reed, 1999; Nagpal et al., 2000; Rogg et al.,

2001; Fukaki et al., 2002), or in proteins implicated in the turnover of AUX/IAA proteins, such as AXR1 and TIR1 (Lincoln et al., 1990; Ruegger et al., 1997) in *Arabidopsis*, produce changes in gravitropic responses and lateral root formation similar to that of the *dgt* mutation. However, the pleiotropic *dgt* phenotype is not completely phenocopied by any known *Arabidopsis* auxin-related mutant. LeCYP1/DGT regulates a subset of *AUX/IAA* expression in a tissue- and developmental stage-specific manner (Nebenführ et al., 2000; Balbi and Lomax, 2003), suggesting that cytosolic CYP may act as an upstream regulator of *AUX/IAA* gene expression. Regulation of primary auxin-response gene expression involves removal of the AUX/IAA transcriptional repressor from the auxin response factor (ARF)/IAA dimer and rapid turnover of the AUX/IAA protein via ubiquitin-dependent proteasome pathway (Tiwari et al., 2001; Zenser et al., 2001). Therefore, LeCYP1/DGT is speculated to regulate auxin action at least in part by regulating the expression of specific *AUX/IAA* genes. Currently, at least 29 and 11 *AUX/IAA* genes have been identified in *Arabidopsis* and tomato, respectively (Liscum and Reed, 2002; Nebenführ et al., 2000). The specificity of that regulation may be achieved by differential dependence on cyclophilln isomers and/or other types of PPIases. The *Arabidopsis* genome and tomato EST database searches revealed that tomato and *Arabidopsis* cytosolic-type CYP/ROCs have at least two and five isoforms, respectively (Chapter 3). However, the role of each cytosolic CYP/ROC isoform in regulation of *AUX/IAA* gene expression *in planta* remains unknown. This study utilized information from the completely sequenced *Arabidopsis* genome, the availability of numerous auxin-related *Arabidopsis* mutants, and a reverse genetics approach to investigate the putative role(s) of

cytosolic cyclophilins *in planta*.

4.3 Materials and Methods

4.3.1 Plant Materials

Arabidopsis seeds were surface-sterilized in 20% household bleach containing 1% SDS for 20 min and rinsed with sterile, double-distilled water. Seeds were then sown onto sterile germination medium [0.5X MS medium (Murashige and Skoog, 1962) containing 1% sucrose, 1X vitamin B5 mix (100 mg/L myo-inositol, 10 mg/L thiamine-HCl, 1 mg/L nicotinic acid, and 1 mg/L pyridoxine-HCl; Gamborg et al., 1968), and 0.3% Phytagel (Sigma)]. Plates were placed at 4°C in the dark for 2 d to promote germination, and then were transferred to a growth chamber at 21°C with a 16 h light/8 h dark or 24 h dark photoperiod cycle. Unless otherwise stated, all chemicals were from Sigma-Aldrich.

4.3.2 Sequence and Phylogenetic Analyses

Cyclophilin protein sequences were downloaded from the *Arabidopsis* genome database (ftp://ftpmips.gsf.de/cress/) and the Tomato Gene Index (http://www.tigr.org/tdb/lgi). Peptide signals and targeting motifs were determined by TargetP v1.01 (http://www.cbs.dtu.dk/services/TargetP/) and predictNLS (http://cubic.bioc.columbia.edu/predictNLS/). Prior to performing distance analysis, the petidyl-prolyl *cis-trans* isomerase (PPIase, Pfam number:

PF00160) domain of each CYP was identified by Pfam 8.0 search (http://pfam.wustl.edu/hmmsearch.shtml). An alignment of selected PPIase domain sequences was generated with the ClustalX program (Thompson et al. 1997). The matrix of distances was subjected to a cluster analysis using the Neighbor-Joining program within the ClustalX program. For statistical analysis, 1000 bootstrap replications were performed. The consensus tree was established by using the TreeView program (Page, 1996; version 1.6.6, available at http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

4.3.3 Mutant Isolation and Phenotype Analysis

The *Arabidopsis roc* mutants were identified using the Salk Institute Genome Analysis Laboratory (SIGNAL) database (http://signal.salk.edu/cgi-bin/tdnaexpress) and seeds were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). Wild-type (Col-0) and *roc* mutant seedlings were grown vertically for four days on 0.5X germination medium in the dark. The plates were rotated 90° and the seedlings were incubated for another 12 or 24 h in the dark. Seedlings were scanned and the curvature and length of hypocotyls were measured using ImageJ software (version 1.29X; http://rsb.info.nih/gov/ij/).

4.3.4 GFP Expression Plasmid and Transformation

To construct the GFP-LeCYP1 fusion, *LeCyp1* coding region was directionally cloned into the *EcoR*I and *Kpn*I sites of the GFP-expressing plasmid

pMGFP (Ivanchenko et al., 2000). A *LeCyp1*-specific primer pair, 5'-GGA ATT CAG AGA AAT GGC AAA TCC AAA AGG TA-3' and 5'-GGG GTA CCA GAC TGA TAA AAC TAG ATC ATC AT-3', containing *EcoR*I and *Kpn*I sites, respectively, was used to amplify *LeCyp1* coding regions with *Pfu* polymerase (Stratagene). Bioloistic bombardment and transient expression of the construct in maize leaves was performed as described by Ivanchenko et al. (2000). Immature leaves were dissected from 1 to 2-month-old maize plants (inbred line W22). Sections of 5 to 15 mm were taken from the base of the leaf blade and placed, abaxial side up, on MS medium (Life Technologies) containing 3% (w/v) sucrose and 1.5% (w/v) Phytagar (Life Technologies). Transformations were performed with a PDS-1000 helium biolistic system (Bio-Rad) using the manufacturer's protocol with a tungsten 17 microcarrier and a helium pressure of 1100 psi. Transient expression of GFP fluorescence was observed using confocal laser scanning microscopy (Leica DM IRBE with a 100X/NA1.4 objective) equipped with the TCS 4D software and scanning package. GFP fluorescence was imaged using an Ar/Kr laser with the standard Leica FITC settings. Further image processing was done in Photoshop 7 (Adobe).

4.3.5 *AtIAA* Expression Assays

Arabidopsis thaliana plants Landsberg erecta (Ler) and Wassilewskija (Ws) harboring the $P_{IAA3/SHY2}$:: *GUS* (Tian et al., 2002) and P_{IAA28}:: *GUS* (Rogg et al., 2001) constructs, respectively, were plated on germination media as described. Seven-day-old etiolated or light-grown seedlings were transferred onto

germination media containing the indicated concentration of 1-naphthalene acetic acid (1-NAA) and/or cyclosporin A (Fluka). The seedlings were grown further for five days in the same light conditions. For sanglifehrin A (kindly donated by Dr. Gerhard Zenke, Novartis Pharma AG) treatments, five-day-old light-grown seedlings of *Arabidopsis thaliana* plants Landsberg erecta (Ler) harboring $P_{IAA3/SHY2}$*:: GUS* were transferred onto germination media containing the indicated concentrations of cyclsorin A or sanglifehrin A. The seedlings were grown further for two days in the same light conditions. Histochemical staining was performed as described by Guivarc'h et al. (1996) with slight modifications. Tissues were fixed in 90% (v/v) acetone:water for 20 min, rinsed in 50 mM sodium phosphate buffer, pH 7.2, containing 2 mM potassium ferricyanide and 2 mM potassium ferrocyanide, and then transferred to the same solution containing 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc). Tissues were vacuum-infiltrated for 5 min and then incubated at 37°C for 16 h in the same solution. Chlorophyll was extracted by using 80% ethanol prior to determining GUS expression patterns.

4.4 Results

4.4.1 Sequence and Phylogenetic Analyses of Plant Cyclophilins

To identify the candidate *Arabidopsis* ortholog for LeCYP1/DGT, we analyzed 28 genes annotated or identified as cyclophilin in the *Arabidopsis* genomic database (Chapter 3). Phylogenetic analysis of plant CYP domains

revealed that five genes (*AtRoc1, 2, 3, 5,* and *6*) are delimited in the cytosolic CYP clade. The PPIase domain of the five *Arabidopsis* and two tomato cytosolic CYP/ROCs share 73 to 81% amino acid identity and 83 to 91% similarity with very low e-values (at least $<e^{-78}$, Table 4.1). The high conservation of the PPIase domain is retained in other types of CYPs such as AtROC4, 7, 11, and 18 (Table 4.1 and Figure 4.1). Among the five cytosolic AtROCs, ROC1, 5, and 6 are present within regions that are microsyntenic to the LeCYP1/DGT region in tomato (Oh et al., 2002). Based on the protein sequence similarities between LeCYP1/DGT and the AtROCs (Table 4.1), any cytosolic AtROC, other than AtROC2, could be the functional ortholog of LeCYP1/DGT.

The PPIase domain primary sequence is highly conserved throughout the cyclophilin family with the exception of a seven amino acid gap and an extra amino acid residue in AtROC4 and AtROC18/SQN, respectively (Figure 4.1). When the PPIase domain of the cytosolic CYPs are aligned against other types of plant CYPs (Figure 4.1), including AtROC4 (Lippuner et al., 1994), AtROC7 (Jackson and Söil, 1999), AtROC11/CYP5 (Grebe et al., 2000), and AtROC18/CYP40/SQN (Berardini et al., 2001), the PPIase domain of each non-cyotsolic CYP/ROC exhibits 74 to 78% similarity to LeCYP1/DGT (Figure 4.1). Using ClustalX alignment, a Neighbor-Joining tree of cytosolic CYP/ROCs was constructed with 1000 bootstrap repetitions (Figure 4.2). While the percent bootstrap values clearly support two subgroups in the cytosolic CYP clade, poorly supported branches within Group 1 indicate that any cytosolic AtROC, other than AtROC2, could be the functional ortholog to LeCYP1/DGT.

Table 4.1 Comparison of tomato and *Arabidopsis* cyclophilins

	AGI/ Accession	Size [b]	Subcellular localization Signal[c]					I/S/E [d]
			CP	MT	SP	Other	NLS	
AtROC1	At4g38740	172	0.089	0.089	0.165	**0.867**	-	$80/89/2e^{-84}$
AtROC2	At3g56070	176	0.056	0.051	0.538	**0.608**	-	$76/83/5e^{-78}$
AtROC3	At2g16600	173	0.097	0.095	0.150	**0.825**	-	$81/91/3e^{-86}$
AtROC4	At3g62030	260	**0.927**	0.084	0.013	0.049	-	$70/78/1e^{-61}$
AtROC5	At4g34870	172	0.134	0.106	0.228	**0.682**	-	$79/88/7e^{-83}$
AtROC6/CYP2	At2g21130	174	0.172	0.055	0.167	**0.849**	-	$81/91/4e^{-86}$
AtROC7	At5g58710	204	0.012	0.107	**0.951**	0.068	-	$70/77/1e^{-69}$
AtROC11/CYP5	At2g29960	201	0.009	0.046	**0.981**	0.126	-	$71/78/1e^{-70}$
AtROC18/SQN	At2g15790	361	0.087	0.077	0.202	**0.867**	-	$62/74/1e^{-60}$
LeCYP1	P21568	171	0.102	0.089	0.230	**0.809**	-	
LeTC102127		175	0.162	0.051	0.266	**0.780**	-	$73/83/2e^{-74}$

BL2SEQ was used for BLAST comparison of LeCYP1 to each *Arabidopsis* CYP in Biology Workbench (http://workbench.sdsc.edu). The scoring matrix, gap opening penalty, and gap extension penalty of the BLAST analysis were PAM70,10, and 1, respectively.

a. AGI code is indicated for each *Arabidopsis* CYP; The accession number for proteins deduced from ESTs are not provided.
b. Number of amino acid residues.
c. Peptide signals were determined by TargetP v1.01 (Emmanuelson et al., 2000; http://www.cbs.dtu.dk/services/TargetP/) and predictNLS (Cokol et al., 2000; http://cubic.bioc.columbia.edu/predictNLS/). The location with the highest score, in bold and underlined, is the most likely one according to TargetP. Abbreviations: CP, chloroplast; MT, mitochondria; SP, secretory pathway; NLS, nuclear localization signal.
d. I/S/E = identity (%) / similarity (%) / e-value

Figure 4.1. ClustalX alignment of tomato and *Arabidopsis* cyclophilins. Black boxes denote conserved amino acid residues (>70%) in 2 tomato (Le) and 9 *Arabidopsis* (At) cyclophilin sequences. The PPIase domain of each CYP is indicated with tildes (~).

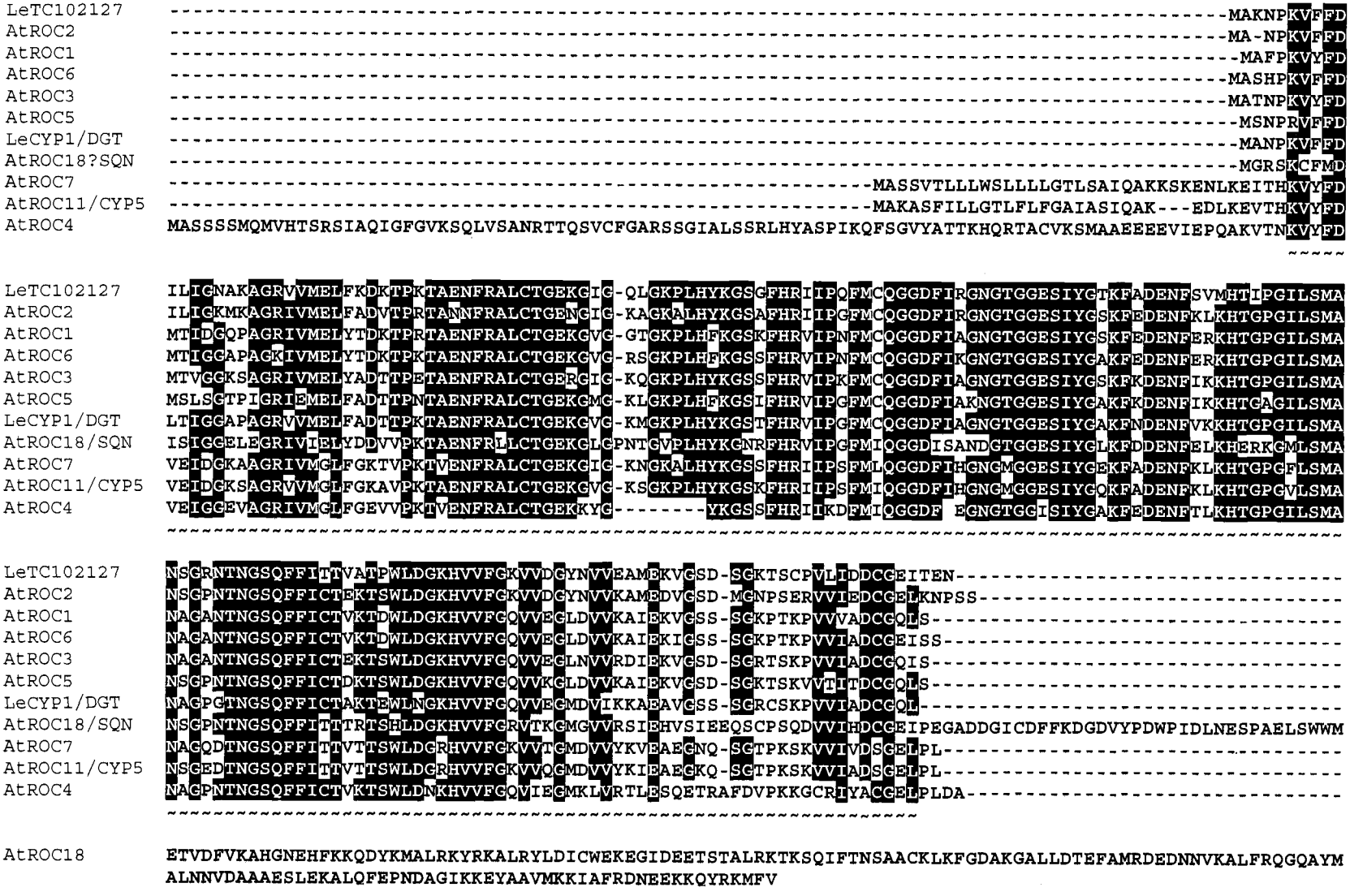

LeTC102127
AtROC2
AtROC1
AtROC6
AtROC3
AtROC5
LeCYP1/DGT
AtROC18?SQN
AtROC7
AtROC11/CYP5
AtROC4
LeTC102127
AtROC2
AtROC1
AtROC6
AtROC3
AtROC5
LeCYP1/DGT
AtROC18/SQN
AtROC7
AtROC11/CYP5
AtROC4
LeTC102127
AtROC2
AtROC1
AtROC6
AtROC3
AtROC5
LeCYP1/DGT
AtROC18/SQN
AtROC7
AtROC11/CYP5
AtROC4
AtROC18

Figure 4.2. Phylogenetic tree of cytosolic cyclophilins in tomato and *Arabidopsis*. ClustalX alignment of the PPIase domains was used to construct an unrooted phylogenetic tree by the Neighbor-Joining method using ClustalX and TreeView software. Percent bootstrap values are indicated on each branch. The scale represents a distance of 0.1 units.

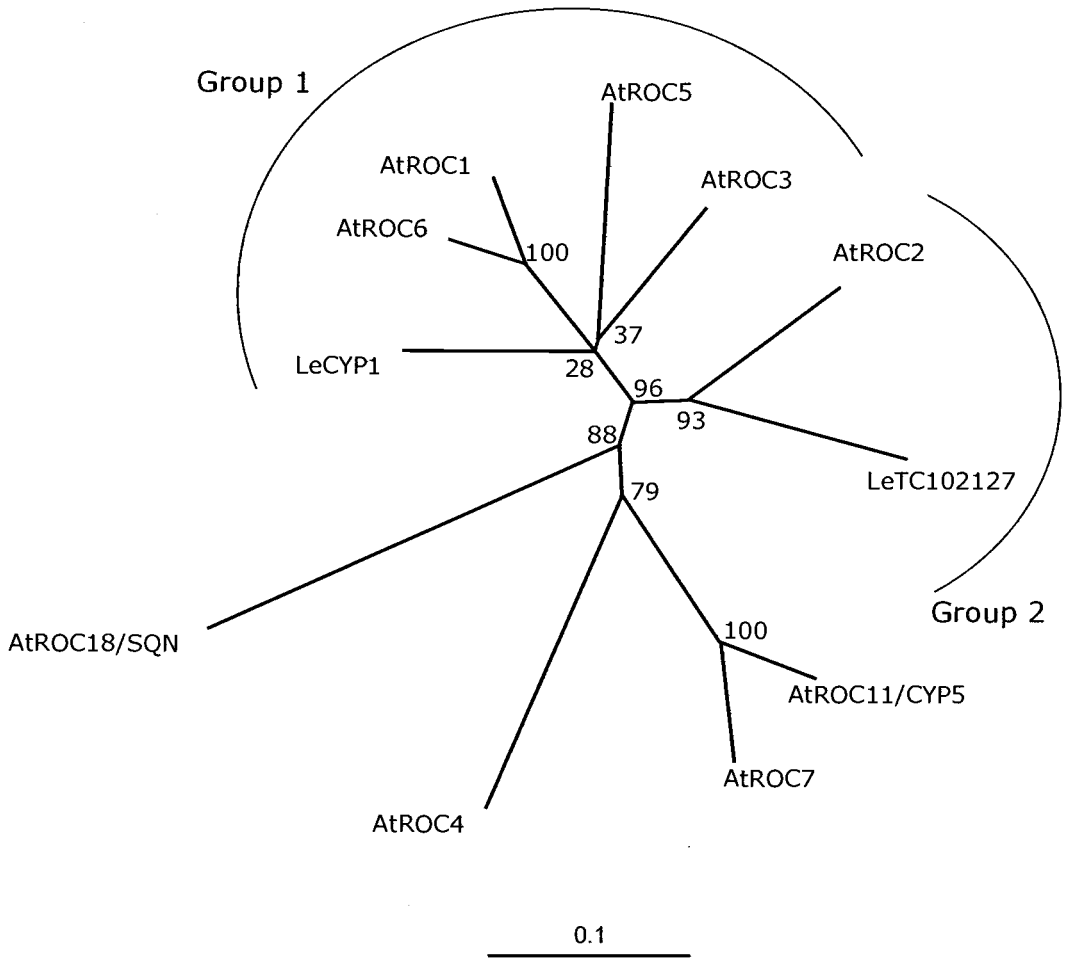

Group 1
AtROC5
AtROC1
AtROC3
AtROC6
100
AtROC2
37
LeCYP1
28
96
93
88
LeTC102127
79
Group 2
AtROC18/SQN
100
AtROC11/CYP5
AtROC7
AtROC4
0.1

Figure 4.2

4.4.2 Isolation and Characterization of *AtROC* T-DNA Insertion Mutants

Mutations in the *LeCyp1/DGT* gene result in a dramatic phenotype, including lack of lateral roots, reduced hypocotyls and internode length, and retarded gravitropic response. To determine if any of the *Arabidopsis* ROC proteins carry out a function similar to LeCYP1/DGT, plants carrying mutations in different *Roc* genes were analyzed to see whether they display a phenotype similar to *dgt*. Eleven T-DNA-tagged lines, each carrying an insertion in the 5′ untranslated region (UTR), exon, or 3′ UTR of a cytosolic *Cyp/Roc* gene, were identified from the Salk Institute T-DNA Express database (data not shown). A The database arbitrarily defines the UTRs and promoter sequences as 300 bp upstream (5′) or downstream (3′) of the open reading frame and 1000 bp upstream from the 5′-UTR sequence, respectively. Only the *Atroc2-1* and *Atroc3-1* mutant alleles have T-DNA insertions in the coding sequence. All other *Atroc::T-DNA* alleles have T-DNA insertions in either the UTR or promoter sequence.

Five mutations, *Atroc1-1*, *2-1*, *3-1*, *3-2*, and *6-1*, were chosen for phenotypic analysis with respect to presence or absence of lateral roots, hypocotyl length, and gravitropic response in etiolated seedlings (Figure 4.3). Since *Atroc5* mutant was not available in SIGNAL database among four Group 1 *AtRoc* genes, *Atroc5* was not included in phenotypic analysis. *Arabidopsis* wild-type and *roc* mutants were similar with respect to numbers of lateral roots and primary leaves and hypocotyl length. Light-grown wild-type and mutant plants have almost identical numbers of lateral root and leaves at 22 days (Figure 4.4A) and the hypocotyl elongation is similar in 5-d-old etiolated wild-type and mutant plants

Figure 4.3. *roc::T-DNA* alleles used for analyzing the effect of mutagenesis of *Arabidopsis* cyclophilin genes. (A) *Atroc1.* (B) *Atroc2.* (C) *Atroc3-1* and *3-2.* (D) *Atroc6.* Arrowheads in T-DNA symbols represent the direction of T-DNA insertion.

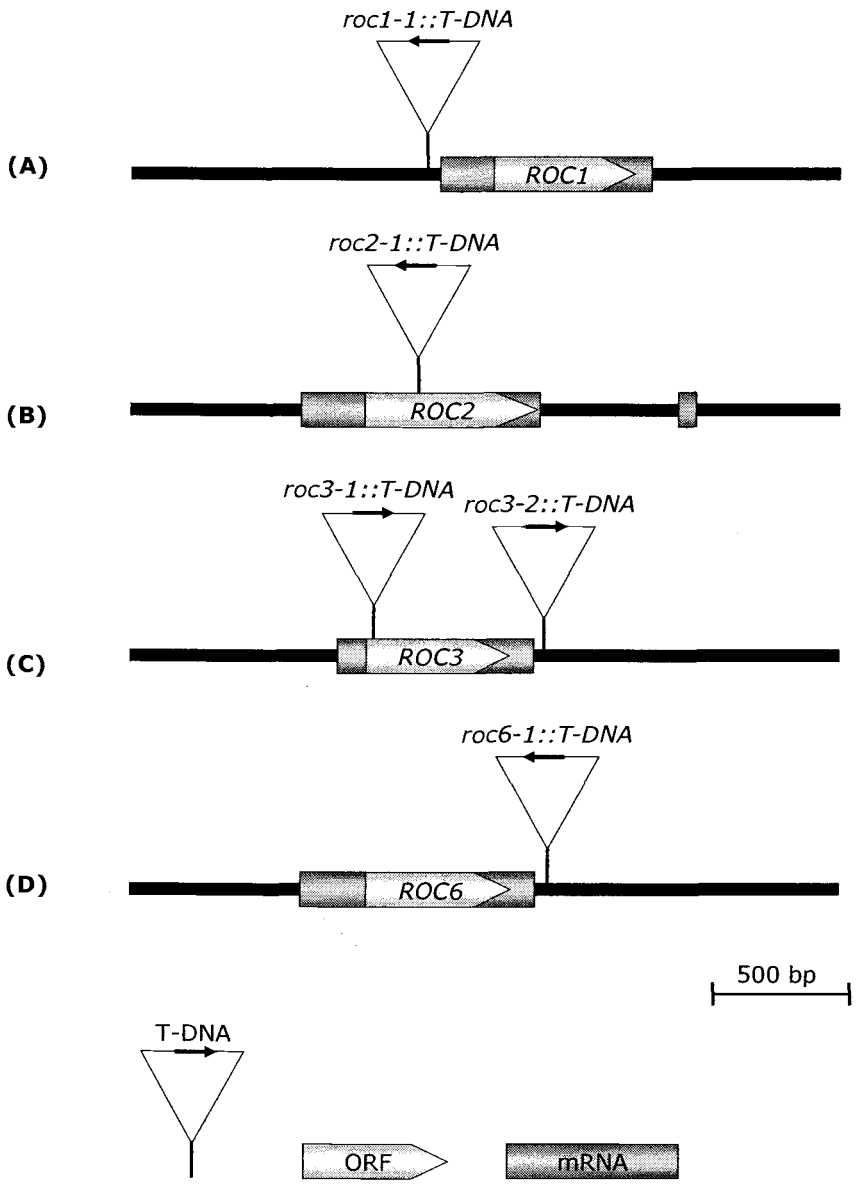
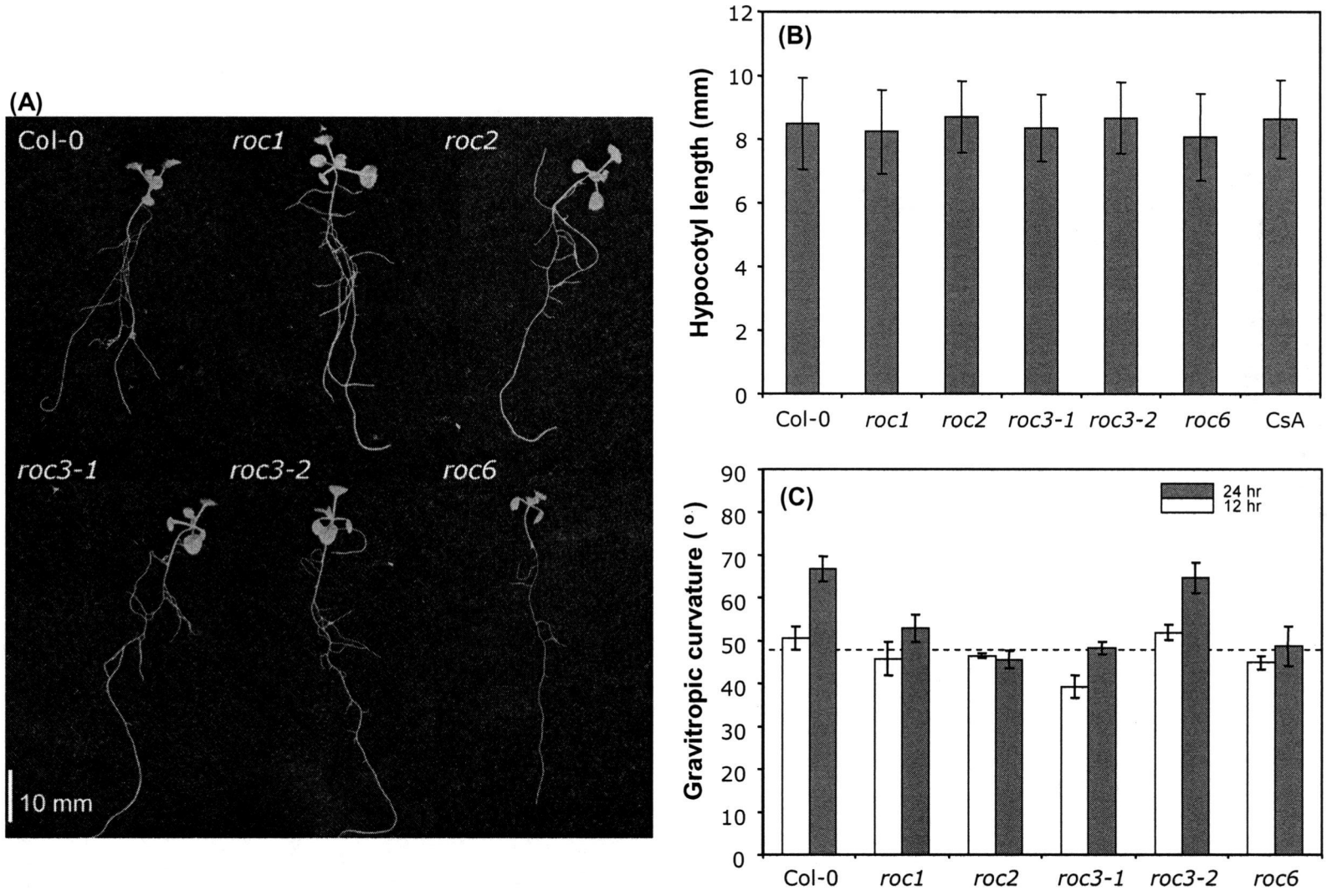

roc1-1::T-DNA
(A)
ROC1
roc2-1::T-DNA
(B)
ROC2
roc3-1::T-DNA
roc3-2::T-DNA
(C)
ROC3
roc6-1::T-DNA
(D)
ROC6
500 bp
T-DNA
ORF
mRNA

Figure 4.3

Figure 4.4. Phenotype of the *Arabidopsis roc* mutants. (A) Lateral root initiations of 22-d-old *roc* mutants grown on germination medium. All *roc* mutants analyzed appeared to be similar to wild-type seedlings in overall phenotype and number of lateral roots. (B) Hypocotyl lengths of 5-d-old etiolated seedlings were measured. Vertical bars represent standard deviations. There was no significant difference between the mean values of wild-type, *roc* mutants, and CsA–treated (10 μM) wild-type seedlings ($n = 60$ to 90; t- test, $p > 0.05$). (C) The gravitropic responses of 4-d-old wild-type and mutants measured to 12 or 24 h gravistimulation in the dark. The dotted line represents the mean value of the gravitropic responses of wild-type seedlings treated with 10 μM CsA after 24h. The gravitropic response of the mutants was stastically different from the wild-type response ($p < 0.05$) by t-test, except for *Atroc3-2*.


(A)
Col-0
roc1
roc2
roc3-1
roc3-2
roc6
10 mm
(B)
Hypocotyl length (mm)
0
2
4
6
8
10
12
Col-0
roc1
roc2
roc3-1
roc3-2
roc6
CsA
(C)
24 hr
12 hr
Gravitropic curvature (°)
0
10
20
30
40
50
60
70
80
90
Col-0
roc1
roc2
roc3-1
roc3-2
roc6

(Figure 4.5B). Hypocotyl elongation and lateral root formation were also not affected by CsA treatment of wild-type seedlings (Figure 4.4B). However, the gravitropic responses of 4-d-old etiolated *Atroc* mutant plants was reduced to the level of wild-type plants treated with 10 μM CsA with the exception of *Atroc3-2::T-DNA* (Figure 4.4C).

4.4.3 LeCYP1 Translocation Without Nuclear Localization Signal

The TargetP program (Emanuelson et al., 2000) was used to identify any putative targeting signals contained within cytosolic AtROC/CYPs and LeCYP1/DGT, and the related tomato homeolog LeTC102127 (Table 4.1). Among the larger cyclophilns, AtROC4 contains a chloroplast targeting signal and AtROC7 and AtROC11 contain secretory pathway signals, but none of the smaller, putative cytosolic cyclophilins contain targets to major organelles or nuclear localization signals. Interestingly, the carboxy-terminus of the LeCYP1/DGT sequence contains a putative, cryptic prenylation motif CxxL, where C is Cys, x is usually an aliphatic residue, and L is Leu, (Hayman and Miernyk, 1994). Prenylation motifs are found in small GTPase proteins located in the cytoplasm as well as in the plasma membrane (Ivanchenko et al., 2000; Molendijk et al., 2001). Other cytoplasmic CYP/ROCs have the cryptic CxxL, motif followed by a few extra amino acids at the C-terminal end (Figure 4.1).

To test whether LeCYP1/DGT could be targeted to the nucleus without a nuclear localization signal, biolistic bombardment of a green fluorescent protein (GFP) fusion of LeCYP1, GFP::LeCYP1, was used in transient expression analysis in maize leaf epidermal cells. The GFP::LeCYP1 fusion protein was predominantly

distributed in the nucleus whereas there was no visible nuclear localization of the GFP control (Figure 4.5, comopare A-C with D-F). However, fusion proteins were also identified in the crenulated area of maize plasma membrane as well as in the cytoplasm in vesicles or aggregates that circulate with cytoplasmic streaming (Figure 4.5A-C).

4.4.4 Cyclosporin A differentially affects expression of AUX/IAA genes

Since LeCYP1/DGT affects a subset of *LeIAA* genes (Nebenführ et al., 2000; Balbi and Lomax, 2003), we tested the effect of CsA, a CYP inhibitor, on *AUX/IAA* gene expression in *Arabidopsis* using GUS reporter fusions of *AtIAA3/SHY2* and *AtIAA28*. In wild-type *Arabidopsis*, light-responsive*AtIAA3/SHY2* expression is up-regulated by auxin application (Tiwari et al., 2001), whereas *AtIAA28* mRNA levels decrease slightly in response to exogenous auxin treatment (Rogg et al., 2001). We compared the expression of these genes in response to 10 μM CsA in the presence or absence of exogenous auxin. Figure 4.6 shows the X-gluc staining of 12-day-old wild-type seedlings carrying the $P_{IAA3/SHY2}$*::GUS* or P_{IAA28}*::GUS* transgene (Tian et al., 2002; Rogg et al., 2001). The seedlings were grown for 7 days in light or dark and subsequently placed on germination medium in the presence or absence of 0.5 μM 1-NAA and/or 10 μM CsA. After 5 d incubation, expression of the $P_{IAA3/SHY2}$*::GUS* and P_{IAA28}*::GUS* constructs was examined by whole mount GUS staining.

CsA appeared to affect the gene expression pattern of *AtIAA3/SHY2* but not that of *AtIAA28*. *AtIAA3/SHY2* expression was repressed in hypocotyls of 12-

Figure 4.5. LeCYP1/DGT is located in both the nucleus and cytoplasm. (A-C) Three confocal planes of a maize cell expressing GFP::LeCYP1 fusion proteins. rrowheads in (C) indicate the aggregated form of GFP::LeCYP1 protein. (D-F) Three confocal planes of the GFP control. N: nucleus; V: vacuole; horizontal bar = 10 μm.

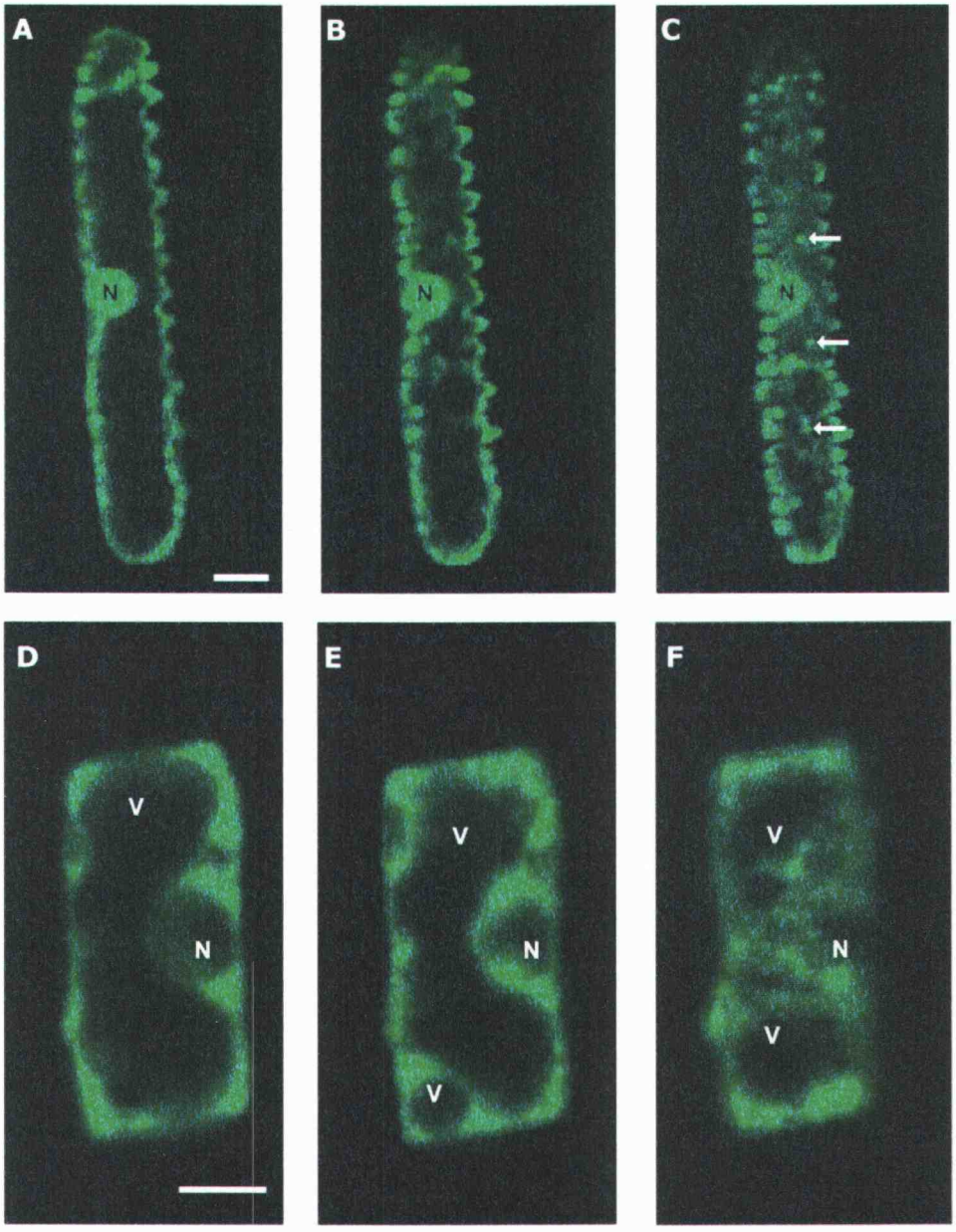


Figure 4.5

Figure 4.6. Histochemical analysis of $P_{IAA3/SHY2}$::*GUS* and P_{IAA28}::*GUS* expression. Fifteen to twenty 7-d-old seedlings grown in dark or light conditions were further incubated on germination medium containing 0.5 μM 1-NAA and/or 10 μM CsA. Auxin-induced *IAA3* expression is repressed by CsA whereas auxin-induced *IAA28* expression is unaffected. D: dark-grown seedling; L: light-grown seedling; Vertical bars: 5 mm.

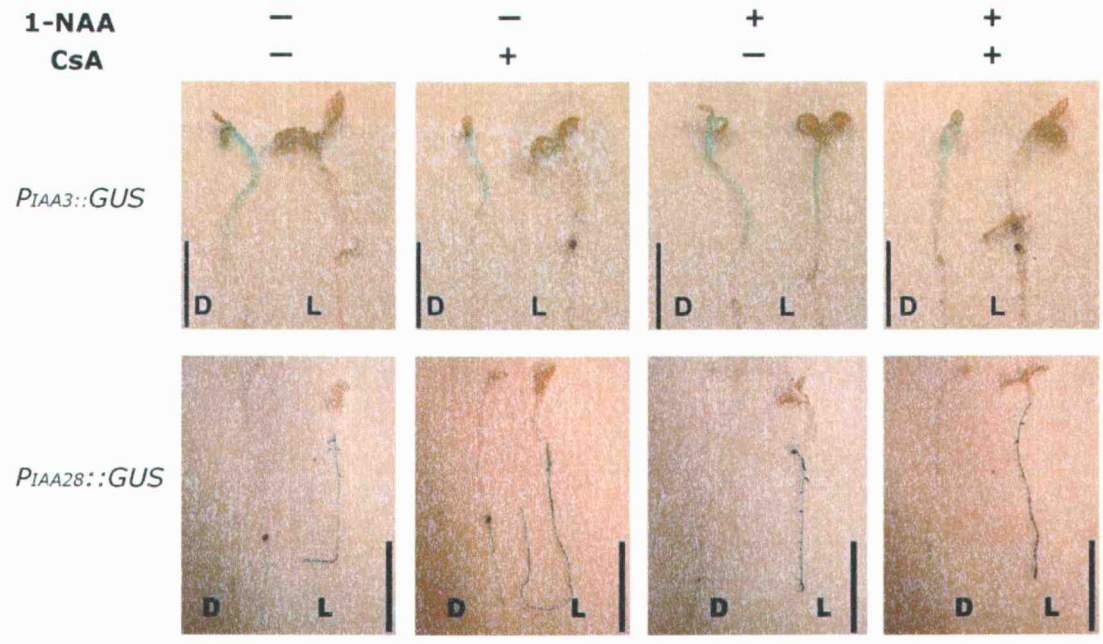


Figure 4.6

d-old light-grown seedlings. Application of exogenous auxin induced *AtIAA3/SHY2* expression, which could be inhibited by treatment with CsA. *AtIAA3/SHY2* was expressed in hypocotyls of etiolated seedlings, regardless of treatment. The *AtIAA28* promoter construct was expressed under all treatment conditions. However, roots expressed *AtIAA28* more strongly in etiolated than those of light-grown seedlings. The *IAA28* expression did not appear to be responsive to exogenously-applied auxin or CsA.

Another immunosuppressive drug, sanglifehrin A (SFA) binds to CYPs with a higher affinity than CsA (Sanglier, 1999) and inhibits PPIase activity, but not calcineurin activity (Zenke et al., 2001). Five-day-old, light-grown *Arabidopsis* seedlings, harboring *PIAA3/SHY2::GUS* constructs, were treated with CsA and SFA for two days to test whether calcineurin-like activity is involved in the expression of *IAA3/SHY2* (Figure 4.7). *GUS* expression driven by the *IAA3/SHY2* promoterwas detected in the apices, hypocotyls, and cotyledon petioles of light-grown 7-d-old seedlings in the absence of exogenous auxin (Figure 4.7) but it was not detected in 12-d-old seedlings (Figure 4.6). Exogenous application of 10 μM SFA partially repressed the expression of *PIAA3/SHY2::GUS* constructs whereas CsA completely represses *IAA3/SHY2* expression at the same concentration.

4.5 Discussion

Our exciting discovery that the *DGT* gene encodes LeCYP1, a previously unknown component of auxin signaling (Chapter 3), now permits investigation into the role(s) of CYP(s) in auxin signal transduction. Since similar proteins

Figure 4.7. Histochemical analysis of $P_{IAA3/SHY2}$*::GUS* expression in the presence or absence of CsA and SFA. 5-d-old light grown seedlings were grown for 2 d on germination medium containing the indicated concentration of CsA or SFA. Horizontal bars represent 5 mm.

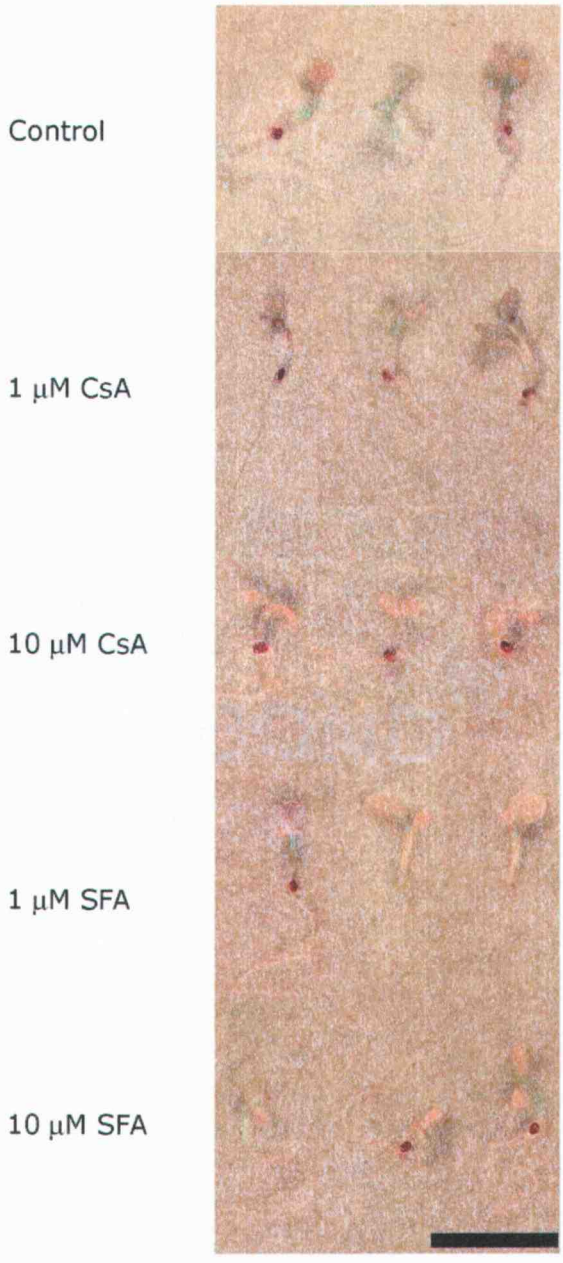


Figure 4.7

having similar functions are encoded by orthologous genes between different species (Peng *et al.,* 1999) and since many mutants of potential target proteins are available in *Arabidopsis*, I sought to characterize the phenotype of orthologous *Atroc* mutants. The *Arabidopsis* genome encodes 28 *Cyp/Roc* genes including five cytosolic *Cyp/Roc* genes while the tomato genome may encode as least 10 *Cyp/Roc* genes including two cytosolic *Cyp/Roc* genes (Chapter 3). The unrooted Neighbor-Joining phylogenetic tree clearly delimited (with 96% bootstrap support) two groups of putative cytosolic CYP/ROCs, Groups 1 and 2, suggesting that Groups 1 and 2 CYP/ROCs diverged before speciation. This hypothesis is also supported by previous phylogenetic analyses (Chou and Gasser, 1997; Chapter 3). However, the poor bootstrap differentiation of different members of the Group 1 CYP/ROCs hampers identification of the functional ortholog(s) of LeCYP1/DGT in *Arabidopsis.* Searches of *Arabidopsis* T-DNA insertion mutants identified 11 *Atroc::T-DNA* mutant alleles in *Atroc1, 2, 3,* and *6.* Five T-DNA-insertion alleles were chosen to determine any phenotypic differences and to identify the functional ortholog. However, our results demonstrated that, although most *Atroc* mutants exhibited a slight change in gravitropic response, they did not display obvious phenotypic differences from wild-type (Figure 4.5). These results may be due to compensation by other cytosolic CYP/ROC family members within the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000). Single or double knockout mutants of three brassinosteroids (BR) response mutants (*bee1, 2,* and *3*) do not have any obvious developmental or hormone response phenotypes whereas the *bee1 bee2 bee3* triple mutant had a light-grown developmental phenotype that resembled a weak BR response mutant (Friedrichsen et al., 2002).

Likewise, creating multiple knockout *cyp/roc* mutants may destroy potential functional redundancy and produce obvious phenotypic differences in *Arabidopsis*. Also, further phenotypic analysis of the T-DNA-tagged lines, with respect to auxin responsiveness, adventitious rooting, pigmentation, and auxin-regulated gene expression, need to be performed to identify more subtle similarities with the tomato *dgt* mutant.

Phenotypic similarities of the *Ataux/iaa* mutants, together with the demonstration that the *dgt* lesion alters the expression of subsets of *LeAUX/IAA* genes (Nebenführ et al., 2000; Balbi and Lomax, 2003), indicates that LeCYP1/DGT selectively operates in common pathways with some AUX/IAA proteins that operate in negative transcriptional feedback loops during auxin-induced transcription (Tiwari et al., 2001; Zenser et al., 2001). However, information about the subcellular localization and target of cytosolic CYP/ROCs is still unknown in plants. In mammalian cells, cytosolic CYPs, in association with CsA, inhibits CaN activity required for the nuclear import of nuclear factor of activated T-cells (NFAT, Luo et al., 1996). Alternatively, CPR1P, a cytosolic CYP of yeast, regulates the nuclear export of the cytoplasmic zinc finger protein ZPR1 (Ansari et al., 2002), suggesting the possible localization of at least some cytosolic-type CYPs in the nucleus. In addition, nuclear localization of cytosolic CYPs has been reported in mammalian cells (Ryffel et al., 1991; Montague et al., 1997). These findings suggest that LeCYP1/DGT might be translocated to the nucleus, even without having a nuclear localization signal (NLS), as determined by signaling motif prediction programs (Table 4.1). Our demonstration that transiently overexpressed GFP::LeCYP1 is detected in the nucleus, cytoplasm, and

plasma membrane (Figure 4.3) supports this hypothesis and suggests multiple roles of LeCYP1/DGT with respect to auxin signaling. Interestingly, the fusion protein was not evenly distributed in the cytosol but formed aggregates associated with cytoplasmic streaming, possibly contained within cytoplasmic inclusion bodies (Stacey et al., 1999). Whether the subcellular localization patterns of LeCYP1/DGT are unique within plant cytosolic CYP/ROC proteins and whether interactions between LeCYP1/DGT and its protein partners depends upon its subcellular localization remains unknown.

The AUX/IAA proteins play critical roles as transcriptional repressors in auxin-induced gene expression. Expression of AUX/IAA proteins in tomato is sensitive to exogenous application of the CYP inhibitor, CsA (Chapter 3). Therefore, we tested whether the expression of promoter::GUS constructs of *AtIAA3/SHY2* and *AtIAA28* genes were also affected by CsA. Differential repression of the AUX/IAA gene expression in response to CsA (Figure 4.6) supports the selective regulation of AUX/IAA proteins by CYPs. This may be due to substrate specificity of CYP upstream of *AUX/IAA* gene expression. There is emerging evidence that some individual PPIase domains of the cyclophilin, FKBP, and parvulin families have restricted substrate-protein specificity (reviewed in Hunter, 1998) and recognize different motifs or domains in a substrate protein (Deng et al., 1998). For example, the *Drosophila* cyclophilin, NinaA, is required for correct localization of only two of the numerous *Drosophila* eye rhodopsins (Baker et al., 1994). Similarly, the VirD2 protein of *Agrobacterium* requires different domains for interacting with AtROC1 and AtROC5 (Deng et al., 1998).

Calcineurin, the primary target of CYP action in mammalian and fungal

cells, contains catalytic (A) and regulatory (B) subunits. The B subunit acts as a Ca^{2+} sensor protein that, like calmodulin, contains four Ca^{2+}-binding EF-hand motifs. Although no plant gene has been identified with a regulatory subunit that resembles animal calcineurin (reviewed in Luan et al., 2002), calcineurin B subunit-like proteins (CBLs) are encoded by a multigene family of at least 10 members in *Arabidopsis* (Luan et al., 2002). There is growing evidence that CBL activities are involved in plant signaling in response to environmental stresses. For example, yeast Ca^{2+}-dependent calcineurin expressed in tobacco appears to mediate salt stress signaling (Pardo et al., 1998). Ca^{2+}-dependent phosphatase activity is inhibited by CYP-CsA complexes in the guard cell cytoplasm (Luan et al., 1993). CBL could be the target of CsA since AtCBL1 interacts with the rat calcineruin A subunit in the yeast two-hybrid system and complements a yeast calcineurin B mutant (Kudla et al., 1999). However, the involvement of calcienurin-like activity in auxin signaling still remains unknown.

Sanglifehrin A (SFA) belongs to a novel family of immunophilin-binding ligands (Sanglier et al., 1999). Sanglifehrin A binds to CYP with 100-fold higher affinity than CsA and inhibits PPIase activity. Unlike CsA, the cyclophilin-sanglifehrin A complex has no effect on calcineurin (Zenke et al., 2001). Therefore, SFA was used to determine if the CYP/ROC action that regulates *AtIAA3/SHY2* expression requires CBL activity. The *AtIAA3/SHY2* expression appeared to be partially suppressed by the SFA application (Figure 4.7), suggesting that CBL or CBL-like activity may be required for AUX/IAA gene expression by regulating the nuclear targeting of transcription factors, similar to how calcineurin directs the nuclear targeting of NFAT upon cytokine elicitation in mammalian cells. CBLs have

been shown to interact with a single family of protein kinases, CBL-interacting kinases (CIPKs), which mediate signal transduction pathways for Ca^{2+}, abscisic acid, and cold stress (Shi et al., 1999; Kim et al., 2000; Kim et al., 2003). The conserved myristoylation site in the N-terminal region of several CBLs suggests that CBLs are localized to the plasma membrane (Liu and Zhu, 1998; Kudla et al., 1999; Kim et al., 2000, Albrecht et al., 2001; Luan et al., 2002). Thus, CBLs are not a tempting hypothetical target protein to be regulated by the nuclear-localized LeCYP1/DGT. However, we observed LeCYP1 localization in the cytoplasm and crenulated areas of the plasma membrane in maize cells (Figure 4.3). Two RHO GTPases, AtRAC7 and 8, contain CTAA and CGKN motifs, respectively, at the carboxy-terminus and are found in the cytoplasm as well as in the plasma membrane (Lavy et al., 2002). Targeting analyses of prematurely terminated proteins lacking their last residue demonstrated that the C-terminal CTA and CGK sequence motifs, instead of CTAA and CGKN, respectively, still target the mutant proteins to the plasma membrane. These partial motifs were believed not to require prenylation for the association of AtRAC7 and AtRAC8 with the plasma membrane. The CGKN motif of AtRAC8 required for palmitoylation is highly similar to the CGQL motif of the LeCYP1/DGT protein that may act as a site for palmitoylation instead of prenylation. If the CGQL motif at the carboxy-terminal of LeCYP1/DGT functions as a prenylation or palmitoylation site for plasma membrane targeting, interaction between LeCYP1/DGT and CBL may occur at the plasma membrane (Figure 4.1).

Interactions between CYP/ROCs and many nuclear-targeted proteins such as zinc finger transcription factors (Krummrei et al., 1995), hormone receptors

(Rycyzyn and Clevenger, 2002) and histone acetylase complexes (Arévalo-Rodriguez et al., 2000) in mammalian and yeast cells suggest that CYP/ROCs may regulate the nuclear import or export of auxin response factors, the transcriptional repressor AUX/IAA proteins, or other unknown transcription factors involved in auxin-regulated gene expression. Interestingly, the tobacco PSA1 protein, a functional proteasome subunit, is localized in the nucleus and antisense expression of *NtPSA1* resulted in reduced apical dominance and resistance to exogenously-applied auxin (Bahrami et al., 2002). This suggests that ubiquitin-dependent degradation of transcriptional repressor AUX/IAA proteins is mediated by a nuclear-localized proteasome. It also suggests that the nuclear-localized CYP/ROC protein may play a role in the ubiquitin-dependent protein degradation step or in substrate modification such as phosphorylation prior to proteasomal degradation (Colón-Carmona et al., 2000).

The predominant nuclear localization of the LeCYP1/DGT protein, coupled with the selective CsA-inhibition of *AUX/IAA* gene expression, suggests a role for LeCYP1/DGT in the transcriptional regulation of primary auxin-response genes. However, further studies must be conducted to decipher the role of LeCYP1/DGT in auxin signal transduction. Comparison of subcellular localization of *Arabidopsis* ROC proteins could indicate different functional roles, based on localization. Also, further genetic analyses of multiple knock-out *ROC* mutants, as well as identification of the target or interacting partner protein(s) of LeCYP1/DGT, is required to elucidate the role of CYP action in auxin signaling.

4.6. ACKNOWLEDGEMENTS

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5. CONCLUSIONS

Plant growth and development are regulated by plant hormones that perceive environmental signals and transduce the signals to the endogenous developmental machinery during the plant's life cycle. In particular, auxins regulate a variety of processes at the cellular and organ level, such as cell division, elongation, differentiation, tropic responses, and apical dominance. Those processes are established by an auxin gradient through the primary axis of the plant, which controls the architecture of the entire plant. Thus, auxins are often considered a plant 'morphogen'. How the auxin signal is perceived and transduced into cellular and organismal level responses is one of the most fundamental questions for understanding plant and growth development. Our knowledge of the auxin-response-mechanism components and their actions in auxin homeostasis, perception, and auxin-response gene expression is being expanded by molecular and genetic analyses of auxin-related mutants. Although remarkable progress has been made in understanding auxin-induced gene regulation involving the transcriptional activators and repressors, ARFs and AUX/IAAs, respectively, as well as the ubiquitin-dependent degradation of AUX/IAA proteins, there are still many missing links in the mechanism of auxin action (Chapter 1).

The single-gene auxin-resistant *dgt* mutant of tomato provides a unique opportunity to fill gaps between previously identified components of auxin signaling. Based on the pleiotropic, auxin-related phenotype of *dgt* and other physiological data, the hypothesis has been developed that the *dgt* lesion affects not auxin perception but a select subset of auxin response mechanisms. Our primary objective was to identify the *DGT* gene sequence in order to better

understand DGT-mediated auxin signaling. During the course of this work, I developed a novel and alternative method to expedite conventional high-resolution mapping, microsynteny-based comparative mapping, utilizing the known genome of *Arabidopsis* to identify genetic markers in the less well-characterized tomato genome. This microsynteny-based approach will be valuable for positional cloning of genes within other important crop species whose complete genomic information is not available. Furthermore, it will facilitate the analysis of gene evolution and divergence from an ancient progenitor. Using map-based cloning in conjunction with microsynteny-based comparative mapping (Chapter 2 and 3), I successfully determined that the *DGT* gene encodes a cyclophilin. DNA sequence and immunoblot analyses of the three *dgt* mutant alleles, along with complementation studies, confirmed the identity of the *DGT* gene (Chapter 3). Transiently-overexpressed GFP::LeCYP1/DGT protein in maize cells revealed that at least a portion of the LeCYP1/DGT protein is localized to the nucleus (Chapter 4). Taken together with the differential regulation of *AUX/IAA* gene expression by LeCYP1/DGT, this raises the possibility that LeCYP1/DGT plays a role in the transcriptional regulation of early auxin-induced genes in the nucleus.

Cyclophilins serve as the receptor for some immunosuppresive drugs and have been studied extensively in the field of animal and medical sciences. The following proposed models for cyclophilin action within plant systems are based on the results of this dissertation study, previously published results, and results obtained from the study of mammalian and yeast systems.

First, by controlling conformational changes, cyclophilins may regulate the activity of proteins, such as protein phosphorylation-dephosphorylation switches

or calcineurin B-like protein (CBL), involved in controlling the proper subcellular localization of transcription factor(s) and/or other signaling component(s) of polar auxin transport or signaling. Based on the observation that CsA has greater inhibitor effect on *AtIAA3/SHY2* expression than SFA (Chapter 4), the ROC/CYPs appear likely to require CBL or CBL-like activity for regulation of primary auxin gene expression. If so, CBL-interacting protein kinases (CIPK) may be the first target component in DGT-mediated auxin signaling, since CBL and CIPKs have been implicated in various signal transduction pathways (Liu and Zhu, 1998; Kudla et al., 1999; Albrecht et al., 2001). Under this hypothesis, cytoplasmic and membrane-bound cyclophilins, rather than predominantly nuclear cyclophilin, are likely to interact with CBL and/or membrane-bound CIPK.

Second, nuclear LeCYP1/DGT protein may interact with auxin response factors and/or other unidentified components of the transcription machinery to directly regulate gene expression. Or, LeCYP1/DGT may play a role in the ubiquitin-dependent protein degradation step or substrate modification, such as phosphorylation, prior to proteasomal degradation of the rapidly-turned over auxin transcription repressors (Colón-Carmona et al., 2000). To date, no evidence supports this suggested model in plants. However, in yeast, cytosolic CYP/CPR1P interacts with nuclear proteins such as zinc finger proteins and histone deacetylase complexes (Ansari et al., 2002; Arévalo-Rodriguez et al., 2000), all of which are involved in the regulation of gene expression. Interestingly, the histone deacetylase SIN3–RPD3 complex, a global transcriptional regulator, is antagonistically regulated by two different types of PPIases, CPR1 and ESS1. In *ess1* mutants, hyperactive SIN3–RPD3 complexes deregulate expression of genes

involved in cell cycle control, causing mitotic arrest. CPR1 reduces complex activity with mitotic targets and suppresses the cell cycle arrest of *ess1* mutants (Arévalo-Rodriguez et al., 2000). The antisense expression of *AtHD1*, a homolog of the *RPD3* global transcriptional regulator gene in yeast, results in developmental pleiotropy in *Arabidopsis* (Wu et al., 2000; Tian and Chen, 2001). Although the pleiotropic phenotype induced by the antisense-expression of *AtHD1* is not similar to the *dgt* phenotype, it is feasible that LeCYP1/DGT may regulate a global transcriptional regulator, as CPR1 and ESS1 do in yeast.

Third, since the protein fusions with GFP are observed in both the cytoplasm and plasma membrane (Chapter 4), LeCYP1/DGT may act as a chaperone to control the timely folding of proteins and/or insertion into membranes and membrane compartments. For example, following photoaffinity-labeling with the auxin azido analog [^{3}H]5-N_3IAA, two polypeptides were isolated in membrane preparations of wild-type plants but were found to be greatly reduced in *dgt* plants (Hicks et al., 1989). These labeled proteins may have been targeted to the plasma membrane by membrane-bound and/or cytosolic LeCYP1/DGT since greatly reduced amounts of labeled proteins were recovered from membrane preparations of treated *dgt* plants. The function of the two polypeptides in auxin signaling remains unknown. Additionally, there is some reported evidence that endoplasmic reticulum (ER)-type cyclophilin may be involved in auxin signaling. Two ER-type cyclophilins of *Arabidopsis*, AtROC7 and AtROC11/CYP5, interact with protein phosphatase 2A and GNOM, respectively, proteins reportedly involved in polar auxin transport (Jackson and Söil, 1999; Grebe et al., 2000; Geldner et al., 2003). Phenotypic analysis of these two *ROC*

gene mutants should be pursued to confirm the role ER-type cyclophilins play in auxin signaling.

To differentiate between these three hypotheses, identification of protein partners that interact with LeCYP1/DGT is crucial. Genetic and genomic information from *Arabidopsis* was utilized to differentiate between the hypothetical models proposed for LeCYP1/DGT. I initated experiments to identify a functional ortholog(s) of LeCYP1/DGT in *Arabidopsis* and to characterize individual *Atroc/cyp* mutants (Chapter 4). Preliminary phenotypic analyses of the currently available *Atroc/cyp* mutants, along with phylogenetic analysis of AtROC proteins, were not conclusive in determining a functional ortholog to LeCYP1/DGT. This may be due to the redundant nature of *Roc/Cyp* genes in the *Arabidopsis* genome. Perhaps, RNAi technology, a more efficient post-transcriptional gene silencing method (Hamilton and Baulcombe, 1999; Bass, 2000), or creating multiple *cyp/roc* mutants by crossing single *cyp/roc* alleles can be applied to silence multiple cyclophilins at once in *Arabidopsis*. Further genetic, biochemical, and molecular analyses of *Arabidopsis roc/cyp, cbl*, and putative target protein mutants are required to better understand the role of cyclophilin in auxin-regulated plant growth and development, such as cell proliferations, vascular tissue differentiation, tropic responses and so forth. The *LeCyp1/Dgt* gene, as a novel player in auxin signaling, provides a new opportunity to investigate the mystery of auxin signal transduction.

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