ANALYSIS OF PHYSIOLOGICAL FUNCTION(S) OF

A LACCSKE-LIKE MULTICOPPER OXIDASE IN *ARABIDOPSIS THALIANA*

by

CHIEH-TING WANG

(Under the Direction of Jeffrey F. D. Dean)

ABSTRACT

Laccase and related laccase-like multicopper oxidases
(LMCOs) have been studied in plants for more than a century, yet
our understanding of their physiological function(s) remains
limited. The work described in this dissertation was directed
toward understanding of the physiological function of one
particular *Arabidopsis* LMCO gene, At2g30210, by measuring its
enzyme activities, patterns of tissue-specific expression,
subcellular localization, effects of loss-of-function mutations,
and regulation by external factors. Heterologously expressed
At2g30210 LMCO showed phenoloxidase active, but no ferroxidase
activity. RT-PCR and transcriptional profiling showed that the
At2g30210 LMCO gene is expressed primarily in root tissues.
Histochemical analyses of transgenic, GUS-expressing plants
revealed that At2g30210 transcripts were preferentially
expressed in developing endodermis of the root elongation zone.

Analyses of subcellular localization indicated that the At2g30210-YFP fusion product was localized to the cell periphery. A loss-of-function At2g30210 mutant displayed phenotypic responses to sugar availability and salt levels consistent with alterations in endodermal function. The At2g30210 LMCO gene was not regulated by changes in iron levels. However, the gene was up-regulated in seedlings grown on MS medium lacking sucrose, a response that was consistent with a phenotype observed in knockout mutants of this gene. These results altogether suggest a possible physiological function for the At2g30210 LMCO gene product in endodermal cell development, possibly in formation of the lignin-like phenolic polymers observed in the specialized cell walls that constitute the Casparian strip in endodermal cells.

INDEX WORDS: laccase, laccase-like multicopper oxidase, LMCO, phenoloxidase, ferroxidase, Casparian strip, GUS, knockout mutant, sugar, salt stress, qPCR, gene expression, regulation, transformation, heterologous expression, suberin, lignin, *Arabidopsis thaliana*, endodermis

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To Shu-Chen, Erin, and Ethan, my love and future

I love you

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Multicopper oxidases

Multicopper oxidases (MCOs) typically contain four copper atoms per protein molecule and catalyze oxidation reactions in which electrons are removed from reducing substrates and transferred to oxygen to form water. In general, MCOs possess three spectroscopically distinct copper centers. These centers are called Type-1 (or blue), Type-2 (or normal) and Type-3 (or coupled binuclear), although in some MCOs Type-2 and Type-3 centers may be organized into a trinuclear cluster (Solomon et al. 1996). Type-1 copper (blue copper) centers exhibit intense spectral absorption at about 600 nm, owing to a charge-transfer reaction between Cu^{2+} and a coordinated cysteine residue. Type-2 copper centers show little or no visible light absorption, and function as a one-electron acceptor. Type-3 copper centers contain two copper atoms that absorb at 330 nm, and function as a two-electron acceptor (Solomon et al. 1996).

Well-known MCOs include laccases in fungi and plants, ascorbate oxidase in plants, ceruloplasmin in mammals, and the Fet3 ferroxidase in yeast. In addition to these enzymes, there are a number of other proteins, which are similar to the multicopper oxidases, including the copper-resistance protein A

(copA) from bacteria; the bilirubin oxidase, sulochrin oxidase,
dihydrogeodin oxidase, and tyrosinase enzymes from various
fungi; and the phenoxazinone synthase enzyme found in several
Streptomyces species (Messerschmidt 1997, Brouwers et al. 2000).
All these enzymes are involved in the oxidation of organic
compounds (Solomon et al. 1996; Brouwers et al. 2000).

Ceruloplasmin is unique among the multicopper oxidases in
that it contains two additional Type-1 centers (Solomon et al.
1996). Although isolated ceruloplasmin is able to oxidize
aromatic substrates, it oxidizes ferrous iron with much higher
affinity (Harris et al. 1995). Ceruloplasmin has been implicated
in iron metabolism because of its catalytic oxidation of Fe^{2+} to
Fe^{3+} (ferroxidase activity) (Harris et al. 1995, Mukhopadhyay et
al. 1998). Its main function is thought to be in mobilization of
cellular iron. Mutations leading to a loss of ceruloplasim
activity disrupt mammalian iron homoeostasis (Harris et al.
1995, 1999, 2004). Hephaestin, a ceruloplasmin homologue
involved in releasing iron from intestinal enterocytes into the
circulatory system (Vulpe et al. 1999), can oxidize ferrous iron
as well as *p*-phenylenediamine, a typical laccase substrate (Chen
et al. 2004).

Fet3p is a MCO required for a high-affinity iron uptake
system in yeast (Askwith et al. 1994, Stearman et al. 1996). A
model for this high-affinity (Km = 2µM) iron uptake system is

shown in Figure 1.1. In the model, Fe^{2+} generated by cell surface ferrireductases is converted into Fe^{3+} by the Fet3p, and is subsequently transferred to an Fe-binding site on the associated permease protein, Ftr1p (Eide 1998, Shi et al. 2003). Interaction between Fet3p and Ftr1p is required for exact protein targeting, and in the absence of any Ftr1p, Fet3p fails to properly localize to the plasma membrane in yeast (Shi et al. 2003). The Fet5p/Fth1p complex, a homologue of the Fet3p/Ftr1p complex, plays a role in releasing iron from the yeast vacuole to the cytoplasm (Urbanowski and Piper 1999).

The discovery that the Fet3 protein plays a key role in yeast iron metabolism has had a great impact on our understanding of iron metabolism across a spectrum of eukaryotes, in particular supporting the function of the plasma MCOs, ceruloplasmin and hephaestin, in vertebrates (Askwith and Kaplan 1998, Harris et al. 1999, Attieh et al. 1999, Vulpe et al. 1999). Recently, Harris et al. (2004) showed that injection of yeast Fet3p could restore iron homeostasis in phlebotomized mice having a deletion of their endogenous ceruloplasmin genes. This analysis strongly supports the conservation of function for copper-containing proteins in eukaryotic iron metabolism.

Ascorbate oxidase, which catalyzes the oxidation of ascorbate to dehydroascorbate, is found mainly in higher plants, and is especially abundant in members of the *Cucurbitaceae* (e.g.,

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cucumber, squash, and melon; Ohkawa et al. 1989). Since first discovered by Szent-Gyorgyi (1928), this enzyme has undergone extensive biochemical study, and numerous genes encoding ascorbate oxidases have been isolated and sequenced (Messerschmidt 1997). Ascorbate oxidase is one of the few multicopper oxidases that have been well characterized by X-ray crystallography (Messerschmidt et al. 1992). Immunohistochemical localization in green zucchini revealed that ascorbate oxidase is distributed ubiquitously over vegetative and reproductive organs (Chichiricco et al. 1989). Although ascorbate oxidase has been characterized in detail with regard to enzyme structure and expression, its *in vivo* role in plants remains unclear. The enzyme has been suggested to play a role in cell expansion (Lin and Varner 1991), in regulation of the cell cycle (Arrigoni 1994, Diallinas et al. 1997), and in plant oxidative defense systems (Foryer et al. 1994).

Laccases and laccase-like multicopper oxidases (LMCOs) are common enzymes in nature, and they are widespread in plants and fungi, as well as in some bacteria and insects. They are structurally homologous to ceruloplasmin and ascorbate oxidase, and are interesting as models for understanding multicopper oxidases (Messerschmidt 1997). Laccase is a polyphenol oxidase (*p*-diphenol:oxygen oxidoreductases, EC 1.10.3.2), first reported in Japanese lacquer tree (*Rhus vernicifera*) sap by Yoshida

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(1883), and characterized as a metal-containing oxidase by Bertrand (1895). Although some enzymes in the MCO family oxidize specific, identified substrates, and have been named accordingly, most of the better known MCOs, including the "laccases," are capable of oxidizing multiple potential substrates, and this has created significant confusion in enzyme nomenclature (Mayer and Harel 1979). Indeed, the name "laccase" should be reserved for those members of the MCO family that are found in plant saps containing the unsaturated alkylcatechols ("laccol," also known as "urushiol") that are their natural substrates (Du et al. 1984). However, the name has frequently been used to identify any monomeric MCO that contains four copper atoms in a specific arrangement of Type-1, Type-2 and Type-3 electromagnetic centers and displays some level of phenoloxidase activity (Messerschmidt 1997). Recently, many more "laccases" have been identified from genomic sequence data by sequence similarity with known laccase genes, rather than biochemical characterization. Due to the multiplicity of functions likely performed by the numerous MCOs appearing in plants, "laccase-like multicopper oxidases" or "LMCOs" has been proposed as a more appropriate for these newly discovered four-copper MCOs (Hoopes and Dean 2004; McCaig et al. 2005).

Prokaryote LMCOs

Only a few bacterial LMCOs have been described so far. The first bacterial LMCO was detected in the plant root-associated bacterium, *Azospirillum lipoferum* (Givaudan et al. 1993), where it was shown to be involved in melanin formation (Faure et al. 1994). The purified phenoloxidase was later confirmed as a LMCO (Alexandre and Bally 1999, Diamantidis et al. 2000). Melanization related to a bacterial LMCO has also been observed in the marine bacterium, *Marinomonas mediterranea* (Solano et al., 2000), but no functional role has yet been assigned to this enzyme.

A laccase-like enzyme activity was found in spores of a *Bacillus sphaericus* strain (Claus and Filip 1997). The spore protein, CotA, of *Bacillus subtilis* has also been recognized to be a LMCO (Hullo et al. 2001). Mutants in the gene encoding CotA lost the ability to produce a brownish spore pigment. The oxidation of Mn^{2+} by laccase-like spore proteins of a marine *Bacillus* strain has been shown from mutants in genes coding for multi-copper oxidases that had lost their metal-oxidizing activities (van Waasbergen et al. 1996). The involvement of similar enzymes in manganese oxidation by various *Pseudomonas* species has also been suggested (Brouwers et al. 1999, Francis and Tebo 2001). The *yac*K gene product of *E. coli* has features typical of a MCO and has ferroxidase activity (Kim et al. 2001).

The crystal structure of this protein showed three repetitive pseudoazurin domains in conjunction with a Type-1 Cu^{2+} in domain 3 and a trinuclear Cu^{2+} cluster between domains 1 and 3 (Roberts et al. 2002). The authors suggested a role for the enzyme in the uptake of and tolerance to copper and iron cations.

Fungal LMCOs

LMOCs from fungi are the most diverse and extensively studied members of this class of enzymes. The large number of fungi producing LMCOs and the diversity of LMCO isoforms they produce suggest that these enzymes play a host of important roles in fungal biology (Thurston 1994, Mayer and Staples 2002). The involvement of LMCOs in fungal morphogenesis is probably the best established of the numerous putative functions given for these enzymes. Biochemical and genetic evidence that a LMCO gene product in *Aspergillus nidulans* catalyzes the final step in spore pigment formation, the conversion of a yellow polyketide precursor into a dark green, polymeric product, suggests that the LMCO is important for structural development of fungal spores (Law and Timberlake 1980, Aramayo and Timberlake 1990). LMCOs have also been proposed to participate in fungal morphogenesis in *Armillaria* spp. (Worral et al. 1986), *Lentinus edodes* (Leatham and Stahmann 1981) and *Volvacea volvacea* (Chen et al. 2004).

LMCOs are also thought to be involved in lignin degradation by certain white-rot fungi, although the presence of both peroxidases and LMCOs has been an obstacle in elucidating their precise roles in lignin biodegradation (Ranocha et al. 2000). Many ligninolytic fungi, such as *Phlebia radiata*, *Phanerochaete chrysosporium*, and *Rigidiporus lignosus* secrete both classes of enzyme. *Pycnoporus cinnabarinus*, however, has become a model species to demonstrate the role of LMCOs in ligninolysis since it produces LMCOs only, and not any of the peroxidases (lignin peroxidase and manganese peroxidase), commonly associated with lignin degradation (Eggert et al. 1996). This fungus is perfectly capable of hydrolyzing lignin polymers under normal conditions, whereas a LMCO-deficient *P. cinnabarinus* mutant was unable to hydrolyze synthetic lignin (DHP) polymers (Eggert et al. 1997).

The reaction mechanism by which LMCOs catalyze lignin degradation is not completely understood. Studies indicate that LMCOs may oxidize the terminal phenolic units in lignin, but they are unable to directly oxidize non-phenolic lignin substructures due to their low redox potentials (Eriksson et al. 1990). Thus, small organic compounds or metals that can be oxidized and activated by fungal LMCOs, e.g., veratryl alcohol (Nishizawa et al. 1995), 3-hydroxy-anthranilic acid (Eggert et al. 1996), or Mn^{2+} (Schlosser and Hofer 2002), have been proposed

to mediate radical-catalyzed depolymerization of non-phenolic lignin structures. Non-physiological redox-mediators have also been used in biotechnological processes to increase the oxidation potential of LMCOs for similar purposes (Li et al. 1999).

LMCOs of various fungi may also be involved in virulence mechanisms (Thurston 1994, Mayer and Staples 2002). For example, the grapevine grey mould, *Botrytis cinerea*, produces a LMCO that is necessary for pathogenesis, and the role of this LMCO is suggested to be in detoxifying antifungal metabolites produced by the host (Bar-Nun et al. 1988). LMCOs are also important for pathogenesis in the chestnut blight fungus, *Cryphonectria parasitica*, and the human pathogen, *Crytococcus neoformans* (Mayer and Staples 2002). In *C. neoformans*, synthesis of melanin, an important virulence factor for this fungus, was shown to be dependent on a single, copper-dependent LMCO (CNLAC1), a role confirmed using knock-out strains of the fungus (Salas et al. 1996, Williamson et al. 1998). In mice, however, Liu et al. (1999) suggested that the ferroxidase activity of this LMCO may protect *C. neoformans* from macrophages by oxidizing phagosomal iron to Fe^{3+} with a resultant decrease in antifungal hydroxyl radical formation.

Plant LMCOs

The occurrence of LMCOs in plants has for a long time appeared more limited than in fungi. The first laccase was identified in sap from *Rhus vernicifera*, the Japanese lacquer tree, by Yoshida (1883). Since then, LMCOs have been described in various *Rhus* species (Keilin and Mann 1939, Nakamura 1958) and a few other plant species (Mayer and Staples 2002, McCaig et al. 2005). Cell suspension cultures of sycamore maple (*Acer pseudoplatanus*) were shown to produce and secrete a LMCO (Bligny and Douce 1983, Driouich et al. 1992, Sterjiades et al. 1992), and loblolly pine (*Pinus taeda*) was shown to produce at least eight LMCOs, all expressed predominantly in differentiating xylem (Sato et al. 2001). Five distinct LMCOs were shown to be expressed in the xylem tissues of poplar (*Populus trichocarpa*) (Ranocha et al. 1999), and four closely related LMCOs were identified in xylem tissues of yellow-poplar (*Liriodendron tulipifera*) (LaFayette et al. 1999). LMCOs have also been reported in other species, including *Zinnia elegans* (Liu et al. 1994), tobacco (*Nicotiana tabacum*)(Kiefer-Meyer et al. 1996), peach (*Prunus persica*)(Mayer and Staples 2002), and Sitka spruce (*Picea sitchensis*) (McDougall 2000). Recently, McCaig et al. (2005) used LMCO sequences culled from GenBank and the *Arabidopsis thaliana* genome to construct a gene phylogeny that clearly divided plant LMCOs into at least six distinct classes

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(Figure 1.2). These studies together suggest that LMCOs are widespread amongst angiosperms, and quite probably in gymnosperms as well.

Despite the fact that LMCOs were first discovered in plants over 100 years ago, relatively little is known about the role these enzymes play *in vivo.* Many plant LMCOs appear to be cell wall-associated enzymes that are relatively difficult to purify. Consequently, solid biochemical and molecular data are difficult to come by (Ranocha et al. 1999). Wound healing through oxidative polymerization of alkylcatechols in lacquer tree sap (true laccase) had long been the only strongly supported physiological function advanced for plant LMCOs (Mayer and staples 2002; McCaig et al. 2005). The sap from *Rhus* is used for a wide variety of purposes in Asia, including the manufacture of ornamental lacquerware in Japan, and consequently its production is of considerable economic importance (Dean and Eriksson, 1994).

Plant LMCOs have also been proposed to play a part in lignin biosynthesis (Dean and Eriksson 1994; Dean et al. 1998; Mayer and Staples 2002). Freudenberg and co-workers were the first to suggest a role for laccase in lignification based on studies using a fungal LMCO (Freudenberg, 1950; Freudenberg and Dietrich, 1953; Freudenberg, 1959). Later, however, Nakamura (1967) claimed that such enzymes were likely not responsible for

lignification on the basis that a laccase purified from Japanese lacquer tree sap could not polymerize the lignin precursor, coniferyl alcohol. Moreover, a histochemical study using syringaldazine demonstrated peroxidase, but not LMCO, activity in lignifying xylem from a variety of tree species (Harkin and Obst 1973). On the basis of these two negative results, LMCOs were dropped from consideration as oxidative catalysts for lignification, and peroxidases were proposed as the sole enzyme responsible for lignin polymerization (O'Malley et al. 1993, Olson and Varner 1993).

In the early 1990's, characterization of the LMCO purified from cell-suspension cultures of sycamore maple prompted a reevaluation of the role this enzyme might have in lignification (Bligny and Douce 1983, Sterjiades et al. 1992, Driouich et al. 1992). Sterjiades et al. (1993) showed that the LMCO purified from sycamore maple cells was capable of oxidizing the lignin precursors, or monolignols (sinapyl alcohol, coniferyl alcohol, or *p*-coumaryl alcohol), to form water-insoluble dehydrogenation polymers (DHPs). In addition, a LMCO isolated from loblolly pine xylem was shown to produce DHPs from coniferyl alcohol, and the activity was localized to lignifying xylem near the cambium, whereas peroxidase activity was quite evident throughout pine stems and was not specifically localized to tissues undergoing active lignification (Bao et al. 1993). Similarly, a tight

correlation of LMCO activity with the lignification of secondary cell walls in developing primary xylem was also observed using histochemical staining (Liu et al. 1994). These studies clearly showed that LMCO activity is spatially and temporally associated with lignification in plants. It is possible that, as suggested by Sterjiades et al. (1993), LMCOs and peroxidases are separated temporally in their roles as catalysts for lignin deposition. However, transgenic plants with down-regulated LMCOs showed no detectable alteration in lignin content or composition of xylem tissues (Dean et al. 1998, Ranocha et al. 2002). Thus, whether plant LMCOs are direct catalysts for lignin biosynthesis remains unclear.

On the basis of specific enzyme activities associated with plant LMCOs, some other putative functions have been discussed in different reports. Recent demonstration of ferroxidase activity in a LMCO from yellow-poplar (Hoopes and Dean 2004) was taken to suggest that some plant LMCOs might play a role in iron metabolism, similar to the function provided by the Fet3 LMCO in the high-affinity iron uptake system described previously. The plausibility of this argument is enhanced by widespread ferroxidase activity in LMCOs from fungi, such as *Cryptococcus neoformans* (Liu et al. 1999) and *Phanerochaete chrysosporium* (Larrondo et al. 2003), and bacteria, *E. coli* (*yacK*/CueO) (Kim et al. 2001, Singh et al. 2004), as well as the green alga,

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Chlamydomonas reinhardtii (Herbik et al. 2002, La Fontaine et al. 2002). Based on widespread distribution of MCOs involved in iron metabolism in all kingdoms, it is reasonable to think that some LMCOs in plants may take part in iron uptake systems similar to the yeast Fet3 system. LMCOs are also one of several oxidative enzymes with demonstrated capacity to degrade chemical compounds in their immediate vicinity (Boyajian and Carreira 1997). That transgenic *Arabidopsis* over-expressing a secretory LMCO from cotton (*Gossypium arboretum*) exhibited enhanced resistance to several phenolic allelochemicals and 2,4,6-trichlorophenol suggests that plant LMCOs could also play a role in detoxification of organic compounds (Wang et al. 2004). Most of the LMCOs studied in plants can oxidize phenolic compounds and their derivatives, which are related to compounds involved in developmental processes, defense, and signaling. Thus, LMCOs have the potential to play a host of roles in whole plants. However, in most cases, definitive demonstration of physiological functions for plant LMCOs has been difficult because the enzymes are typically able to oxidize a wide variety of phenolic and inorganic substrates. In addition, multiple LMCO gene products may be expressed simultaneously in the same tissues. To overcome these difficulties, and to distinguish LMCO from peroxidase function, the use of combined biochemical and molecular approaches must be applied. Ranocha et al. (1999) were

first to combine both approaches in cloning several distinct LMCO cDNAs and performing biochemical characterization of one of the corresponding gene products.

Structure and catalytic mechanism of LMCOs

Structural studies of LMCOs have so far been limited to fungi, as crystal structures of LMCOs from three fungal species have been determined (Ducros *et al.*, 1998; Piontek *et al.*, 2002; Bertrand *et al.*, 2002; Hakulinen et al. 2002). The protein fold structure of these LMCOs consists of three cupredoxin-like domains (A, B, and C) (Figure 1.3, Hakulinen et al. 2002), which have also been found in ascorbate oxidase (Messerschmidt et al. 1992) and ceruloplasmin (Murphy et al. 1997). All three domains are important for the catalytic activity of LMCOs. There are significant differences in the loops forming the substrate-binding pocket in different LMCOs, suggesting different affinities for different substrates (Hakulinen et al. 2002). The substrate-binding site is located in a cleft between domains B and C, close to the mononuclear copper center in which the Type-1 copper atom is coordinated to two histidine and one cysteine residues.

Multiple sequence alignments of the fungal LMCOs available in public databases have identified distinctive sequence signatures around the conserved copper-binding domains that distinguish fungal LMCOs from plant LMCOs and other multicopper oxidases. The

copper-binding domains consist of four ungapped sequence

segments, L1 to L4, that contain the 12 residues serving as

copper ligands (Figure 1.4) (Kumar et al. 2003). These four

blocks can be found in all MCO amino acid sequences. Two of

these regions are near the C-terminus, separated by 35-75 amino

acid residues. The other two are near the N-terminus and

separated by 35-60 residues (Brouwers et al. 2000, Kumar et al.

2003). Each of the N- and C-terminal regions contains a

conserved HXH motif (Figure 1.4). Eight histidine residues

within the four blocks provide the conserved ligands for the

trinuclear center. The Type-2 copper is coordinated by two of

these histidine residues, and each of the Type-3 copper atoms is

coordinated by three of remaining histidine residues. The

conserved ligands for the Type-1 copper center are located in

the two C-terminal regions and consist of one cysteine and two

histidine residues. The bond between the Type-1 copper and S_{cys}

is highly covalent, and is the source of the strong absorption

band around 600 nm that gives these proteins their typical blue

color. In some LMCOs the Type-1 copper atoms are also

coordinated with an axial ligand provided by a methionine,

leucine, or phenylalanine residue. The Type-1 center cysteine

residue is flanked by two of the histidines coordinating the

trinuclear cluster, which provides a proximal link between the

Type-1 center and the trinuclear cluster. Through this Cys-His

link the Type-1 center passes electrons from four sequential one-electron oxidations to the trinuclear cluster (Solomon et al. 1996, Brouwers et al. 2000). The trinuclear cluster is the site of the four-electron reduction of oxygen to water, probably through a peroxide intermediate (Solomon et al. 1996).

Molecular biology of plant LMCOs

As discussed previously, biochemical analyses have not yet provided clear functions for plant LMCOs. Thus, genetic analysis will be critical for demonstrating the function of specific plant LMCOs. The first plant LMCO gene was isolated from sycamore maple (LaFayette et al. 1995). Since then, the number of LMCO genes sequenced in plants has increased considerably. Using the sycamore maple LMCO DNA sequence (APU12757), a search of the GenBank database revealed LMCO genes in at least 15 plant species, drawn from non-vascular plants, conifers, and flowering plants. However, a significant number of these sequences are only partial stretches of putative LMCO genes that have been found in genome-wide sequencing projects, and have been annotated on the basis of their sequence homology with known LMCOs. The number of LMCO genes for which the corresponding protein products have been experimentally characterized is significantly lower.

Heterologous expression systems would be helpful for getting large amounts of LMCO protein for biochemical analysis. *E. coli* has been used routinely for heterologous gene expression; however, there have been no reports of successful use of this system to express plant LMCOs. In contrast, suspension-cultured tobacco cells have been used successfully to express plant LMCOs (Dean et al. 1998). LaFayette et al. (1999) demonstrated activity from a yellow-poplar LMCO cDNA heterologously expressed in tobacco cells. Subsequently, ferroxidase activity was demonstrated in a LMCO produced by tobacco cells transformed with another LMCO cDNA cloned from yellow-poplar (Hoopes et al. 2004). These results aside, faster progress could be made in studies of plant LMCOs if an easier system for heterologous expression were available.

To attack the question of LMCO function in plants, Ranocha et al. (2002) generated four populations of transgenic hybrid poplar, each expressing an individual poplar LMCO gene in the antisense (AS) orientation. This was the first in-depth characterization of genetic manipulation of LMCOs in plants, although preliminary data for similar studies of *L. tulipifera* (Dean et al. 1998) and *Arabidopsis* (Halpin et al. 1999) LMCOs had been reported. Surprisingly, none of these studies showed reproducible alterations in lignin content or composition. Only one of the populations of poplar (lac3AS) exhibited quantitative

differences in global phenolic metabolism accompanied by perturbations in xylem fiber cell wall structure. The authors concluded that the product of the lac3 gene is essential for normal cell wall structure and integrity in xylem fibers, although a direct correlation between LMCO gene expression and lignification could not be assigned (Ranocha et al. 2002).

The analysis of mutants is a very powerful tool for revealing the role of a particular gene in the physiological and developmental processes of plants. For many woody plants, such as trees, it is difficult to isolate or screen mutants due to the long life cycle and a dearth of genetic information. In contrast, *Arabidopsis*, a model plant, grows quickly and has a wide array of available genetic and genomic resources. Consequently, it has become a valuable model for study of plant-specific gene functions, as well as fundamental processes common to all higher organisms at the molecular and cellular levels.

The completed *Arabidopsis* genome sequence provides new ways of addressing biological questions, ranging from molecular genetics to evolution, from an integrated perspective (Bouché and Bouchez 2001). The *Arabidopsis* genome encodes 17 LMCO genes that exhibit a wide variety of expression patterns in different tissues, and can be separated into 6 distinct phylogenetic groups (McCaig et al. 2005). A preliminary report described a phenotype associated with reduced expression of an *Arabidopsis*

LMCO (an antisense mutant of the At5g03260) as complex and characterized by chlorosis and poor growth, but having no detectable changes in lignin quantity or composition (Halpin et al. 1999). Recently, the *Arabidopsis* TT10 gene, which encodes a putative LMCO (At5g48100), was identified by a mutant phenotype having a pale brown seed coat at harvest. The authors suggested that the TT10 LMCO in *Arabidopsis* serves as a flavonoid oxidase in developing seed coats (Pourcel et al. 2005).

Recently, well-developed bioinformatics systems have been developed to provide online access to whole genome datasets of gene expression data. With these tools researchers can quickly obtain large amounts of information on the expression patterns of specific gene(s) in which they are interested. For example, Figure 1.5 shows the expression profiles of the 17 *Arabidopsis* LMCOs at different stages of development.

In addition, several hundred thousand T-DNA and transposon insertion lines generated in multiple laboratories have been made available to the research community (Bouché and Bouchez 2001). Such mutants provide powerful tools for revealing the role of particular genes in physiological and developmental processes. Several groups have initiated programs for the systematic sequencing of the flanking DNA in various insert populations, which has greatly accelerated the process of identifying mutants related to specific genes. Most of the

information associated with these *Arabidopsis* collections can be accessed through the TAIR website (http://www.arabidopsis.org/links/insertion.jsp).

Iron metabolism in plants

In plants, iron is taken up from the soil into the root and then distributed throughout the plant, crossing a variety of cellular and organellar membranes. To compete for limited iron in the surrounding environment, plants have developed several mechanisms to enhance iron acquisition. Under iron-deficiency stress, all plants, except grasses, use a reductive strategy, similar to that of yeast, to acquire iron. This strategy, referred to as Strategy I, starts with reduction of Fe^{3+} by a plasma membrane-bound Fe^{3+}-chelate reductase. This is followed by transport of Fe^{2+} across the root epidermal cell membrane via divalent metal transporters. The roots of Strategy I plants also release protons under conditions of iron-deficiency, thereby lowering the rhizosphere pH and increasing the levels of soluble Fe^{3+}.

The FRO2 gene, which encodes the Fe^{3+}-chelate reductase in *A. thaliana*, was cloned based on the sequence of its yeast homologues, FRE1 and FRE2 (Robinson et al. 1999). FRO2 is only expressed in roots and its mRNA levels are induced by iron deficiency. Loss-of-function mutants in FRO2 result in decreased

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Fe^{3+}-chelate reductase activity, chlorosis, and poor growth under low-iron conditions. Fe^{3+}-chelate reductase from pea, FRO1, encodes a protein that is 55% identical to *Arabidopsis* FRO2 (Waters et al. 2002).

The IRT1 gene was the first ferrous ion transporter cloned from plants using functional complementation of a yeast strain defective (fet3fet4 mutant) in iron uptake (Edie et al. 1996). In addition to iron, IRT1 also displays manganese and zinc uptake activities when expressed in yeast (Korshunova et al. 1999). The IRT1 transcript and protein accumulate in *Arabidopsis* roots in response to iron deficiency, supporting a role for IRT1 in iron uptake in planta (Eide et al. 1996, Vert et al. 2002, Connolly et al. 2002). IRT1 knockout mutants are chlorotic and have a severe growth defect when grown in soil (Henriques et al. 2002, Varotto et al. 2002, Vert et al. 2002). Another putative iron transporter gene, IRT2, was isolated using the same strategy of expressing *Arabidopsis* cDNA of IRT1 homologues in the fet3fet4 yeast mutant defective in iron uptake (Vert et al. 2001). Although IRT2 is closely related to IRT1, its function as an iron transportor has not yet been demonstrated.

The NRAMP gene family, which has seven members in *Arabidopsis*, encodes another class of metal transporters. NRAMP1 and NRAMP2 are respectively up-regulated and down-regulated in roots experiencing iron deficiency (Curie et al. 2003, Thomine et al.

2000). Over-expression of NRAMP1 in transgenic *Arabidopsis*
plants results in increased resistance of the plants to iron
toxicity. However, there is no data to indicate whether these
transporters play a role in iron uptake.

In contrast to Strategy I plants, Strategy II plants,
including all of the grasses, release Fe^{3+}-binding compounds,
low-molecular-mass secondary amino acids (mugineic acids, MAs)
also known as "phytosiderophores" (PS), into the surrounding
soil. These chelators bind iron and are then taken up into the
root by active transport (Fett et al. 1998, Mori 1998). The
biosynthesis pathway for PS has been extensively studied and has
been almost completely deciphered in barley (Mori 1999, Negishi
et al. 2002). The maize mutant, *yellow stripe 3 (ys3),* which
shows interveinal chlorosis characteristic of iron deficiency,
has been associated with a defect in MAs secretion (Basso et al.
1994). Another maize mutant, *yellow stripe 1* (*ys1*), is
deficient in Fe^{3+}-phytosiderophore uptake, leading to the
suggestion that YS1 is a Fe^{3+}-phytosiderophore transporter (Curie
et al. 2001). Interestingly, a family of eight genes in
Arabidopsis that encode proteins sharing ca. 80% similarity with
YS1 has been identified. This surprising finding suggests that
metal-chelated transporters may actually be found in all plants,
and not just grasses (Curie and Brait 2003).

Chloroplasts are likely the strongest sink for iron in plants. Plastids contain ferritin, an iron storage protein, and iron-ferritin complexes represent more than 90% of iron found in the pea embryo axis (Marentes et al. 1998). In *Arabidopsis*, four ferritin genes (*AtFer1-4*) have been cloned, and all four ferritin proteins are predicted to be targeted to the plastids. Gene *AtFer2* is the only gene of the four to be expressed in seeds, whereas *AtFer1, AtFer3,* and *AtFer4* are expressed in various vegetative organs, but not in seeds. Waldo et al. (1995) showed that ferritin mineralization iron loading occurs after protein localization into the plastid, which indicates that the plastid must have an active system to take up iron for incorporation into ferritin. Iron transport into the plastids is, therefore, of major importance in plant iron metabolism. However, the mechanism of iron uptake into chloroplasts and/or plastids at the molecular level is unclear. Further information on subcellular localization of high-affinity transporters of iron may play a critical role in addressing iron uptake into subcellular compartments. A summarized model for iron acquisition systems in plants is shown in Figure 1.6. In it, we propose an additional transporter class (3), which uses a high-affinity iron uptake system similar to those found in yeast and animal systems. Such transporters could be located in different

membrane systems to control iron fluxes between different subcellular compartments.

Aims of the study

The main goal of the work described in this dissertation was to extend our understanding of the physiological function(s) of the LMCOs in plants. *Arabidopsis* was used as a model system and a single LMCO gene was selected for characterization via both biochemical and genetic approaches. The initial hypothesis for the study was that the At2g30210 LMCO might function as a Fet3 ortholog, i.e. working as a ferroxidase in high-affinity iron-uptake pumps. Specific objectives were as follows:

(1) Clone a full-length cDNA for the At2g30210 gene;

(2) Heterologous expression, as well as phenoloxidase and ferroixdase activity analysis, of the heterologous expressed LMCO gene product;

(3) Test tissue-specific expression of the gene;

(4) Test subcellular localization of the LMCO gene product;

(5) Investigate LMCO knockouts for clues to the physiological function(s) of At2g30210 gene; and

(6) Study the regulation of the LMCO gene in response to various environmental perturbations.

The results of this research are detailed in the following chapters:

Chapter 2 presents the cloning of a full-length At2g30210 cDNA from *Arabidopsis* and heterologous expression of the cloned gene. Phenoloxidase and ferroxidase activities of the gene product were tested and the gene expression pattern was examined in multiple tissues.

Chapter 3 presents the identification and characterization of At2g30210 mutants. An At2g30210 promoter:reporter gene fusion was used to demonstrate tissue-specific patterns of gene expression at the cellular level in transgenic *Arabidopsis*. Subcellular localization of the At2g30210 protein using a chimeric At2g30210-EYFP fusion protein was also demonstrated.

Chapter 4 presents studies of the regulation of the At2g30210 gene in response to different metal, sugar, and salt stresses. An alternative function for the At2g30210 protein, other than as a ferroxidase involved in iron metabolism, is proposed.

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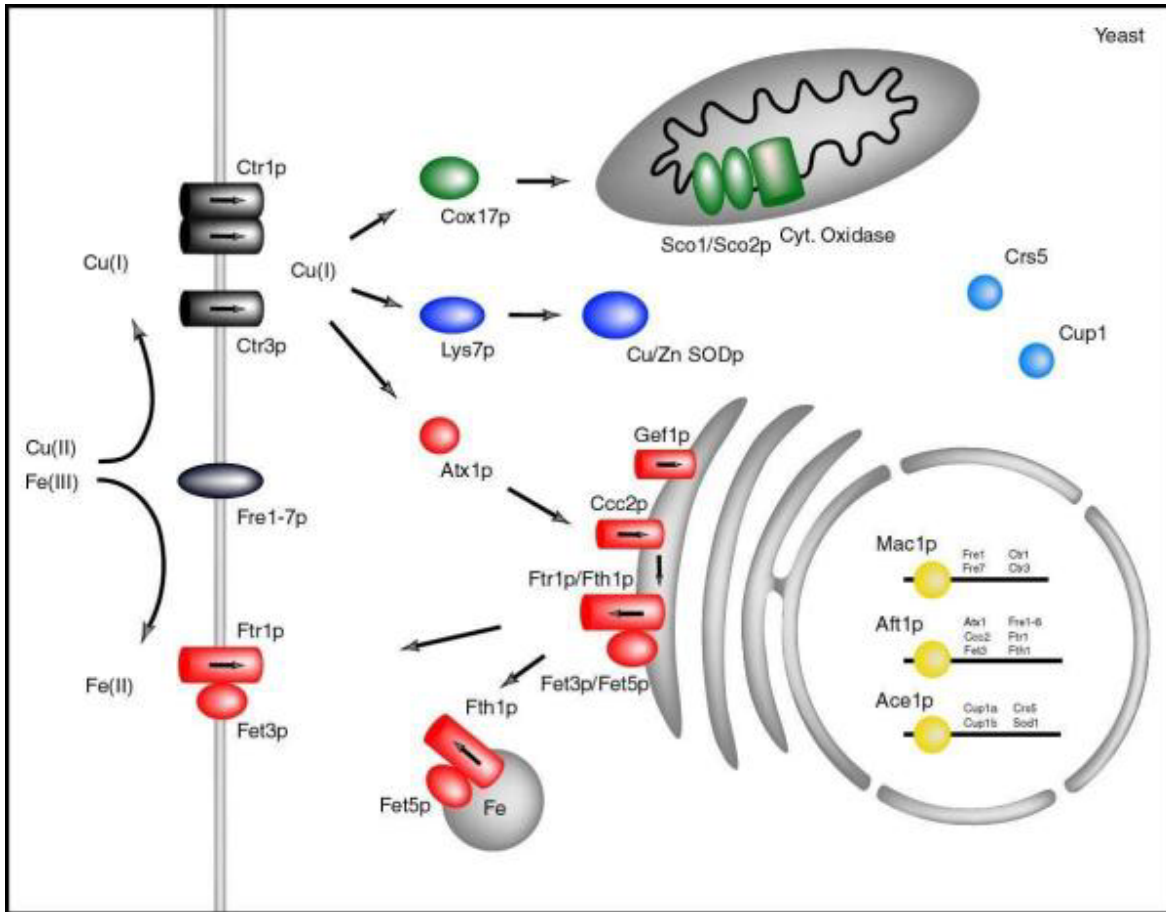   toolbox. Plant Physiol 136:2621-2632

Figure 1.1. A model for copper and iron uptake systems in yeast. (picture taken from Department for molecular genetics, University Medical Center Utrecht, Netherlands http://genomics.med.uu.nl/~harm/research)

Figure 1.2 Neighbor-joining phylogeny depicting relatedness of plant LMCOs. *Arabidopsis* genes are shown in boxes. Note that *Arabidopsis* genes are spread across all groups and there are corresponding homolog genes on chromosome 2 and chromosome 5 from 4 of the phylogenetic groups (1,2,3,and 4). (from McCaig et al. 2005).
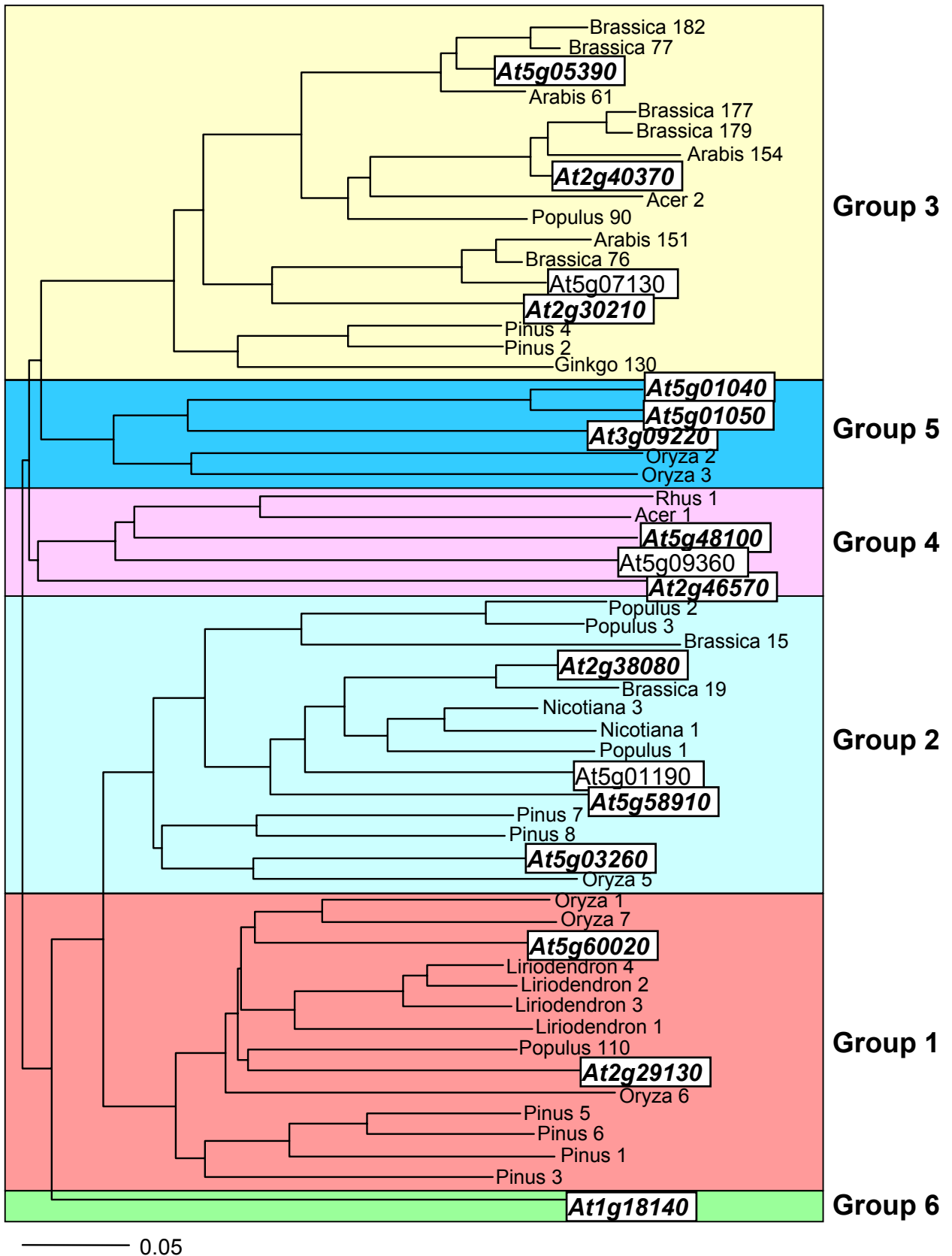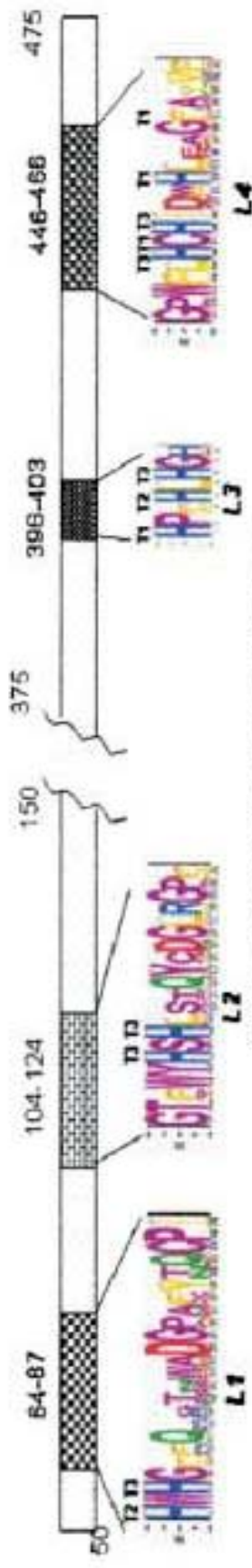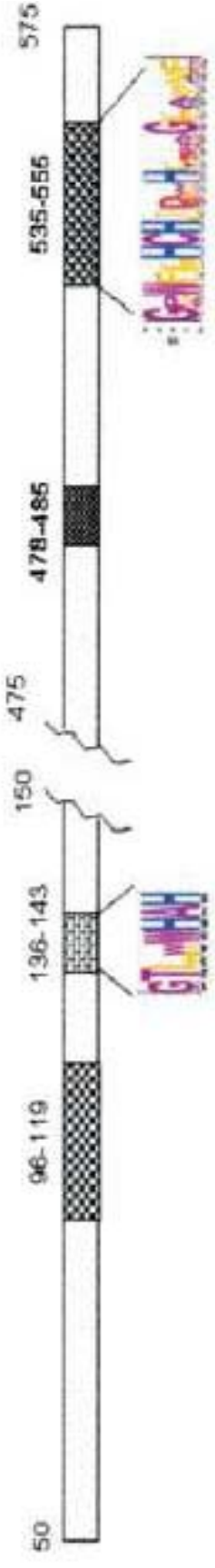
Figure 1.2.

Figure 1.3. Three-dimensional structure of the LMCO from *Melanocarpus albomyces*. Domains A, B,and C, are colored red, green, and blue, respectively. The four copper atoms are shown as yellow balls and carbohydrates as grey sticks. (modified from Hakulinen et al. 2002) .

Figure 1.4. An overview of the conserved domains identified by multiple sequence alignment for LMCOs. Consensus sequence obtained after multiple sequence alignment of (**A**) 64 fungal LMCO sequences; (**B**) 40 plant LMCO sequences; (**C**) consensus sequence from (**A**) and (**B**). The conserved regions are shaded if the length of the sub-sequence is greater than 7 residues. The corresponding regions, which are conserved both in plant and fungal laccases, are shaded in the same pattern. Sequence logos are shown for selected regions, L1–L4, which represent the frequency of occurrence of each amino acid at a particular position in the ungapped region of the aligned protein sequences. Height of a letter in the logo represents the information content at that location. (Picture adapted from Kumar et al. 2003)

(A) Conserved regions in fungal laccases

(B) Conserved regions in plant laccases
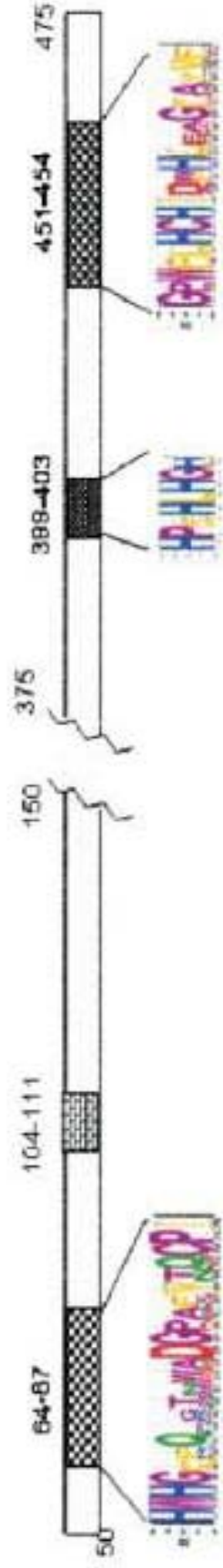
(C) Conserved regions in fungal and plant laccases

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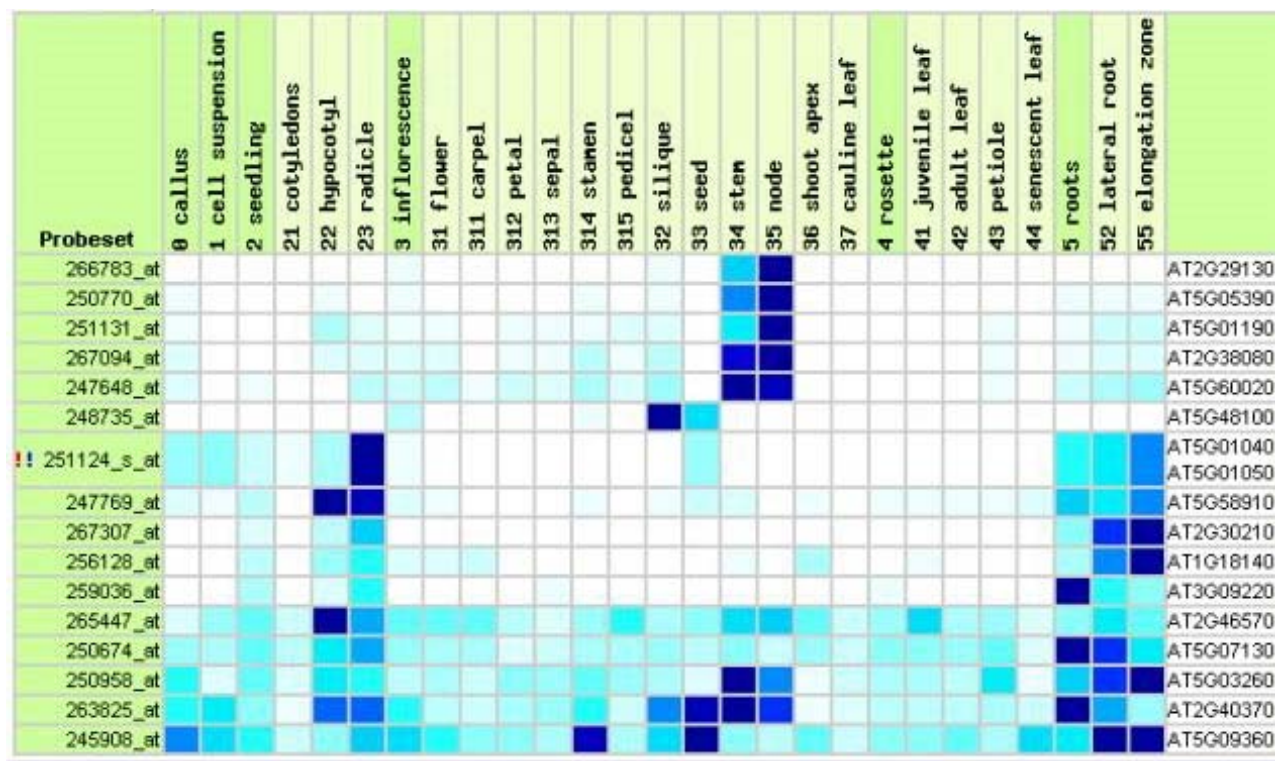
Figure 1.5. An expression profile for *Arabidopsis* LMCOs. Data were collected from online microarray studies using the *Meta-Analyzer* on the GENEVESTIGATOR web server (https://www.genevestigator.ethz.ch) (Zimmermann et al. 2004). The blue/white color indicates the averaged signal intensities. Similar profiles were clustered together.
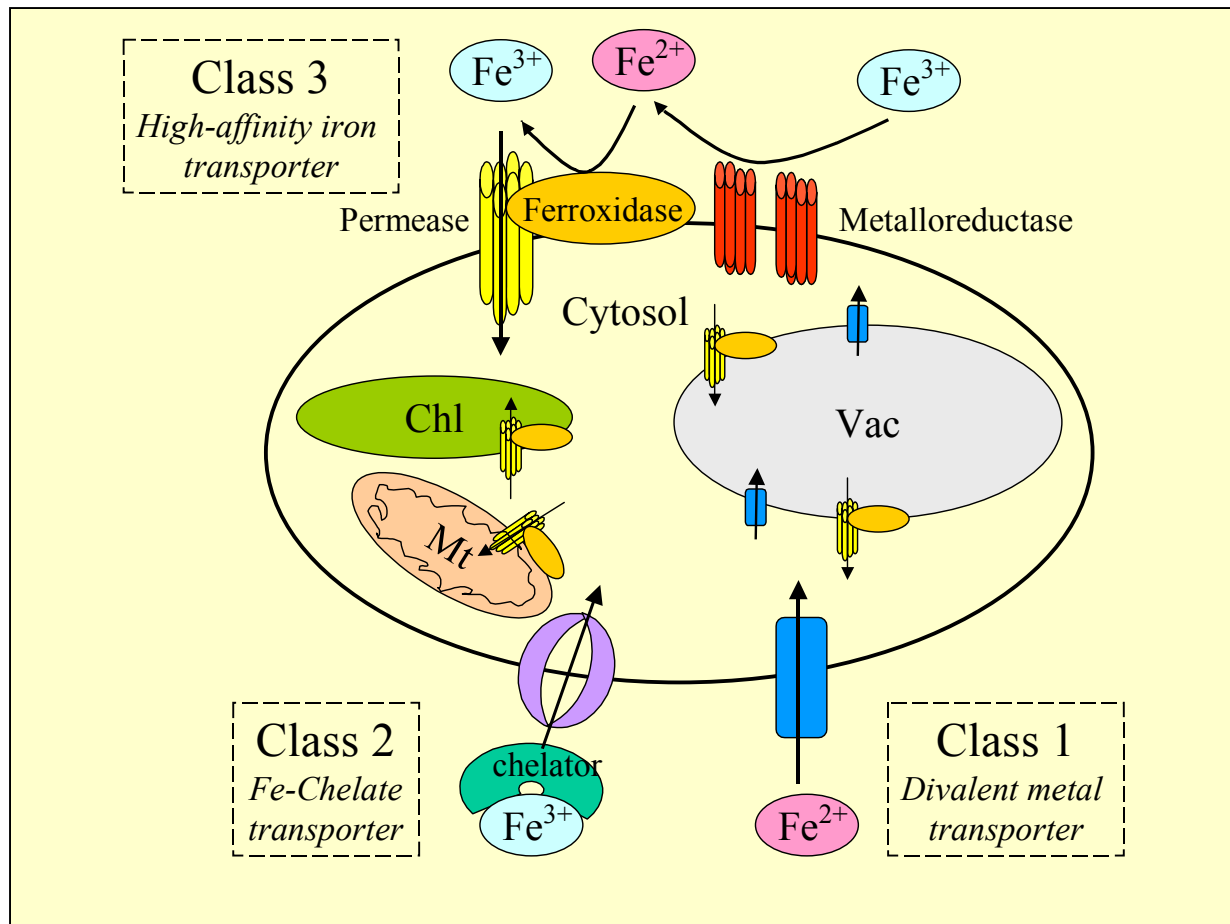
Figure 1.6. Iron acquisition systems in plants. In order to acquire sufficient iron, plants utilize multiple iron acquisition and transport systems. Chl: chloroplast, Mt: mitochondria, Vac: vacuole.

CHAPTER 2

CLONING, CHARACTERIZATION, AND EXPRESSION ANALYSIS OF A LACCASE-LIKE MULTICOPPER OXIDASE GENE FROM *ARABIDOPSIS THALIANA*[1]

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[1] Wang, C. -T. and J.F.D. Dean. To be submitted to *Plant molecular biology Today.*

Abstract

Laccases or laccase-like multicopper oxidases (LMCOs) have been found in a wide range of living organisms, yet in many cases, their physiological functions remain unclear. Because of its completed genome sequence, *Arabidopsis* provides a good system in which to study the physiological function of LMCOs. Here we report the cloning, characterization, and expression analysis of one particular LCMO gene from *Arabidopsis thaliana*. Analysis of the gene structure and predicted amino acid sequence revealed that the At2g30210 LMCO possesses the conserved copper binding domains that characterize multicopper oxidases. Heterologously expressed LMCO showed phenoloxidase activity, but no ferroxidase activity. RT-PCR and transcriptional profiling showed that the At2g30210 LMCO gene is expressed primarily in root tissues. Putative physiological functions for this specific LMCO are discussed in the context of these results.

Introduction

Laccases, or *p*-diphenol:oxygen oxidoreductases (EC 1.10.3.2), belong to an enzyme superfamily called the multicopper oxidase (MCO), which also includes ascorbate oxidase (E.C. No. 1.10.3.3) from plants, the yeast FET3 protein, and ceruloplasmin (E.C. No. 1.16.3.1) from vertebrate animals (Messerschmidt and Huber, 1990; Ryden and Hunt, 1993). Laccases or laccase-like multicopper oxidases (LMCOs) have been found in a wide range of living organisms, including bacteria, fungi, insects, and plants. Fungal LMCOs are the most extensively documented group of enzymes in this superfamily, and their functions have been shown to be associated with a variety of physiological processes, including lignin degradation, morphogenesis, and pathogenicity (Thurson, 1994; Mayer and Staples 2002). In plants, LMCOs encoded by multigene families were observed in *Arabidopsis thaliana*, rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*)(Kiefer-Meyer et al. 1996), poplar (*Populus trichocarpa*)(Rancha et al. 1999), yellow-poplar (*Liriodendron tulipifera*)(Lafayette et al. 1999), Sitka spruce (*Picea sitchensis*)(McDougall 2000), and loblolly pine (*Pinus taeda*)(Bao et al. 1993; Sato et al. 2001), yet their physiological functions remain unclear.

Although early studies suggested that laccase and laccase-like oxidase activities were associated with lignification in

plants based on the association of their expression often tightly coordinated with lignin deposition in vascular tissues (O'Malley et al. 1993; Dean and Eriksson 1994), definitive evidence to support a role for LMCOs in this process has not yet been demonstrated.

Previous studies of plant LMCOs have focused either on enzymological or molecular genetic aspects independently, and confirmed links between the corresponding gene products has been limited. The first complete DNA sequence reported for a plant LMCO was for a cDNA isolated from sycamore maple (*Acer pseudoplatanus*) (LaFayette et al. 1995). The capabilities of LMCOs from this species to polymerize lignin precursors and localize to the cell wall of lignifying vascular tissues in *Acer* stems were demonstrated earlier (Sterjiades et al. 1992, Driouich et al. 1992). Ranocha et al. (1999) were the first to report in a single study the cloning of several distinct LMCO cDNAs from poplar together with the biochemical characterization of one of the corresponding gene products. Recently, the same group was able to demonstrate a linkage to vascular tissue development by genetic modification of 3 independent LMCO genes. Although the results did not clearly point to a role for an LMCO in lignification, they did suggest that the product of at least one LMCO gene is essential for structural integrity of xylem fiber cell walls (Ranocha et al. 2002).

Roles in iron uptake and metabolism have been associated with some LMCOs. For instance, FET3 encodes a multicopper oxidase required for a high-affinity iron uptake system in yeast (Askwith et al, 1994). Recent identification of a ferroxidase activity in a LMCO from yellow-poplar (Hoopes and Dean 2004) suggests that at least some LMCOs in plants have the capacity to take part in iron metabolism systems similar to the FET3 system found in yeast.

Because of its completed genome sequence, *Arabidopsis* provides a good system in which to study the physiological function of plant LMCOs. Previous work from our lab (McCaig et al. 2005) and data available from public databases showed that the 17 LMCO genes in *Arabidopsis* exhibit a wide variety of expression patterns in different tissues (Figure 2.1). One particular LMCO gene (At2g30210) showed strong expression in young roots, a tissue not thought to be highly lignified. To develop a better understanding of the physiological functions of plant LMCOs, we cloned, characterized, and measured expression of the At2g30210 LCMO gene from root tissues of *Arabidopsis thaliana*. Its likely physiological function(s) are discussed on the basis of these results.

Materials and Methods

RNA isolation and cDNA synthesis

Wild-type *A. thaliana* (var. Columbia) seeds were sterilized in 50% EtOH for 1 minute, transferred to 50% bleach and 0.1% Tween for 10 minutes, and washed three times in sterile water. Surface sterilized seeds were germinated and grown for two weeks in liquid Murashige and Skoog medium (Invitrogen, Carlsbad, CA) containing sucrose (20g/L), with shaking at 22°C under constant illumination. Roots were harvested from 2 week-old seedlings, immediately frozen in liquid nitrogen, and stored at –80°C until used. For tissue-specific analyses, different tissues were collected from 8-week old plants grown in soil.

Total RNA was extracted by the method of Durbin et al. (2000), or using Trizol reagent according to manufacture's instructions (Invitrogen). For cDNA synthesis, 5µg of total RNA was reverse transcribed using 200 units of Superscript II reverse transcriptase (Invitrogen) with an adapter primer (AP) in a 50µl 3' RACE (Rapid Amplification of cDNA Ends) reaction, according to the manufacturer's instructions (Invitrogen). For tissue-specific analysis, cDNA pools from different tissues were first diluted 10-fold before amplification with a pair of PCR primers (1F, 5'-ATGGAGTCTTTTCGGCGATT-3' and 1016R, 5'-TCGGAGACGGTTGGTGAAAG-3'). The ubiquitin, UBQ10, gene was used as an internal control to show that the same amount of cDNA was

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loaded in each PCR reaction. The amplification product for the UBQ10 gene was a 483 bp fragment obtained using the UBQ10 primers (UBQ1: 5'-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3', and UBQ2: 5'-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3')(Weigel and Glazebrook 2002).

Cloning of the At2g30210 cDNA

The coding region of the At2g30210 gene was amplified using the forward primer, 1F, and the reverse primer 5'-TTAGCATCTTGGAAGATCCA-3' (1694R). A different forward primer, 5'- GAACATCACGTCCATCAATTC-3' (76F), along with the reverse primer (1694R) was used to amplify the coding region lacking the signal peptide. The PCR reaction mixtures in a total volume of 50 µl, consisted of 1µl cDNA template from a 20µl cDNA synthesis reaction using 5µg of total RNA, 5µl reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 7.0, and 1.0% Triton® X-100), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM each of forward and reverse primer, and 2.5 units proof-reading Taq DNA polymerase (Promega, Madison, WI). The reaction mixtures underwent an initial 3-minute denaturation at $94^\circ C$, then 30 cycles at $94^\circ C$ (1 minute), $55^\circ C$ (1 minute) and $72^\circ C$ (2 minutes 30 seconds), and a final extension time of 10 minutes at $72^\circ C$. The PCR products were resolved on 0.8% agarose gels and stained with ethidium bromide. Aliquots (4µl) of the RT-PCR products were used for cloning into the cloning vector (PCR2.1)

following instructions provided with the cloning kit
(Invitrogen). Putative positive clones were first analyzed by
digestion with EcoRI restriction enzyme, and then sequenced
using an automated DNA sequencer (ABI 310 or Licor 4200).
Sequences were matched exactly to the confirmed genomic sequence
in GenBank.

5'- and 3'- RACE

5'-RACE was used to obtain the 5' untranslated region (UTR)
of the At2g30210 gene. In brief, 5µg total RNA isolated from
seedling roots was reverse transcribed using the gene-specific
reverse primer (1694R) and Superscript II as previously
described. The resultant cDNA was used as template for PCR
employing the proof-reading polymerase (Promega), in combination
with the secondary reverse primer (1061R) and an abridged anchor
primer provided by the RACE kit manufacturer (Invitrogen). The
PCR product was subsequently cloned into the pCR2.1 vector and
sequenced. The potential secondary structure of the 5'UTR was
predicted using the RNAfold program at a web interface server
(http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Hofacker et
al. 1994).

To capture the sequence of the 3'UTR of the At2g30210 gene,
3' RACE was performed as described above, and the forward primer
(76F) and reverse primer provided by the manufacturer (AUAP),

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were used to amplify the 3'UTR region. PCR products were cloned into the pCR2.1 vector and sequenced as described previously.

Expression of At2g30210 in *E. coli*

The pTrcHis Xpress^{TM} Protein Expression System (Invitrogen) was used to express the LMCO protein in *E. coli*. The At2g30210 cDNAs, one with the full-length coding sequence (1F) and the other lacking the signal peptide (76F), were subcloned from the cloning vectors into the pTrcHis expression vector digested with the EcoRI restriction enzyme. The resultant constructs were used to transform *E. coli* Top10 (Invitrogen) or BL21-CondonPlus RP (Stratagene, La Jolla, CA) cells. Correct orientation of the At2g30210 cDNA inserts with respect to the Trc promoter was confirmed by restriction analysis and sequencing. For expression, 50 ml of SOB medium (20g tryptone, 5g yeast extract, 0.5g NaCl, 0.186g KCl in 1 liter water, pH 7.0) containing ampicillin (50μg/ml) was inoculated with 0.3ml of an overnight culture, and incubated at 37 ^{o}C with vigorous shaking to reach OD$_{600}$=0.6. Inducing agent, IPTG, was added to a final concentration of 1 mM, and cultures were continued for 4 hours. Cells were harvested by centrifugation at 6000 x g for 5 minutes and lysed in using the freeze-and-thaw method described in the Xpress Purification manual (Invitrogen).

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Recombinant proteins were purified using the Xpress System Protein Purification (Invitrogen). In brief, cells were resuspended in 10mL of guanidine lysis buffer (6M guanidine, 20mM sodium phosphate, 500mM NaCl, pH 7.8), and slowly rocked for 10 minutes at room temperature, followed by sonication on ice with three 5-second pulses at high intensity. The insoluble debris was removed by centrifugation at 3000 x g for 15 minutes, and the supernatant was loaded onto a ProBond^{TM} resin column. The column was washed with a series of denaturing washing buffers (8M urea, 20mM sodium phosphate, 500mm sodium chloride) at various pHs (7.8, 6.0, 5.3), and eluted with the denaturing washing buffer at pH 4.0. The eluted fractions were collected in 1.5 ml tubes and aliquots (10μl) were loaded onto gels for SDS-PAGE and immunoblot analysis.

Expression of At2g30210 in tobacco cells

The cloned full-length coding region (1F) was excised from PCR2.1 in an XbaI/SpeI digest and inserted into XbaI-digested pKYLX80 vector under the control of a dual-enhanced CAMV 35S promoter. Orientation was confirmed by restriction analysis and sequencing. The resultant construct was precipitated onto gold particles (1 μm) and introduced into BY2 cells using a PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA) equipped with 11 Mpa rupture disks according to the manufacturer's instructions. Four-day old cultured tobacco cells

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were used as targets for particle bombardment as described by LaFayette at al. (1999). Transformed cells were selected on solid MS medium containing 200mg/L kanamycin and then transferred to new plates containing the chromogenic laccase substrate, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) to screen for phenoloxidase activity.

Cell lines positive for ABTS oxidation were maintained on solid medium containing kanamycin. After several transfers, cells were harvested and lysed in buffer containing 50 mM NaOAc (pH 6.5), 1 mM phenylmethethylsulfonyl fluoride (PMSF), 10 mM NaCl, 1 mM CuSO4 and 10 % glycerol using an OMNI Macro homogenizer (Omni International, Inc., Gainesville, VA). Cell debris was removed by centrifugation at 20,000 x g and 4°C for 30 min, and supernatant was filtered through Miracloth (Calbiochem-Novabiochem Corp., San Diego, CA). Supernatants were tested directly for phenoloxidase activity without further purification. To test for ferroxidase activity, the supernatant was adjusted to 50% saturation ammonium sulfate and stirred overnight at 4 °C. Insoluble material was removed by centrifugation at 20,000 x g and 4 °C for 30 min, and supernatant was filtered through Miracloth. The resultant enzyme pool was desalted and concentrated by ultrafiltration against a 30 kDa cutoff membrane (Amicon, Beverly, MA), and stored at -80 °C until use.

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Protein and enzyme assays

Protein was quantified using the Bio-Rad protein assay reagent (Bio-Rad) against bovine serum albumin (BSA) standards. Phenoloxidase activity was determined from the increase in absorbance at 420 nm due to the oxidation of 5 mM ABTS in 100 mM sodium acetate buffer (pH 5.0) (Hoopes and Dean 2004). Ferroxidase activity was assayed using ferrous sulfate (100 μM) as the electron donor in 100 mM sodium acetate buffer (pH 5.0) and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a specific chelator to bind ferrous iron remaining at the end of the reaction (De Silva et al. 1997). The assay was also run in a modified reaction using 1μM apo-tranferrin as Fe^{3+} acceptor to promote reactions. Protein boiled for 20 minutes was used as a negative control. Reactions were carried out in disposable cuvettes containing 100μM ferrous sulfate in 100 mM sodium acetate buffer (pH 5.0). The reactions were quenched by addition of ferrozine to a final concentration of 1.5 mM after incubation at room temperature. The rate of Fe^{2+} oxidation was calculated from the decreased absorbance at 560 nm.

Gel electrophoresis

SDS-PAGE was performed according to the protocol of Laemmli (1970). For phenoloxidase activity zymogram, crude extracts were mixed 1/1 (v/v) with 2x Laemmli sample buffer containing no

reducing agents, and loaded directly onto gels without heat denaturation. After electrophoresis, gels were stained for phenoloxidase activity using 1,8-diaminonapthalene (DAN) as described previously (Hoopes and Dean 2001). For ferroxidase zymograms, concentrated and semi-purified protein was tested as described (Hoopes and Dean 2004) using the *E. coli yacK* ferroxidase as a positive control (Kim et al. 2001).

Results

Cloning and characterization of the At2g30210 gene from *A. thaliana*

Although the At2g30210 gene was predicted from genomic sequence information deposited in GenBank, and available from The Arabidopsis Information Resources (TAIR) Database, no full-length cDNA was available to confirm the predicted gene. To clone the full-length cDNA, a PCR strategy relying on primers based on the genomic sequence was applied. A full-length coding region cDNA (primer 1F, 1714 bp product) and a second cDNA (primer 76F, 1639bp product) lacking the predicted signal peptide were amplified and cloned, respectively, from cDNA pools derived from 14-day old seedling root tissues. In addition, four different lengths of 3' untranslated region (3'UTR), ranging from 179 to 230 bps, with the consensus AAUAAA sequence and poly(A)+ tail were also cloned. A search of the *Arabidopsis* EST

database identified one sequence (Accession No. BX839990)

containing a putative 5' untranslated region (5'UTR), although

that 5'UTR was not used in predicting the At2g30210 gene model.

To clone the 5'UTR and confirm the transcription initiation

site, a 5'-RACE protocol was used to recover an additional 47

bases of sequence upstream of the translation initiation codon.

Comparison of sequence data for the cDNAs with genomic DNA

allowed us to generate a gene structure (Figure 2.2), which

agreed with the predicted gene model, except for the 47 bp UTR

upstream of the translation initiation codon. In the 5'UTR

region, an $(AG)_3$ repeat flanked the 5' side of the translation

initiation codon. Various lengths of AG repeats were also

reported in the 5'UTRs of four different LMCO genes isolated

from *Liriodendron tulipifera*. In addition, the predicted

secondary structure (Figure 2.3) of this 5'UTR suggested two

hairpin structures, similar to iron-response elements (IREs)

found in ferritin and transferrin receptor genes in animals.

This data suggested possible regulation of the At2g30210 LMCO at

the post-transcription level.

Amino acid analysis showed that At2g30210p contains 570 amino

acids with a predicted molecular mass of 64 kDa, and possesses

the conserved copper binding domains that characterize

multicopper oxidases. The Amino acid sequence also contains a

putative glycosyl hydrolase family 1 signature near the carboxyl

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terminus. The At2g30210 protein has a predicted isoelectric

point at pH 9.67 and harbors 7 potential N-linked glycosylation

sites. Analysis of the inferred protein sequence using SignalP

indicated that At2g30210p has a functional signal sequence and

may be targeted to the secretion pathway. Furthermore, the N-

terminus of the signal peptide contained twin arginine residues,

a feature that could possibly target At2g30210p to the TAT (Twin

Arginine Translocation) protein transport system, which has been

reported to transport folded proteins across membranes in

bacteria and plastids (Cristobal et al. 1999, Berks et al.

2000).

Heterologous expression of the At2g30210 gene product in *E. coli*

Previous studies showed that at least eight *Arabidopsis* LMCO

genes are simultaneously expressed in young root tissues (McCaig

et al. 2005). To obtain large quantities of individual proteins

for enzymatic activity analyses, the recombinant At2g30210

proteins were expressed first in *E. coli*. The full-length cDNAs

with (1F) and without signal peptides (76F) were placed in

constructs having a 6x His-tag at the N-terminus, and these were

expressed under control of the *E. coli* Trc promoter. The *E.

coli* strain, TOP10, was used first for expression studies, but

low levels of expression suggested that codon usage differences

between *E. coli* and *Arabidopsis* might be a problem (Table 2.1).

To improve expression, another strain, BL21-CondonPlus RP

(Stratagene, La Jolla, CA), was transformed with the same constructs. This *E. coli* strain contains extra copies of the *argU* and *proL* genes, which encode tRNAs that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively. These are low usage codons in prokaryotes. After screening for positive colonies, recombinant protein was detected by western blot in cell lines carrying the shortened construct (76F) (Figure 2.4), but no expressed enzyme could be detected for the full-length construct (1F). Further analysis showed, however, that the recombinant protein accumulated in insoluble aggregates so that purification could only be accomplished under denaturing conditions. Unfortunately, we were unable to detect any phenoloxidase activity using the laccase substrates, ABTS or 1,8-diaminoaphthalene (DAN). We manipulated various expression conditions to obtain active enzyme, including culturing at low temperature, adding copper, and changing inducer (ITPG) concentration, but no active product was recovered.

Heterologous expression of the At2g30210 gene product in tobacco BY2 cells

To overcome the difficulties encountered with the *E. coli* expression system, a tobacco (BY2) cell-suspension culture system was used to express the At2g31020 gene. The full-length coding region (1F) of the At2g30210 cDNA was inserted into a

plant expression vector (pKYLX80) under the control of a dual-enhanced CaMV 35S promoter. Tobacco cells were transformed with this vector using particle bombardment. Laccase activity was demonstrated in three cell lines identified from amongst 56 kanamycin-resistant tobacco calli by testing the ability of cells to oxidize the laccase substrate, ABTS (Figure 2.5A). No freely soluble laccase activity was detected in the culture medium, as was previously seen in transformed tobacco cells expressing the sycamore maple LMCO gene (Dean et al. 1998). Proteins extracted from calli of the three resistant cell lines were compared with a control cell line transformed with the pKYLX80 vector. One of these three lines (Fig 2.5A, #2) exhibited a novel H_2O_2-independent phenoloxidase activity using an in-gel detection method with 1,8-diaminonaphthalene (DAN), a substrate specific for laccase and peroxidase (Hoopes and Dean 2001). Endogenous phenoloxidases were also detected as bands of activity in lanes containing extracts from control BY2 cells (Figure 2.5B). The apparent molecular mass of the recombinant LMCO product was ~64 kDa, as estimated by SDS-PAGE. Phenoloxidase activity of the cell line was also measured in a liquid assay using ABTS as substrate, and more than 3-fold greater activity was detected in the highest expressing kanamycin-resistant line compared to control cell lines (data not shown). The growth rate of cell lines expressing the

Arabidopsis LMCO was no more than half that of cell lines transformed with empty vector (data not shown). The same observation was previously reported for tobacco cells expressing a LMCO gene from yellow-poplar (Hoopes and Dean 2004).

Recently, ferroxidase activity associated with a plant LMCO gene was demonstrated *in vitro* (Hoopes and Dean 2004), and similar activity has been seen with LMCOs from other organisms (Kim et al. 1999; Herbik et al. 2002). To test whether the At2g30210 LMCO harbored ferroxidase activity, cell lysates were partially purified by ammonium sulfate precipitation and ultrafiltration. Three independent tests for ferroxidase activity were conducted using a zymogram approach, but no such activity was detected (Figure 2.6A). In addition, a liquid assay using ferrous sulfate as the electron donor also showed no detectable ferroxidase activity for the At2g30210 protein, as judged in comparison to extracted protein from control and transgenic tobacco cell lines (Figure 2.6B).

Tissue-specific expression of the At2g30210 gene

Clues to the physiological function(s) of a gene can sometimes be inferred from its pattern of expression in different tissues. RT-PCR was used to detect the expression of the At2g30210 gene in different *Arabidopsis* tissues. Figure 2.7A shows that At2g30210 is expressed primarily in roots. Several genome-scale transcriptional profiling datasets for *Arabidopsis*

have recently been made available online, including Massively

Parallel Signature Sequencing (MPSS) (http://mpss.udel.edu/at/,

Meyers et al. 2004), and the Genevestigator

(https://www.genevestigator.ethz.ch, Zimmermann et al. 2004).

Figures 2.7B and 2.7C show At2g30210 gene expression patterns in

different *Arabidopsis* tissues as obtained from the MPSS and

Genevestigator databases, respectively. Although a previous

study (McCaig et al. 2005) showed At2g30210 gene expression was

specific to young root tissues, these other techniques found low

level expression in additional tissues. Notably, expression was

seen during seed development (Figure 2.7C). As none of these

tissues are rich in lignin, this data suggests that the gene

product may be involved in physiological roles other than

lignification. In general, the expression patterns observed

using these different approaches agreed with each other,

although the exact tissue samples used in the various studies

were not identical. Attempts at RT-PCR analysis of At2g30210

expression in siliques failed due to problems with RNA isolation

from this tissue, so we were unable to confirm the data for this

tissue obtained from online sources.

Discussion

The completed genome sequence for *Arabidopsis* and advanced

genomic techniques that can provide mass data by analysis of the

entire complement of *Arabidopsis* genes simultaneously provides a great resource for plant biologists, but investigation of the *in vivo* function for every gene in the organism still requires detailed studies specific to each target gene. There are an estimated 25,500 genes in *Arabidopsis* (ARABIDOPSIS GENOME INITIATIVE 2000), but only a small proportion of them have been well-characterized with respect to function. Within the *Arabidopsis* genome, 17 genes have been annotated as LMCOs, but none has yet been experimentally characterized, although most have been included in commercial microarray chips for genome-scaled studies. Here we report isolation of the first full-length *Arabidopsis* LMCO cDNA, and show that the gene structure and enzyme activity are similar to those reported for other plant LMCOs. The completed *Arabidopsis* genome sequence, as well as the diversity of bioinformatics tools and numerous mutant resources available for the species, makes *Arabidopsis* an excellent model in which to investigate the physiological function(s) of LMCOs in plants.

Combining 5′ and 3′ RACE, a PCR-based strategy allowed us to obtain the full-length cDNA sequence of At2g30210. The full-length DNA supports the computationally predicted intron-exon boundaries annotated in the TAIR database, adding only the 5′ UTR and giving the precise sequence of the 3′UTR with polyadenyl tails. A consensus TATA box located at position -25 upstream of

the 5'UTR suggests that the initiation site for At2g30210 transcription occurs at position -47 from the coding sequence. There were 11 ESTs matching At2g30210 in GenBank and only one (BX839990) contained the 5'UTR extending to 30 bp in front of ATG start codon. However, this sequence was not used to predict a 5'UTR for the At2g30210 gene model in the database. The predicted secondary structure of the 5'UTR contains stem-loop structures similar to IREs in animal genes, suggesting possible regulatory significance. Although some members of the LMCO family play roles in iron metabolism, we did not find evidence to support such a function for At2g30210p. Further study to compare the expression level of transcripts and mature protein would be necessary to determine whether the 5'UTR functions in the regulation of this LMCO.

Compared to the 5'UTR, the length of the 3'UTR seems to be more flexible. In general, a consensus polyadenylation signal (AAUAAA) was found 10 to 30 bp upstream from where the primary transcript is cleaved to generate a new 3'-OH end. There were 3 different lengths of 3'UTR with a variety of polyadenyl tails ranging in length from 15 to 23 A residues. Sequence analysis of this region showed a consensus sequence (AAUAAA) for poly (A)$^{+}$ addition 14 bp upstream of the poly(A)$^{+}$ tail in the longest 3'UTR sequence (230 bps). Interestingly, a second sequence (AAUAGA), similar to the poly(A)$^{+}$ signal sequence, was found 12

bp upstream of the poly(A)$^+$ tails of 2 clones that were 44 bp

shorter than the one having the longest 3'UTR, suggesting that

AAUAGA may serve as an alternative signal for poly(A)$^+$ addition.

Additional support for alternate polyadenylation comes from

three ESTs in the *Arabidopsis* EST database (AV538466, AV547158

and AV538596), which terminated their 3' UTRs 24 to 39 bp

downstream of the AAUAGA signal, although the poly(A)$^+$ tails

were not identified. In another case, an EST (Z34656)

containing a longer 3'UTR was identified amongst cDNAs generated

from seven-day-old etiolated Versailles-VB seedlings.   This

longer 3'UTR was 60 bp longer than the longest we identified,

extending 74 bp beyond the poly (A)$^+$ addition site. Further

study will be required to understand whether ecotype or the

etiolation treatment may be responsible for the unusual 3'UTR.

The function of plant LMCOs has been frequently associated

with lignification due to the ability of these enzymes to

oxidize monolignols *in vitro* and their expression in lignifying

tissues (Dean et al. 1994). However, the expression of multiple

isoenzymes (gene products) with overlapping substrate

specificities within specific tissues or organs has been a major

obstacle to elucidating the function of individual plant LMCOs

(Ranocha et al. 1999). Moreover, lignin content and composition

were not changed in transgenic aspen (Ranocha et al. 2002) or

yellow-poplar (Dean et al. 1998) engineered to down-regulate LMCO expression. In other cases, the high levels of plant LMCO proteins expressed in non-lignifying tissues, such as suspension-cultured *Acer* cells and *Rhus* sap, as well as global expression patterns of *Arabidopsis* LMCOs (McCaig et al. 2005), suggest that plant LMCOs must play multiple roles beyond lignification.

Heterologous expression allowed us to characterize the At2g30210 LMCO with minimal interference from endogenous LMCOs. The zymogram using a laccase-specific substrate, DAN, gave strong indication that the gene encoded an active LMCO. Plant LMCOs have been considered for the most part exclusively extracellular enzymes, being purified from the spent medium of sycamore maple cell cultures (Driouich et al. 1992, Sterjiades et al. 1993 et al.), from extracts of loblolly pine and poplar xylem cell walls treated with high-salt buffers (Bao et al. 1993, Ranocha et al. 1999), and secretions from transgenic *Arabidopsis* roots expressing a cotton LMCO (Wang et al. 2004), as well as the sap released by lacquer trees (Keilin and Mann 1939). However, the At2g30210 gene product could only be released from transformed tobacco cells by cell disruption. The same phenomenon was also observed when yellow-poplar LMCOs were expressed in tobacco cells (LaFayette et al. 1999, Hoopes et al. 2004). The authors postulated that a specialized glycochaperonin

and/or secretory pathway may be required for proper folding and
transport of LMCOs to the apoplasm during differentiation, and
the factors for the proper processing were not present in
suspension-cultured cells (LaFayette et al. 1999). On the other
hand, although the At2g30210 gene product was predicted using
two different algorithms (Predotar V1.03 and TargetP V1.01) to
possess a secretory pathway signal peptide and destined for
secretion via the endomembrane system, the At2g30210 protein was
predicted to have a nuclear localization signal by SubLoc, and a
transmembrane domain within the signal peptide made it difficult
to conclude whether this specific LMCO would be secreted.
Efforts are under way to determine more precisely the
intracellular localization site of the At2g30210 LMCO in BY2
cells, as well as in transgenic *Arabidopsis* plants.

   Histochemical approaches have previously demonstrated that
laccase-like phenoloxidase activities are spatially and
temporally associated with lignifying xylem tissue in various
herbaceous and woody species. The At2g302120 gene is expressed
in root tissue, specifically young roots, which contain little
xylem tissue. A study of transcriptional regulation of secondary
growth in *A. thaliana* showed that At2g30210 gene expression is
up-regulated in bark compared to xylem tissues (Oh et al. 2003).
These results suggest that the At2g30210 LMCO may not be
involved in ligninfication of xylem tissue. However, several

reports mention the presence of lignin in conjunction with suberin in endodermal and hypodermal cell walls of developing roots, where the modified cell walls, called the Casparian strip, serve as diffusion barriers that play important roles in response to stress (Zeier et al. 1999; Degenhardt and Gimmler 2000; Yokoyama and Karahar 2001; Ma and Peterson 2003; Karahara et al. 2004).

Another possible function for LMCOs expressed in roots is detoxification of phenolic compounds. LMCOs are one of several extracellular enzymes capable of degrading chemical compounds in their immediate vicinity (Boyajian and Carreira 1997). Wang et al. (2004) showed that transgenic *Arabidopsis* over-expressing a secretory LMCO that is normally expressed in roots of cotton (*Gossypium arboretum*), exhibited enhanced resistance to several phenolic allelochemicals and 2,4,6-trichlorophenol. However, *Arabidopsis* plants in the study showed limited laccase activity in root tissues and concentrated culture medium, and since wild-type plants survived in media containing the same toxic phenolic compounds, the endogenous LMCOs cannot be ruled out as being involved in the detoxification mechanism.

Recent evidence for ferroxidase activity in a LMCO from yellow-poplar suggested another possible function for the *Arabidopsis* enzymes. Plant LMCOs might have the capacity to act in iron-uptake systems similar to the FET3 system in

Saccharomyces cerevisiae (Hoopes and Dean 2004), or the iron-oxidizing activity associated with a LMCO from the pathogenic fungus, *Cryptococcus neoformans* (Liu et al, 1999). An essential role for the LMCO homolog, ceruloplasmin, in cellular iron efflux is critical to maintaining iron homeostasis in vertebrate animals (Harris et al. 1999). Also, haphaestin, a ceruloplasmin homolog, is involved in releasing iron from intestinal enterocytes into the circulatory system (Vulpe et al. 1999). More recently, the ferroxidase activities of LMCOs have also been demonstrated in *E. coli* (Kim et al. 2001), and green algae (Herbik et al. 2002). The result that the At2g30210 gene product could not oxidize ferrous iron *in vitro*, however, implies that it is likely not involved in iron metabolism. It should be noted that a single LMCO from yellow-poplar is the only one from plants that so far shows ferroxidase activity. Phylogenetic analysis (McCaig et al. 2005) showed that the yellow-poplar LMCO segregates from LMCOs shown not to harbor ferroxidase activity. Further study to test the ferroxidase activity in other LMCOs grouped with the yellow-poplar LMCO will help us determine the possible functions of different groups of plant LMCOs.

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Table 2.1. Different codon usages of Arg and Pro and Leu for *E. coli* and the At2g30210 gene. Codon usage is expressed as the fraction of all possible codons for a given amino acid. "All genes" is the fraction represented in all 4,290 coding sequences in the E. coli genome. "Class II" is the fraction represented in 195 genes highly and continuously expressed during exponential growth. Table is modified from Novy et al. (2001).

| Amino acid	codon	Fraction in all genes of E. coli	Fraction in class II genes of E. coli	Fraction in At2g30210	quantity of this codon in At2g30210
Arg	AGG	0.022	0.003	**0.100**	4
Arg	AGA	0.039	0.006	**0.250**	10
Arg	CGG	0.098	0.008	0.100	4
Arg	CGA	0.065	0.011	**0.150**	6
Arg	CGU	0.378	0.643	0.325	12
Arg	CGC	0.398	0.330	0.075	4
Pro	CCG	0.525	0.719	0.160	8
Pro	CCA	0.191	0.153	**0.542**	26
Pro	CCU	0.159	0.112	0.140	7
Pro	CCC	0.124	0.016	0.160	8
Leu	UUG	0.129	0.034	0.179	7
Leu	UUA	0.131	0.055	0.103	5
Leu	CUG	0.496	0.767	0.077	2
Leu	CUA	0.037	0.008	**0.103**	5
Leu	CUU	0.104	0.056	0.264	9
Leu	CUC	0.104	0.080	0.282	11
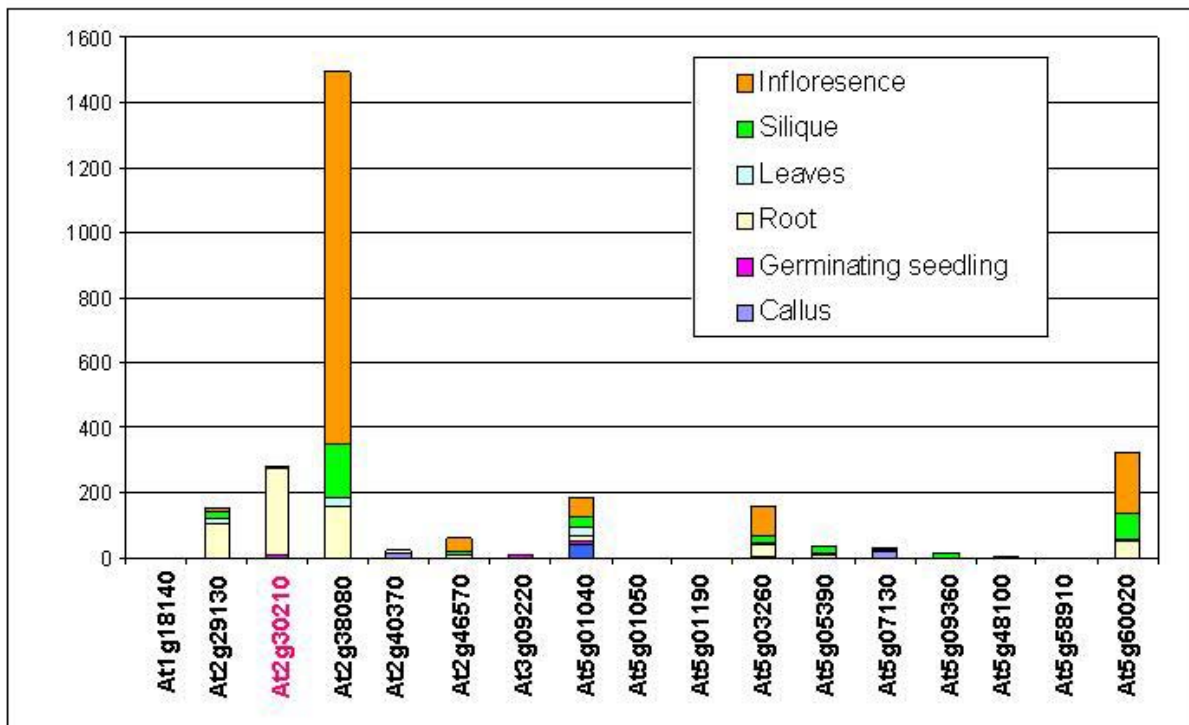
Figure 2.1. Expression profile of LMCOs in *Arabidopsis thaliana.*
LMCO gene expression data in selected *Arabidopsis* tissues
obtained using from massively parallel signature sequencing
(MPSS) was collected from the *Arabidopsis* MPSS website
(http://mpss.udel.edu/at/). Values represent the pooled number
of times the predominant signature tag for each *Arabidopsis* LMCO
gene was seen in MPSS libraries from various *Arabidopsis*
tissues.

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Figure 2.2. Structure of the *Arabidopsis* At2g30210 gene. The Genomic DNA sequence of At2g30210 is shown (A). Introns (gray) and the untranslated regions (blue and underlined) are represented in lowercase letters. A putative TATA box is shown in boldface. Intron positions were based on cDNA sequence compared with genomic sequence. Relative locations of the signal peptide (red) and copper-binding domains (yellow) in the At2g30210 gene are shown (B). Exon color indicates featured domains.

Figure 2.2.

A.

```
1287044  aataaattgt ttaattgcta acgaattcac gaagacctct gttgtttccc gcttagtgtg
1286984  tataaatacc actccttcct tttctccaaa ctcatctcac aaatctccaa cacagagagc
1286924  ttctcacagt gaagcaaaca ATGGAGTCTT TTCGGCGATT CTCCTTGCTA TCCTTCATTG
1286864  CCCTACTTGC CTACTTCGCT TTCCTCGCTT CTGCTGAACA TCACGTCCAT CAATTCGTGg
1286804  taagctagct actaatgtta cattaaagct ttttggtatg ttcttacgtt tatagaattc
1286744  ccttaattta agtttaaacg acgatgactt tagATCACAC CGACACCAGT GAAGAGGCTG
1286684  TGCAGAACTC ACCAAAGCAT CACTGTGAAT GGTCAGTACC CTGGTCCAAC GCTTGTGGTC
1286624  AGGAACGGTG ACTCTCTCGC AATCACTGTC ATCAACAGAG CCCGTTACAA CATTAGTATT
1286564  CATTGgtaaa atatttgacc atcaaattca acaaacatat gttatgttat gttctctgtt
1286504  ttcatacttt gttttttgct ctgtttttttg ggatagGCAT GGAATCAGAC AGCTGCGGAA
1286444  TCCGTGGGCC GATGGTCCAG AGTATATAAC ACAATGTCCG ATCCGTCCAG GACAAACCTA
1286384  CACTTACAGA TTCAAAATCG AGGATCAAGA GGGTACGCTT TGGTGGCACG CTCATAGCCG
1286324  CTGGCTCAGA GCCACGGTCT ATGGTGCTCT CATCATTTAC CCTCGTCTTG GTTCTCCTTA
1286264  TCCCTTCTCT ATGCCCAAAC GTGACATTCC AATTCTTCTT Ggtaaacaac taaacatttt
1286204  gtaacttcta atcacttcaa attatatttt catgatcaca actaataata cttactaaaa
1286144  tagGGGAATG GTGGGATAGA AACCCAATGG ATGTTTTGAA GCAAGCACAA TTTACGGGAG
1286084  CAGCAGCTAA TGTCTCTGAC GCTTACACAA TCAACGGTCA ACCAGGCGAT CTTTACCGCT
1286024  GCTCGCGGGC TGGGACAATC CGTTTTCCAA TTTTCCCCGG GGAGACGGTG CAACTCCGTG
1285964  TCATCAACGC TGGTATGAAC CAAGAGCTCT TCTTCTCAGT CGCCAACCAC CAGTTCACAG
1285904  TTGTAGAAAC TGATTCCGCC TACACGAAAC CATTCACCAC AAATGTCATC ATGATCGGTC
1285844  CTGGCCAAAC CACTAACGTC CTCCTCACGG CAAACCAGAG ACCAGGCCGC TACTACATGG
1285784  CAGCTCGAGC CTACAACAGC GCAAACGCCC CGTTCGACAA CACAACCACT ACTGCTATCT
1285724  TACAATACGT CAACGCTCCA ACAAGACGTG GCCGTGGTCG TGGTCAAATC GCTCCTGTTT
1285664  TCCCAGTCCT CCCCGGGTTC AACGACACCG CAACCGCAAC TGCTTTCACC AACCGTCTCC
1285604  GATACTGGAA ACGAGCTCCA GTACCACAAC AAGTCGACGA GAACCTCTTT TTCACCGTCG
1285544  GATTAGGGCT AATCAACTGT GCCAACCCAA ACAGTCCCCG TTGCCAAGGT CCTAACGGGA
1285484  CCCGATTCGC AGCAAGCATG AACAACATGT CCTTCGTGCT ACCACGAAGT AACTCCGTCA
1285424  TGCAAGCATA TTACCAAGGC ACCCCAGGAA TCTTCACAAC GGATTTTCCG CCCGTTCCAC
1285364  CGGTGCAATT CGATTACACA GGTAACGTTA GCCGCGGGTT ATGGCAGCCC ATAAAAGGAA
1285304  CCAAAGCTTA CAAGCTTAAG TACAAATCTA ATGTTCAGAT TGTGTTACAA GACACTAGCA
1285244  TTGTCACGCC AGAGAATCAT CCCATGCATC TACACGGGTA CCAATTCTAC GTGGTCGGGT
1285184  CAGGTTTCGG TAATTTCAAC CCGAGAACAG ACCCGGCTAG GTTTAACTTA TTTGACCCAC
1285124  CAGAGAGGAA CACCATTGGA ACACCTCCAG GTGGTTGGGT GGCAATTCGG TTCGTCGCTG
1285064  ATAATCCAGg ttagatgatc ttattgactc gaaagcattt gatactttgt aaccataggt
1285004  taaatgatct tattgactca aaaatgaaaa acatattttg atatacttgt ggtgtaatgc
1284944  agGAGCATGG TTTATGCATT GTCACATTGA TTCACATTTG GGATGGGGTT TGGCTATGGT
1284884  TTTCTTGGTA GAGAACGGTC GTGGACAGTT GCAATCGGTG CAGGCTCCAC CATTGGATCT
1284824  TCCAAGATGC TAAtaaaaaa cttctaccgt tggatctaaa gaatttatgg ggtttggttt
1284764  tttgttcttg tttttttaatc aaaaaaagac ttcgtgagtc ttgatatgtt aaagatggac
1284704  aactgctcta ttgaaagtgg ttcaacctttt gttttcgatg tactttttgtt attttcctcg
1284644  taatagagtg agtgttttca tttttccgat taaaaattta ccaaataaaa gctgtgttag
1284584  ttaatcttct ttttctagct gactttccca aacaactttta ttagttatta acttaatatg
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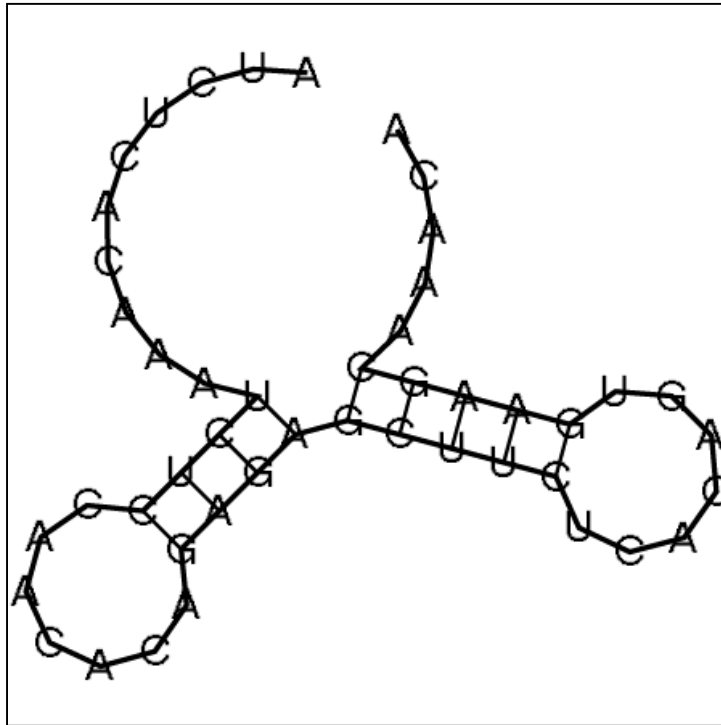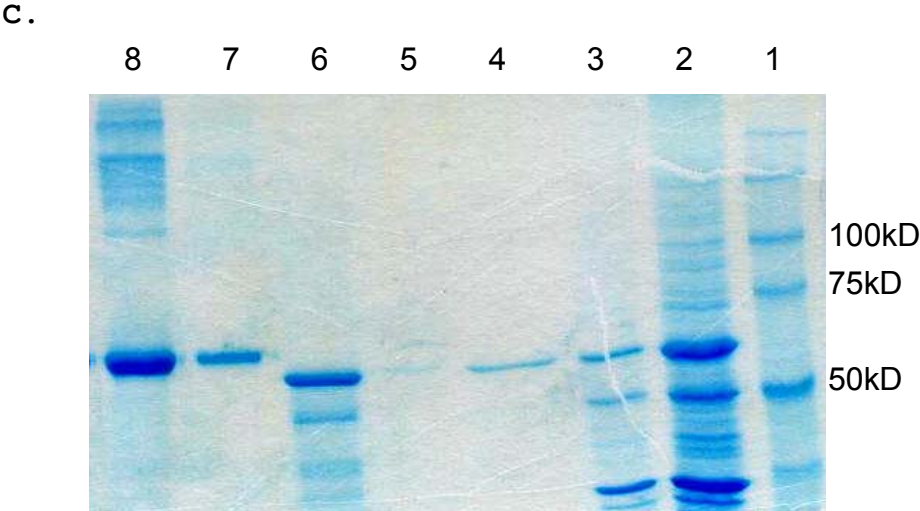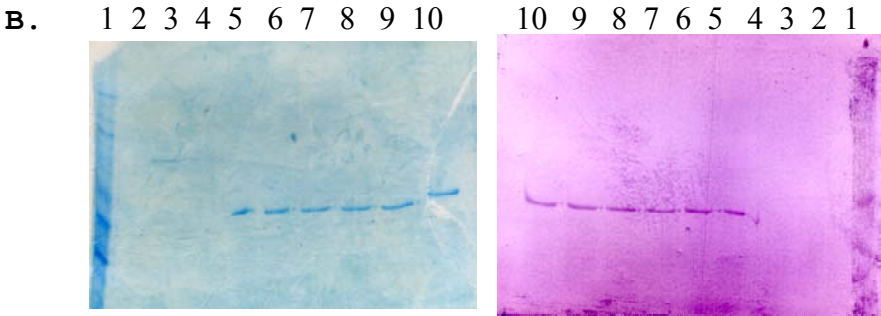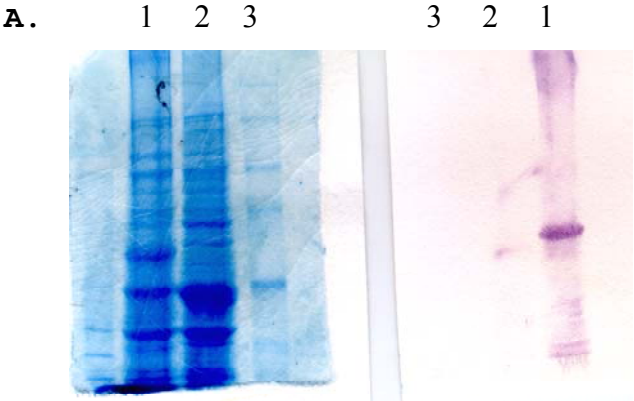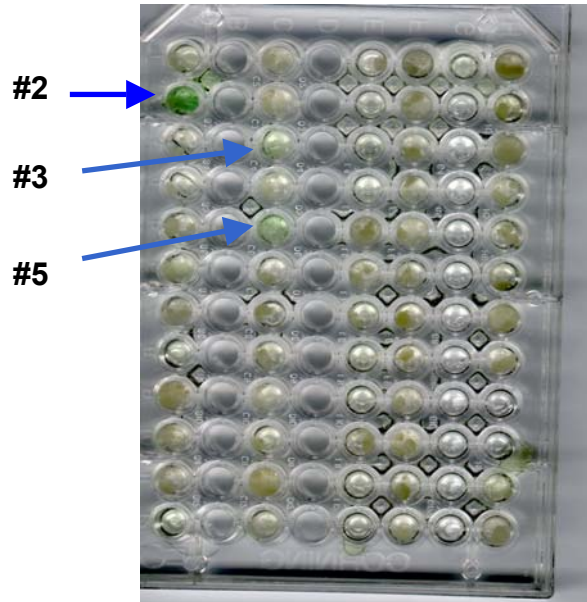
B.

Figure 2.3. Possible secondary structure in the At2g30210 LMCO 5'UTR. An optimal secondary structure based on the minimum free energy was predicted using RNAfold. The 5'UTR sequence was directly submitted to the RNAfold program at a web interface server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Hofacker et al. 1994).

Figure 2.4. Heterologous expression of At2g30210 in *E. coli.* Aliquots (10μl) of crude sample lysates and different purification steps were loaded onto gels for SDS-PAGE and immunoblot analysis, respectively. **A.** Crude extracts from transformed (Lane 1) and control cells (Lane 2) after four hours of induction were analyzed by 10% SDS-PAGE (left panel) and immunoblot with an antibody against Xpress^{TM} tag (right panel). **B.** Purification was performed with an affinity purification column under denaturing conditions using 8M urea buffer. Each step of the purification was collected and analyzed by 10% SDS-PAGE (left panel) and immunoblot (right panel). Lane 1: crude extract: Lane 2: flow through of binding buffer, pH 7.8; Lane 3: flow through of wash buffer, pH 6.0; Lane 4: flow through of wash buffer, pH 5.3; Lane 5~10: a series of fractions eluted using elution buffer, pH 4.0. Samples were analyzed by SDS-PAGE (left). **C.** Lane 1: molecular weight marker; Lane 2: crude lysate of induced cells; Lane 3: unbound fraction of sample through affinity column; Lane 4: pooled final elution using wash buffer (pH 4.0); Lane 5: sample after dialysis against 10mM Tris, pH 8.0, 0.1% triton X-100 overnight at 4 °C; Lane 6: insoluble pellet after dialysis; Lane 7: 1 μg BSA: Lane 8: 5 μg BSA.

Figure 2.4.

A.

1 2 3   3 2 1

B.

1 2 3 4 5 6 7 8 9 10   10 9 8 7 6 5 4 3 2 1

C.

8   7   6   5   4   3   2   1
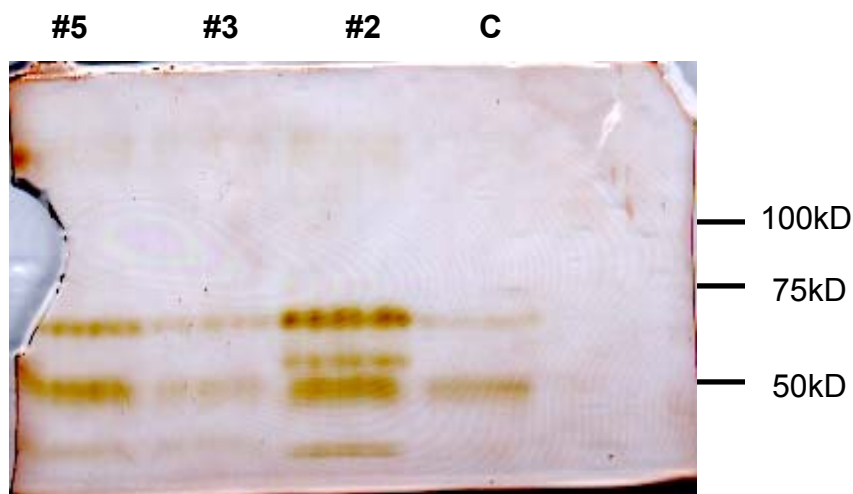
100kD

75kD

50kD

A.

B.

Figure 2.5. Enzyme activity analysis of the At2g30210 LMCO expressed in tobacco cells. **A.** Phenoloxidase activity was demonstrated in three kanamycin-resistant cell lines by testing the ability to oxidize the laccase substrate, ABTS, in liquid assays. **B.** Activity staining of multicopper oxidases separated using SDS-PAGE. Crude protein extracts from three cell lines (2,3,5) transformed with the AT2g30210 gene and a vector control cell line (C) were stained with 1,8-diaminonaphthalene (DAN) for phenoloxidase activities.

Figure 2.6. Testing the At2g30210 protein for ferroxidase activity. **A.** Partially purified protein (20 μg) from one resistant cell line (#2) was used for phenoloxidase staining with DAN (left panel). For detecting ferroxidase activity (right panel), the amount loaded for the sample was 10x that loaded (200 μg, Lane #2) for phenoloxidase staining and an equivalent amount of ABTS-oxidizing activity from the *yac*K protein (Lane *yac*K) was used as a positive control (Kim et al. 2001). Cleared zones representing ferroxidase activity were visualized by applying 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine (ferrozine). **B.** Solution assay for ferroxidase activity was determined using ferrous sulfate as the electron donor and ferrozine as a specific chelator to bind ferrous iron remaining at the end of the reaction. Aliquots of 3 μg protein from tobacco cells transformed with At2g3210 cDNA (#2) or vector alone (Vec) were added to a total 1 mL reaction. Reaction buffer (Buffer) and a boiled sample (Boiled #2) were also included for negative control. Another set of samples were also analyzed in the same buffer with addition of 1 μM apo-transferrin (+Tf), as a Fe^{3+} acceptor to promote reactions.
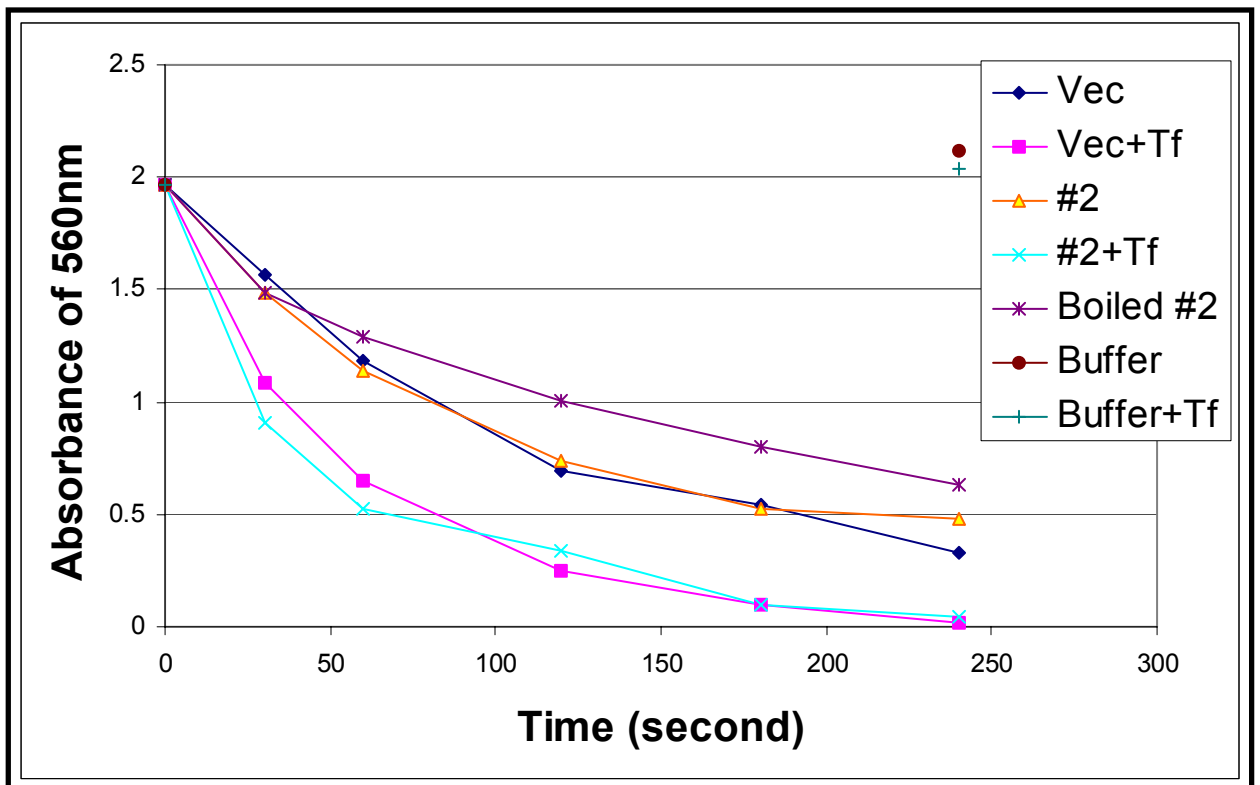
Figure 2.6.

A.
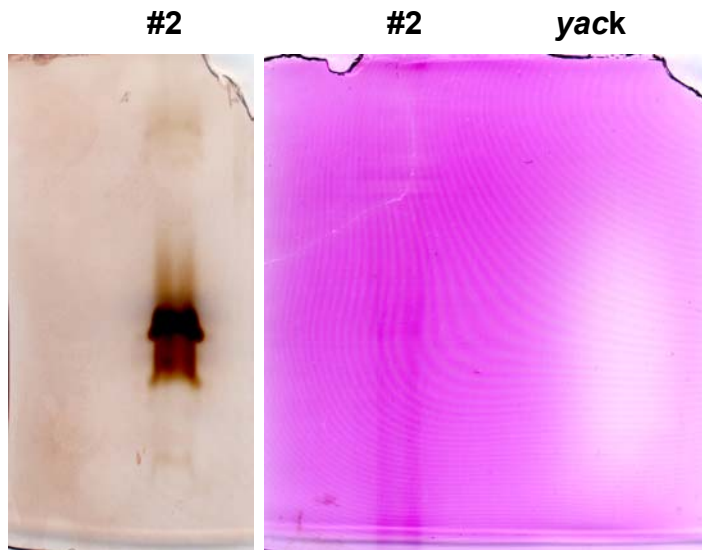
B.

Figure 2.7. Expression profile of the At2g30210 gene in various tissues at different developmental stages. **A.** Messenger RNA isolated from the identified tissues was reverse transcribed, and diluted cDNA product was used as template for each PCR reaction. QUB10 was included to control for equal cDNA input between tissue samples. fl: flowers, sil: siliques, lf: leaves, px: inflorescence, rt: roots, sdl: two weeks old seedling. **B.** Gene expression for the At2g30210 gene in selected *Arabidopsis* tissues as determined using massively parallel signature sequencing (MPSS), as described in Figure 1. **C.** Affymetrix expression data for the At2g30210 gene represented on the ATH1 whole-genome chip. Microarray data for selected tissues were collected from the public database (https://www.genevestigator.ethz.ch) (Zimmermann et al. 2004)

Figure 2.7.

A.

At2g30210

UBQ10

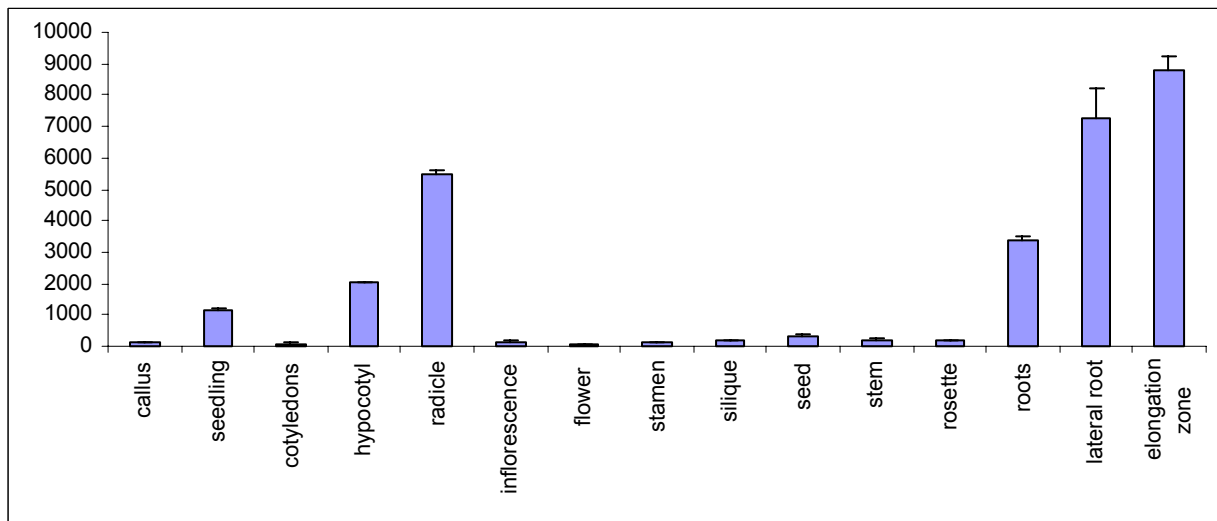fl   sil   lf   px   rt   sdl
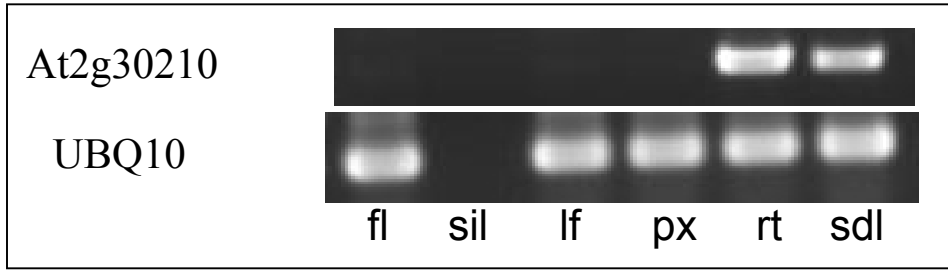
B.
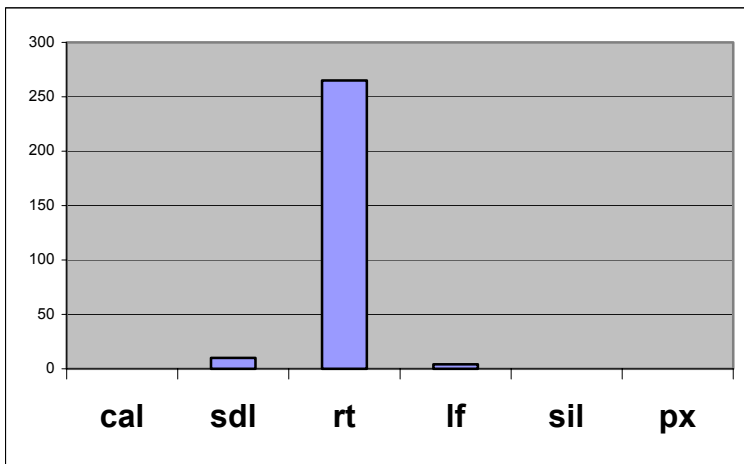
C.

CHAPTER 3

A LACCASE-LIKE MULTICOPPER OXIDASE (LMCO) MAY BE INVOLVED IN ENDODERMIS FORMATION IN *ARABIDOPSIS THALIANA* ROOTS[1]

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Abstract

Laccase and related laccase-like multicopper oxidases (LMCOs) have been studied in plants for more than a century, yet our understanding of their physiological function(s) remains limited. This study analyzed in greater detail the product of the At2g30210 gene, a member of the LMCO family that is predominately expressed in young root tissues of *Arabidopsis thaliana*. To determine the spatial expression pattern of At2g30210 transcripts, a promoter-reporter gene fusion was introduced into *Arabidopsis*. Histochemical analyses of transgenic plants expressing GUS activity revealed that At2g30210 transcripts were preferentially expressed in developing endodermis of the root elongation zone and newly matured roots. Low levels of expression were also detected in hypocotyls, as well as major veins of some cotyledons under some conditions. A chimeric fusion protein comprised of the At2g30210 coding sequence and a modified green-fluorescent protein (YFP) was used to examine subcellular localization of the protein in transgenic plants. Confocal microscopy indicated that the At2g30210-YFP fusion product was localized to the cell periphery, likely extracellularly and possibly to the cell wall. A genetrap insertion (GT7855) mutant of Atg30210 was confirmed by sequence analysis and PCR to have a transposon in the second exon of the gene. RT-PCR of seedling cDNA confirmed loss of

transcript expression. Plants homozygous for the GT7855 knockout mutant were grown under various culture conditions for comparison with wild-type plants. On solid MS medium containing 1% sucrose, the GT7855 mutant showed more lateral root production than wild-type plants. When grown in soil or MS media without sucrose, growth of the mutant was severely repressed. The At2g30210 knockout mutants were also much more sensitive to salt stress than wild-type plants. The results suggest involvement of the At2g30210 LMCO gene product in endodermal cell function, possibly in wall development events leading to formation of the Casparian strip.

Introduction

   Laccase is a member of the multicopper oxidase (MCO) family that also includes yeast FET3 class ferroxidases (De Silva et al. 1995), bacterial metal oxidases (Brouwers et al. 1999; Grass and Rensing 2001), mammalian and avian ceruloplasmin (Musci 2001), and plant ascorbate oxidases (Messerschmidt 1993). Laccases, or more correctly Laccase-like Multicopper Oxidases (LMCOs), have been founded in a wide range of organisms, including bacteria, fungi, insects, and plants, yet their physiological functions remain unclear. Wound healing through oxidative polymerization of alkylcatechols in the sap of *Rhus* species (true laccase) had been the only strongly supported

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physiological function advanced for plant LMCOs (Mayer and
staples 2002; McCaig et al. 2005), yet even the exact mechanism
in that function remains unclear. Plant LMCOs have also been
proposed to assist in lignin biosynthesis in previous reports
(Dean and Eriksson 1994; Dean et al. 1998; Mayer and Staples
2002). Early studies suggested that laccase and laccase-like
oxidase activities were associated with lignification in plants
based on their spatial and temporal correlation with
lignification (O'Malley et al. 1993; Dean and Eriksson 1994,
Ranocha et al. 1999), their capability to oxidize monolignols to
produce dehydrogenation polymers (DHPs)(Sterjiades et al. 1992,
Bao et al. 1993; Ranocha et al. 1999), and the impact on xylem
tissue formation in transgenic plants expressing sense and
antisense LMCO genes (Dean et al. 1998; Ranocha et al. 2002).

Other putative functions attributed to plant LMCOs derive
from their specific enzyme activities. Recent demonstration of
ferroxidase activity in a LMCO from yellow-poplar (Hoopes and
Dean 2004) was taken to suggest that some plant LMCOs might take
part in iron metabolism, similar to the function provided by
FET3 in the high-affinity iron uptake system of yeast. Also,
LMCO is one of several oxidative enzymes with demonstrated
capacity to degrade chemical compounds in their immediate
vicinity (Boyajian and Carreira 1997). That transgenic
Arabidopsis over-expressing a secretory LMCO from cotton

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(*Gossypium arboretum*) exhibited enhanced resistance to several phenolic allelochemicals and 2,4,6-trichlorophenol suggests plant LMCOs could also play a role in detoxification of environmental compounds (Wang et al. 2004). However, in most cases, definitive demonstration of physiological functions for plant LMCOs has been difficult because the enzymes are typically able to oxidize a wide variety of potential phenolic and inorganic substrates. In addition, multiple LMCO gene products may be expressed simultaneously in the same tissues.

The completed *Arabidopsis* genome sequence is expected to provide new ways of addressing biological questions from an integrated perspective, ranging from molecular genetics to evolution (Bouché and Bouchez 2001). Previous study showed that like other plants, the *Arabidopsis* genome encodes multiple (17) LMCOs. The seventeen LMCO genes exhibit a wide variety of expression patterns in different tissues and can be separated into six phylogenetic groups (McCaig et al. 2005). We have taken a combined genetic and biochemical approach to dissecting the physiological functions of the LMCO gene family in *Arabidopsis* using promoter-reporter gene constructs, gene knockouts, and chimeric fusion proteins. Our studies of the At2g30210 LMCO gene point to an extracellular role for the enzyme in development of cell walls in the root endodermis.

Materials and Methods

Identification of At2g30210 insertion mutants

Arabidopsis thaliana mutants were identified by searching the insertion line databases listed on the TAIR server (http:/www.Arabidopsis.org). The Salk_031901 T-DNA insertion line was identified in a collection at the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al. 2003). Annotation information was obtained from the website. PCR analysis was used to confirm the insertion and screen for homozygous transgenic plants using a combination of gene-specific primers (Table 3.1) from the 3' (031901RP) and 5'(031901LP) ends of the At2g30210 gene, as well as the T-DNA left border (Lba1) and right border (RB) primers, respectively. GT3416 and GT7855 were identified from among the gene-trap lines generated using Ac/Ds transposons at Cold Spring Harbor Laboratory (http://genetrap.cshl.org/). Annotation information was obtained from the website. Ds-specific primers (Ds3-4 and Ds5-4) together with At2g30210 primers (1F and 1016R) were used to confirm the insertions, as well as screen for homozygous individuals. All oligonucleotides used in this study were synthesized by Integrated DNA Technologies, Inc (Corvalville, IA) and used without further purification.

Plant materials and Growth conditions

A. thaliana plants were cultivated on soil mixes in growth chambers at 22°C and a 16h light/8h dark illumination cycle. For some experiments, plants were grown in petri dishes on solid MS media (Invitrogen, Carlsbad, CA) supplemented with 1% sucrose and 0.8% phytoagar. For root growth analysis, seedlings were grown on plates set vertically. Tobacco cells were maintained on agar plates as described by Lafayette et al. (1999).

Promoter expression analysis

For promoter activity analysis, 1 kb of genomic DNA upstream of the translational start site and including the first 2 codons was amplified by PCR using Pwo high-fidelity DNA polymerase (Boehringer Mannheim, Indianapolis, IN) with the PMT_F and PMT_R primers. The resultant amplimer was digested with SpeI/SptII and ligated in frame to the 5' end of the GUS gene contained in the pUPC5-GUS vector (from Dr. C Joseph Nairn, University of Georgia, USA). The resultant At2g30210 promoter:GUS fusion was subcloned into the pZP-NPTII binary vector developed by Dr. Nairn, and introduced into wild-type plants by *Agrobacterium tumefaciens*-mediated transformation using the floral dip method (Clough and Bent 1998). Transgenic seedlings were selected on MS medium containing 50μg/mL of kanamycin. Kanamycin-resistant lines were tested for the construct using GUS-specific primers.

GUS histochemical staining was performed on different *Arabidopsis* tissues and stages using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as a substrate (Jefferson et al. 1987). The tissues were stained with the substrate, fixed in FAA (50% ethanol, 10% glacial, and 5% formaldehyde) solution, and then cleared in 70% ethanol before observations. Selected GUS-stained roots were embedded in LRR white resin after ethanol dehydration. Cross sections obtained using a vibratome (TPI, Inc., St. Louis, MO) were observed under a light microscope.

At2g30120 protein localization

For subcellular localization analysis, the coding region of the At2g30210 cDNA was amplified by PCR using Pwo DNA polymerase (Boehringer Mannheim) with primers At2g30210-YFPC-F and At2g30210-YFPC-R. Care was taken to omit the stop codon (TAA) of At2g30210 and fuse the coding sequence in-frame with the EYFP sequence. The At2g30210 PCR product was digested and cloned into the SpeI/SptII sites of the p35S-EYFPC vector provided by Dr. Nairn (University of Georgia, USA). The resultant construct was confirmed by DNA sequencing and restriction digest analysis. The AT2g30210-EYFP fusion construct, under 35S promoter control, was directly transformed into suspension-cultured tobacco cells (BY2) using particle bombardment as described in Chapter 2. After over-night incubation in the dark, cells were observed using fluorescence microscopy with UV illumination (ARC lamp)

and a GFP3 filter (470 nm excitation filter; narrow band pass 525 nm barrier filter).

To generate stable transgenic plants, the construct was subcloned into the pZP-NPTII binary vector and introduced into *Arabidopsis* as described for the promoter:reporter gene construct. Kanamycin-resistant lines were confirmed for transgene presence using At2g30210 gene-specific primers (1F and 1016R) that could distinguish the transgene from the endogenous gene. T_2-generation progeny were used for EYFP localization. EYFP fluorescence from roots of 5-day-old transgenic seedlings was observed using a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany).

Quantitative PCR (qPCR) and RT-PCR experiments

Total RNA was extracted from two-week old seedlings grown in liquid medium. Template cDNA for all samples was made using 5µg of Rnase-free Dnase-treated total RNA and Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The first strand RT reaction was diluted 10 times with water and 10uL of diluted cDNA product was used for each qPCR reaction. Primers specific to At2g30210 (44F and 147R) were used to amplify a 140 bp region upstream of the mapped insertions. The internal control gene, G3PDH, was amplified using the specific primers, G3PDH-F and G3PDH-R. Primer concentration was 300nM for the reactions. DyNAmo SYBR Green

qPCR Mastermix (MJ Research, Watertown, MA) was used for qPCR experiments following the manufacturer's protocol. Samples were replicated three times for the At2g30210 gene and twice for the internal control (G3PDH). A standard curve based on known quantities of plasmid containing the At2g30210 cDNA was determined for each experiment. All experiments were run using an iCycler thermal cycler (Bio-Rad, Hercules, CA) under the following cycling conditions: 95°C for 10 min followed by 40 cycles of 95 $^{\circ}$C for 1 sec and 55°C for 1 min. The instrument was set to measure dye florescence at the end of each cycle. Melting curve analysis was performed at the end of reaction to ascertain the quality of the PCR products. The Cycle Threshold (cT) line was determined manually as the point where the R^2 value for the standard curve was highest.

The same cDNA pools were used for RT-PCR analyses to confirm gene disruption in the insertion lines. To test the Salk_031901 line, 1F and 1694R and 326F and 1694R primer pairs were used in different experiments. For GT7855, the 1F and 1016R pair was used. The PCR products were analyzed on 1% agarose gels stained with ethidium bromide.

Metal ion content

The metal ion concentration in *Arabidopsis* seeds was measured from soil-grown plants. Seeds from three individual plants each of Salk-031901, GT7855, wild-type, and At2g30120-EYFP transgenic

plants were collected. Samples were dried overnight in a desiccator under vacuum, and 0.2 g of dry seeds were digested in 70% HNO_3 using a microwave-assisted method (U.S. EPA, Method 3051). Trace elements were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) at the Environmental Analysis Lab (University of Georgia, USA). Before analysis, solutions were diluted by a factor of ~200.

Results

Isolation and molecular characterization of the At2g30210 mutants

The analysis of mutants is a powerful tool for revealing the role of a particular gene in physiological and developmental processes in plants. To date, several hundred thousand T-DNA and transposon insertion lines have been generated in various laboratories that make them available to the research community. Recently, several groups have initiated programs for the systematic sequencing of insertion sites in various populations (Bouché and Bouchez 2001), and the data are made available through online resources, such as TAIR (http://www.arabidopsis.org/links/insertion.jsp). Using this system, we identified three potential knockout insertion lines from different populations.

A T-DNA insertion line, Salk-031901, was identified in the Salk collection (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al. 2003), and the chromosomal flanking sequence showed that the T-DNA was inserted into the last intron of the At2tg30210 gene (Figure 3.1). To confirm the insertion, unique PCR products were generated using gene-specific primers from the 3' and 5' ends of the At2g30210 gene and the T-DNA left border primer (Figure 3.2A). The T-DNA right border primer together with At2g30210 primers failed to yield any product. This result suggested that the T-DNA was inserted as an inverted repeat within the At2g30210 gene. Similar insertions in which the T-DNA insert has two left borders, one at each end of the insertion site (LB-RB-RB-LB), have previously been reported in the Salk populations (Alonso et al. 2003). Three plants homozygous for the insertion were identified in a screen of 44 individuals using pairs of gene-specific primers that spanned the insertion region. RT-PCR analysis showed that two out of the three homozygous lines no longer produced properly edited transcript as indicated by loss of the ampliner band. The third line showed a weak, but detectable, ampliner band, suggesting that the T-DNA can be spliced out at low frequency (Figure 3.2B). Surprisingly, we were unable to identify kanamycin-resistant seedlings from any of the lines when we tested for co-segregation of the insertion with kanamycin resistance (data not shown). This

result is, however, consistent with a statement in the Salk database indicating that some lines show silencing of the phosphotransferase (NPTII) gene after several generations of growth.

While work with the Salk T-DNA line was proceeding, two additional potential insertions (GT3416 and GT7855, Figure 3.1) were identified among the gene-trap lines generated using Ac/Ds transposons at Cold Spring Harbor Laboratory (http://genetrap.cshl.org/). In that system, the Ds gene-trap element carries as a reporter a β-glucuronidase (GUS) gene having a multiple splice acceptor site, as well as the NPTII gene, as a selectable marker. The gene-trap line, GT7855, has a Ds insertion in the second exon of the At2g30210 gene, 135 bp downstream of the translation start codon, as annotated by chromosomal flanking sequence generated using a TAIL (thermal asymmetric InterLaced) PCR procedure (Martienssen and Springer 1998). The GUS gene is inserted with transcription in the same direction as At2g30210 transcription. The insertion was confirmed from a unique PCR product that was generated using a gene-specific primer and a Ds transposon element primer. We used PCR to identify homozygous seedlings in which no ampliner could be detected using a pair of gene-specific primers, while an amplification product was detected from wild-type genomic DNA (Figure 3.2C). Unfortunately, we were unable to detect any GUS

activity from the reporter gene, even though transcription of the reporter runs in the same direction as the LMCO. However, the insertion line was still a strong knockout mutation as no properly edited At2g30210 transcripts were detected by RT-PCT using primers that spanned the insertion region (Figure 3.2D).

The second putative gene-trap insertion line, GT3416, with a reported Ds insertion in the second exon of the At2g30210 gene, 127 bp downstream of the translation start codon, was also annotated from chromosomal flanking sequence as having the GUS reporter in the same orientation as the At2g30210 gene. Kanamycin-resistant seedlings from this line were transferred to soil and allowed to set flowers. Screening for homozygous individuals using PCR showed that all kanamycin-resistant plants were heterozygous (data not shown), and no significant phenotype was identified. We attempted this screening process three times, but the results were consistent. Whole mount GUS staining showed that the highest activity was observed in young roots, flowers and the abscission zone of siliques, with lesser activity in the pistil and immature seed (Figure 3.3). Very weak activities were also observed in anthers and nodes, but no activity was found in leaves or inflorescences.

Although the GT3416 line had a functional reporter gene with which to map gene expression in various tissues, further analyses failed to confirm that the insertion site was actually

within the At2g30210 gene. Attempts to use the original protocol to perform TAIL-PCR also yielded negative results (data not shown). Further analyses showed that the kanamycin resistance for all progeny from 4 lines failed to co-segregate with the insertion. These results indicated that the insertion may not be associated with the At2g0210 gene after all. Consequently, this line was dropped from further analyses.

Levels of At2g30210 mRNA transcript in homozygous Salk-031901 and GT7855 plants were compared with levels in wild-type plants using quantitative PCR (qPCR). These PCR analyses showed At2g30210 mRNA levels in homozygous GT7855 and Salk-031901 lines to be only somewhat reduced from wild-type levels, 71% and 56% of, respectively. Interestingly, whereas RT-PCR analyses of the Salk line clearly showed low levels of normal transcript being produced, PCR analysis of the 3' end of the transcript suggested instability of the T-DNA modified transcripts (increased turnover), which was not seen in the Ds modified transcripts expressed by the GT7855 line (Figure 3.4).

T-DNA knockouts of At2g30210 show reduced growth and altered root morphology

In the Salk-031901 insertion line, no significant phenotype was observed when seedlings were grown on soil or MS medium (data not shown). A weak phenotype in which the mutants did not develop as much as wild- type germinants was observed when

seedlings were grown on water agar plates (Figure 3.5), although growth was severely repressed in both due to a lack of nutrients. To investigate whether stored nutrients deposited during seed development caused the phenotype, trace element (Fe, Cu, Mn, Zn, and Co) levels were analyzed in seeds from both mutant and wild-type plants. No significant differences were noted between wild-type and mutant seeds (data not shown).

For the GT7855 insertion line, the first phenotype observed was in root architecture. When plants were grown on MS medium supplemented with 1% sucrose, GT7855 produced slightly more lateral roots compared to wild-type plants (Figure 3.6A). However, when grown in soil, the GT7855 line showed severe repression of early development, while the Salk mutant grew nearly as well as wild-type (Figure 3.6B). Since no phenotype was observed when Gt7855 seedlings were transferred to soil after initial growth on MS medium supplemented with sucrose, we manipulated the MS medium formulation to isolate the components affecting the mutant. It proved to be a lack of sucrose, among the major nutrient and minor metal elements, that caused the severe repression of early root development in GT7855 seedlings. The growth of roots was severely repressed after germination on MS medium without sucrose (Figure 3.7). To test whether sucrose was capable of rescuing the mutant grown on soil, two-week-old mutants were watered with a 1 % sucrose solution, after which

GT7855 seedlings developed normally (Figure 3.8A). Testing

showed that glucose was also capable of rescuing the mutant

phenotype, but fructose was not as effective (Figure 3.8B). The

results indicated that sucrose and/or glucose somehow compensate

for loss of the At2g30210 gene product to bring about normal

root growth during the early stages of *Arabidopsis* development.

GT7855 mutant is hypersensitive to salt stress

Salt sensitivity is most evident at germination and during

the early stages of *Arabidopsis* seedling development (Xiong and

Zhu 2002). At 80 mM NaCl, both mutant and wild-type seedlings

stopped growing on MS medium after initial radical emergence

(data not shown). However, when the salt concentration was

lowered to 40 mM NaCl, wild-type and mutant seedlings grew,

albeit poorly and showing obvious symptoms of salt stress, but

the GT7855 plants eventually died after 4 weeks, while wild-type

and the Salk mutant continued to survive (Figure 3.9).

At2g30210 is expressed in the root endodermis

Previous study showed that the At2g30210 gene is primarily

expressed in root tissues (McCaig et al. 2005). Gene expression

patterns at the cellular level can facilitate linking

physiological function of a gene product to the differentiation

state of certain cells or tissue types. Thus, previous studies

suggested that LMCOs might be involved in lignification of

differentiating xylem tissues in stems. To test whether

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exression of the At2g30210 gene was specific to xylem tissues in *Arabidopsis* roots, transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the At2g30210 promoter were generated. Whole mount histochemical staining for GUS activity showed that promoter activity was detected in whole roots and hypocotyls during early developmental stages, but later only in the elongation region behind primary and lateral root tips (Figure 3.10). Higher magnification appeased to show that the At2g30210 gene was preferentially expressed in the developing endodermis of *Arabidopsis* roots (Figure 3.10E). This result is consistent with our previous RT-PCR data and matches well with measurements made using Affymetrix chips (Birnbaum et al. 2003), which showed that expression of this gene is strongly up-regulated in those root developmental zones where the endodermis is initially formed. GUS activity was up-regulated during development of lateral roots similar to the manner seen in the primary root (Figure 3.11).

Some transgenic seedlings grown in liquid MS medium, but not on solid MS agar plates, showed expression of At2g3210 in the major veins of cotyledons, and very low levels of expression were observed in developing pollen sacs if stainign for GUS activitiy was extended to three days (Figure 3.10). However,

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there was no detectable GUS activity in leaf, inflorescence stem, or silique tissues (Figure 3.10 and data not shown).

At2g30210 protein is localized to the cell wall

Ttwo different algorithms (Predotar V1.03 and TargetP V1.01) predicted the At2g30210 protein to possess a secretory signal peptide that targeted the gene product for secretion via the endomembrane system. Extracellular localization would be consistent with a putative function in cell wall formation as has been proposed for other plant LMCOs. However, other software algorithms predicted subcellular localization to mitochondria or the nucleus (http://www.bioinfo.tsinghua.edu.cn/SubLoc/). To determine the precise subcellular localization site for the At2g30210 gene product, we prepared a chimeric protein in which an enhanced yellow fluorescent protein (EYFP) was linked in-frame to the carboxyl-terminal end of the At2g30210 gene product. The resultant construct, driven with a 35S promoter, was transiently expressed in suspension-cultured tobacco cells (BY2), where the protein appeared to be localized to the cell periphery and nucleus (Figure 3.12A). In contrast, fluorescence corresponding to EYFP protein alone was localized in both cytoplasm and nucleus, consistent with the subcellular localization pattern of the control protein reported previously (David and Perrot-Rechenmann 2001). The same construct was subsequently used to stably transform *Arabidopsis*, and confocal laser microscopy of

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intact roots from the resulting transgenic plants displayed fluorescence that appeared to support apoplastic localization of the chimeric protein. EYFP fluorescence was localized to the cell periphery, most likely extracellularly and possibly to the cell wall (Figure 3.12E). We were not able to detect deformation in the pattern when the tissues were subjected to plasmolytic conditions, suggesting that the protein is likely not localized to the cytoplasm or internal plasmalemma (data not shown). These results are consistent with previous studies in which LMCOs were localized to cell walls, where they may be involved in the lignin biosynthesis.

Discussion

The results presented here suggest involvement of LMCOs in development of plant root endodermis. Strong phenoloxidase activity of the heterologously expressed enzyme (see Chapter 2) suggests involvement in the polymerization of phenolic materials. Promoter activity was strongest in the endodermis, a tissue whose specialized cell walls are characterized by development of Casparian bands that contain suberin and the phenolic polymer, lignin (Brundrett et al. 1988, Ma and Peterson 2003). Casparian bands are initially formed very close to the root tip, usually within 10 mm (Ma and Peterson 2003). GUS reporter activity driven by the At2g30210 promoter was initially

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detected in the same region, and expression extended through more developed endodermis cells, where suberin lamellae are formed. The At2g30210-YFP chimeric protein was targeted to the cell periphery, possibly the cell wall, where lignin and suberin are deposited. Finally, and most importantly, observations that the At2g30210 mutant phenotypes showed a sugar requirement during early development and an increased sensitivity to salt stress were consistent with altered function of endodermal cell walls. Endodermal walls are characterized by the Casparian strip, a specialized type of cell wall structure that functions to seal the perimeter of the endodermal walls and, thereby, create an apoplastic transport barrier to water, ions, and sugar between the root cortex and stele (Sattelmacher 2001). Changes in structure or composition of the Casparian strip are known to impact hydraulic conductivity and ion flow (Schreiber et al. 2005).

It is well known that growth of *A. thaliana* is strongly dependent on a supply of exogenous sugar (Gibson 2000). When exogenous sugar is limited, photosynthetic products generated above ground become the sole source of sugars, especially for early stage cotyledons. For mutants lacking the functional At2g30210 gene product, photosynthetic sugar may not be efficiently transported to sink tissues in the root tips because of diffusive leaks along the malformed stele. As a result, root

development in the mutant is impaired, and plants cannot obtain sufficient nutrients from the surrounding soil to continue growth. A similar phenotype was observed in an *Arabidopsis* mutant in which loss-of-function mutants of enzymes involved in sugar-loading into the phloem impaired transport and allocation of sugars to other parts of the plant (Deeken et al. 2002).

Increased salt sensitivity in the At2g30210 mutants is also consistant with impaired Casparian strip function. *Arabidopsis* seed germination is greatly impaired at salt concentrations above 75mM, but low concentrations of NaCl(< 50mM) may actually have a slight stimulating effect on fresh weight increase in seedlings grown in culture medium (Xiong and Zhu 2002). Our data showed that seedlings grown on medium containing 80mM NaCl only developed a very short radical, with mutant and wild-type plants affected to an equivalent degree. However, in medium containing 40mM NaCl, while both wild-type and mutant seedlings showed repressed growth, the mutant was severely chlorotic and eventually died. The ability to restrict Na^+ transport from the root to aerial tissues is one of several important determinants of salt tolerance in plants (Xiong and Zhu 2002). Amongst several steps that control the long distance transport of Na^+ are the radial transport of Na^+ across the root cortical cells and the loading of Na^+ into the xylem. The Casparian bands are impermeable to small ions because of their lignin and suberin

content. This enables the endodermis to serve as a filtration site retarding passive movement of specific ions and solutes between the cortex and the stele. Thus, it plays a critical role in establishing an apoplastic transport barrier in roots (Ma and Peterson 2003). If the At2g30210 mutant is impaired in depositing suberin and/or lignin in endodermal cell walls, the resulting defective Casparian strips could quite conceivably allow solutes to freely pass through to the stele.

The Salk insertion mutant showed a subtler phenotype compared to wild-type and the GT7855 mutant. There are two possibilities to explain this phenomenon. First, the mutation may be leaky because the intron location allows the insert to be spliced out at some low frequency during post-transcriptional processing. This hypothesis is supported by the presence of low levels of mature mRNA as detected using RT-PCR. The other possibility is that the insert results in an enzyme that retains partial function. Stop codons in the insert would result in loss 44 amino acids (the last exon) from the C-terminus. These amino acids encode part of the type-1 copper-binding domain, but the resulting enzyme could still retain partial oxidase activity. The SKU5 gene in *A. thaliana*, a LMCO homologue lacking the type I copper-binding domain, has been shown to be localized to the plasma membrane and the cell wall, and is involved in directed root tip growth (Sedbrook et al. 2002). Similarly, Chen et al.

(2004) recently showed that a truncated hephaestin (a homologue of ceruloplasmin belonging to the MCO family) expressed in sex-linked anemia *(sla)* mice still has measurable, but decreased, oxidase activity. Our analyses of the Salk insert line do not eliminate either possibility. Thus, it may not be surprising that only a subtle phenotype was detected in this mutant.

Recent work on *Arabidopsis* is beginning to shed light on the genetic and molecular mechanisms of endodermal differentiation. Two genes are known to be required for the proper differentiation of root cortex tissues; they are SCARECROW (SCR) and SHORTROOT (SHR) (Ma and Peterson 2003). In the *shr* mutant, the endodermis is missing (Benfey et al. 1993). In the scr mutant, the mutant cell layer has the conformation of cortical parenchyma, but Casparian bands remian present (Di Laurenzio et al. 1996). Plants that over-express SHR result in a multiple-layered endodermis (Helariutta et al. 2000). It is unknown whether the At2g30210 gene is regulated by SHR, the principal transcription factor controlling differentiation of endodermis.

Although our data showed fluorescent-tagged At2g30210 protein localized to cell periphery in both BY2 tobacco cells and transgenic *Arabidopsis*, we did not verify the actual localization of the endogenous At2g30120 LMCO protein to Casparian bands. Our GUS expression data showed that the promoter activity is expressed predominantly in endodermal

cells, which contain Casparian bands, but expression of the chimeric EYFP fusion protein driven from the endogenous At2g30210 promoter may be required to see specific localization to the Casparian bands. Even then, it may turn out that the EYFP domain will disrupt proper localization of the protein.

Suberin is a plant polymer, containing both aromatic and polyester moieties, which functions as a barrier in below ground tissues, wounded surfaces, and a variety of internal organs. Suberin is also a dominant material in Casparian strips. Work by Kolattukudy (1981) demonstrated that suberin contains a core of polyphenolic material resembling lignin, but the inclusion of atypical phenolic subunits, such as various hydroxycinnamic acids, distinguishes this aromatic domain from "true" lignin (Bernards 2002). Although peroxidases have frequently been implicated in the polymerization events related to suberization (Quiroga et al. 2000, Bernards and Razem 2001), no one has previously associated LMCOs with suberizing cell walls. Our results are the first to suggest a possible association between an *Arabidopsis* LMCO and suberin deposition. Future efforts will examine Casparian strip structure and composition in the At2g30210 mutants to verify a direct cause and effect relationship.

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Table 3.1. Oligonucleotide primers used in this study. The order of sequences follows their appearance in the text.

Primer Name	Primer Sequence
031901RP	5' AACCGCAACTGCTTTCACCAA 3'
031901LP	5' CATCGAAAACAAAGGTTGAACCAC 3'
LBa1	5' TGGTTCACGTAGTGGGCCATCG 3'
Ds3-4	5' CCGTCCCGCAAGTTAAATATG 3'
Ds5-4	5' TACGATAACGGTCGGTACGG 3'
1F	5' ATGGAGTCTTTTCGGCGATT 3'
1016R	5' ATGGAGTCTTTTCGGCGATT 3'
PMT-F	5' TCGACTAGTGTTTATGTAGCAGAGGTGTC 3'
PMT-R	5' TCGCTGCAGGCTCCATTGTTTGCTTCACT 3'
EYFPC-F	5' TCGCCTAGGTCGCCACCATGGAGTCTTTTCGGCGA 3'
EYFPC-R	5' TCGACGCGTGCATCTTGGAAGATCCAA 3'
14F	5' GGCGATTCTCCTTGCTATCC 3'
149R	5' GTGATGCTTTGGTGAGTTCTG 3'
G3PDH-F	5' TTATTGTGCTGAAGAAGGTGTTAG 3'
G3PDH-R	5' AGTGGAATCTTGGAAGTGAGG 3'
1694R	5' TTAGCATCTTGGAAGATCCA 3'
326F	5' GACAAACCTACACTTACAGA 3'
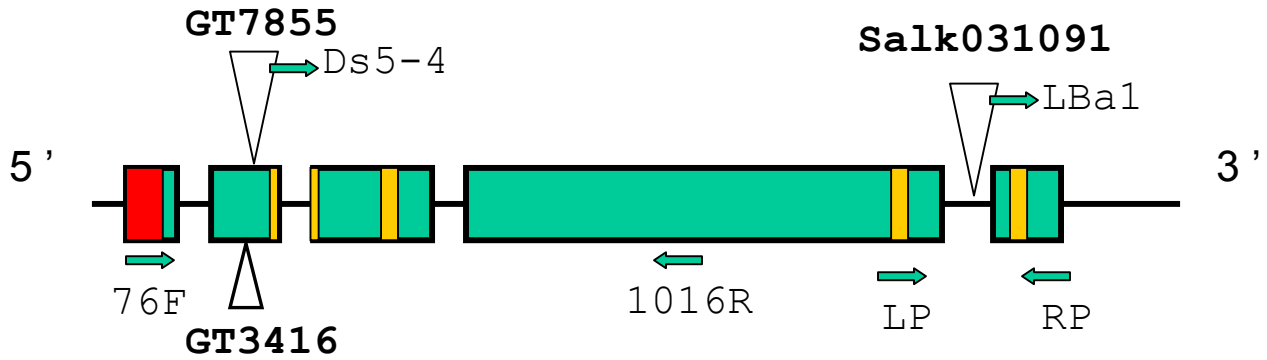
Figure 3.1. Insertion position in the At2g30210 mutants. *A. thaliana* mutants were identified by a search of the insertion line database accessible through TAIR (http:/www.Arabidopsis.org). Arrows show the relative position of primers used to confirm the insertions.

Figure 3.2. Genetic analyses of the At2g30210 insertion mutants. **A.** Three homozygous lines (3,38,41) were isolated from the Salk031901 mutant using gene-specific and T-DNA specific primers. RP and LP: At2g30210 gene-specific primers that spanned the insertion site. LBa1: T-DNA left border-specific primer. **B.** RT-PCR analysis of Salk-031901 mutant with a pair of gene-specific primers (76F and 1694R). **C.** Several homozygous (Ho) lines were isolated from the GT7855 insertion mutant. 76F and 1016R: At2g30210 gene-specific primers that spanned the insertion site. Ds5-4: Ds transposon-specific primer. **D.** RT-PCR analysis of GT7855 mutant with a pair of gene-specific primers (1Fand 1016R). UBQ10 was used as the control to show comparable cDNA loading. Wt: wild type. He: heterozygous. Ho: homozygous.
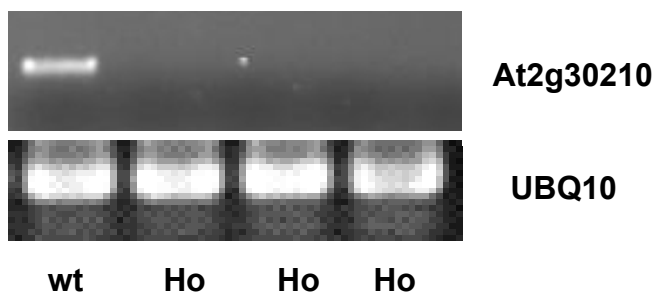
A.

RP/LP   RP/LP/LBa1

1 3 38 41 wt 1 3 38 41 wt

RP/LBa1   LP/LBa1

1 3 38 41 wt   1 3 38 41 wt

B.

At2g30210

UBQ1

3   38   41   wt

C.

76F/1016R

Ds5-4/1016R

He   Ho   wt

D.

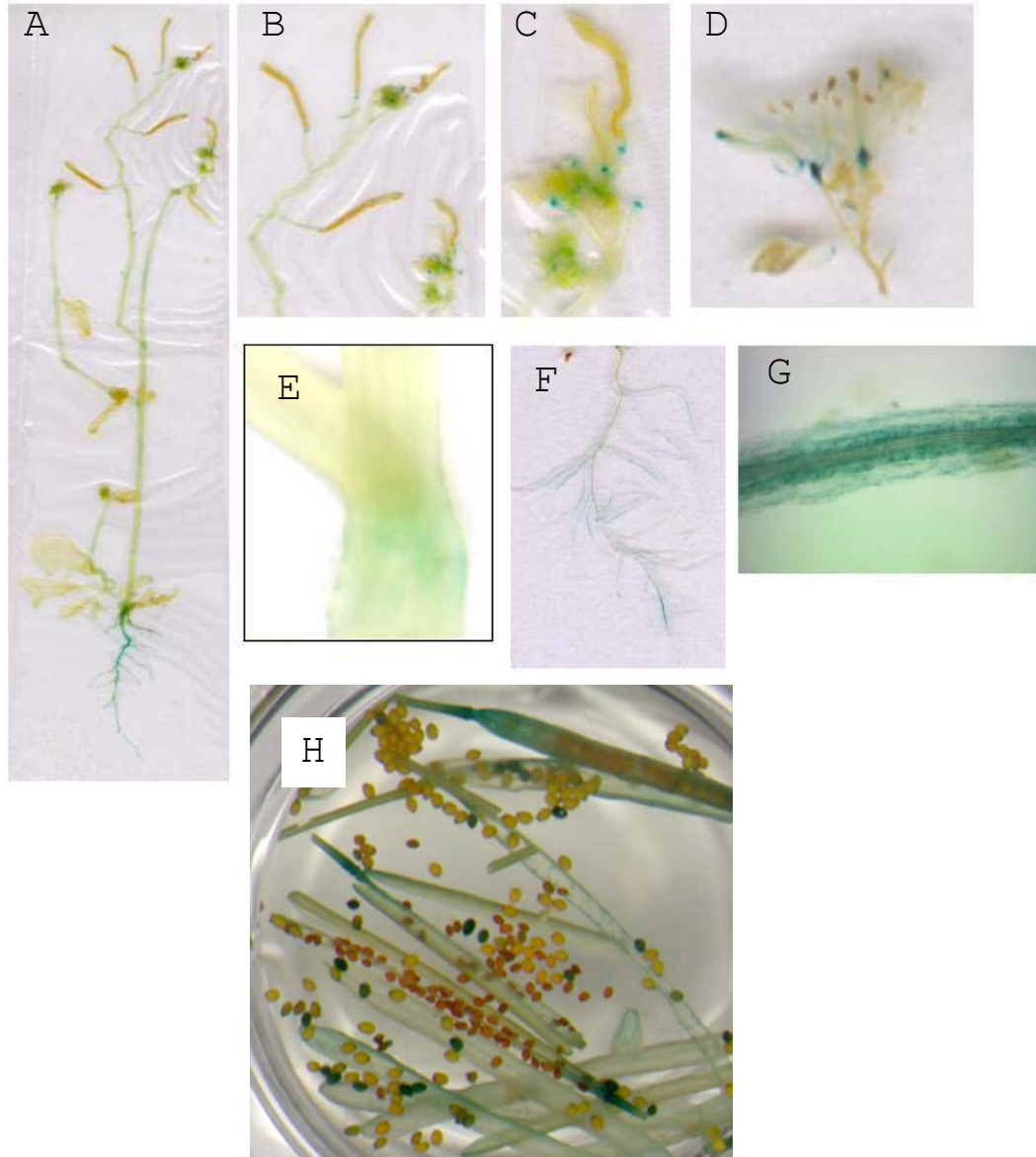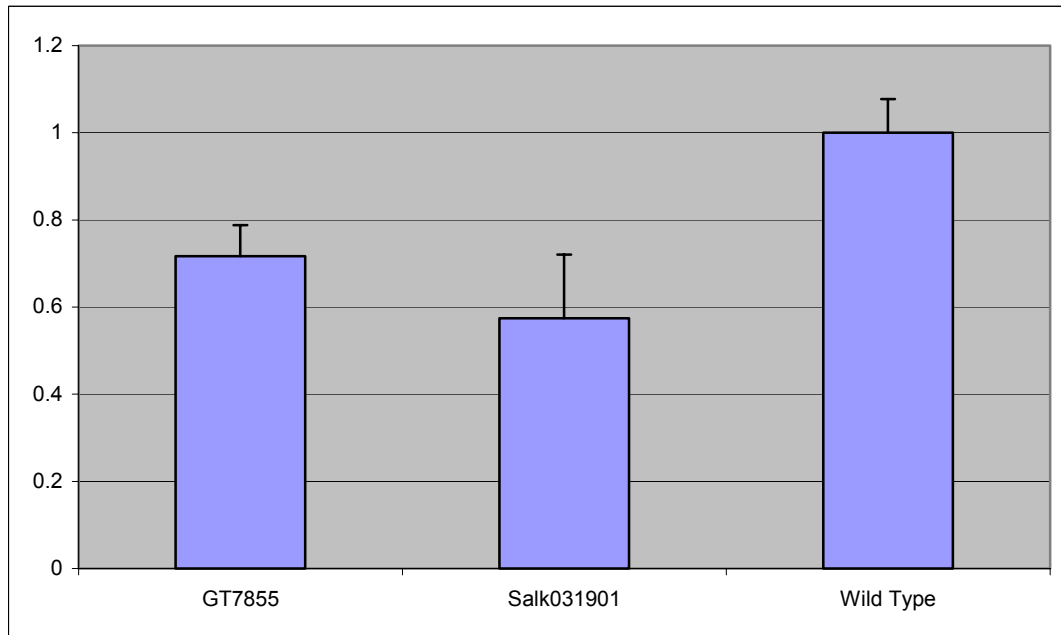At2g30210

UBQ10

wt   Ho   Ho   Ho
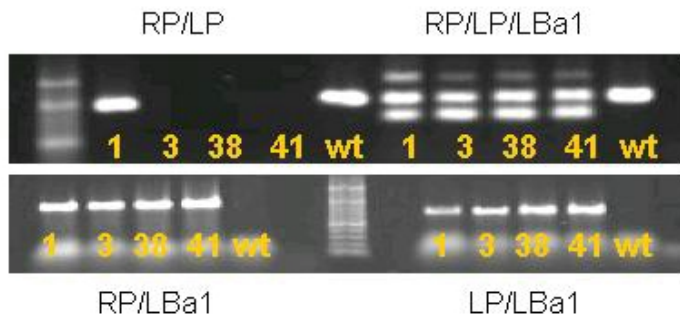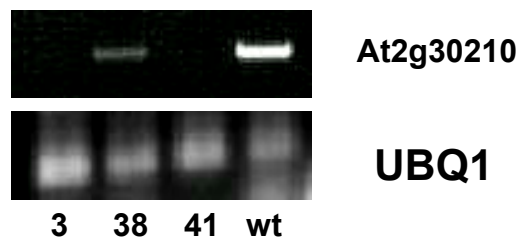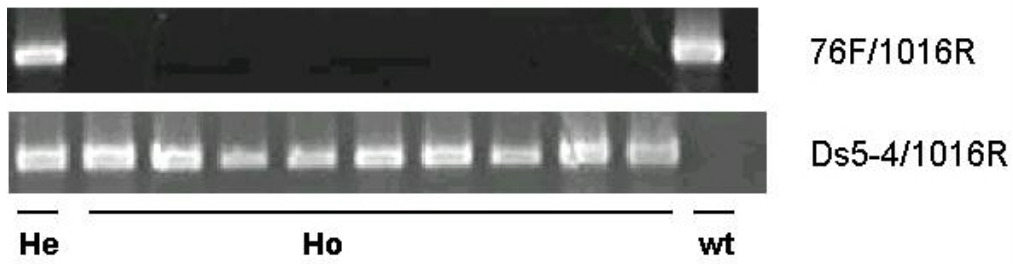
Figure 3.3. GUS expression in tissues from the GT3416 insertion line. Whole mount GUS staining showed that the highest activity was observed in roots (A, F, and G), flowers (c), the abscission zones of siliques (c and D), and developing seed (H), while activity was weak in the node (E).
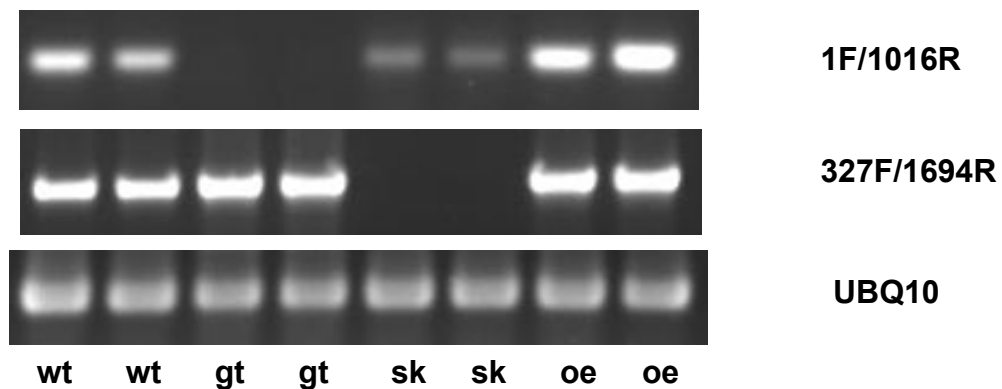
A.

B.

Figure 3.4. Transcription of At2g30210 in wild-type and insertion mutants of *Arabidopsis*. **A.** qPCR analysis showed slightly reduced transcription in both the Salk-031901 and GT7855 lines. Y-axis displays the fold change compared to wild-type plants and error bars represent one standard deviation (S.D.). **B.** RT-PCR analyses using primers spanning the insertion sites showed that the insertions disrupted levels of normal At2g30210 transcriptss. Wt: wild type, gt: GT7855 line, sk: Salk-031901 line, oe: At2g3021-EYFP transgenic plant.
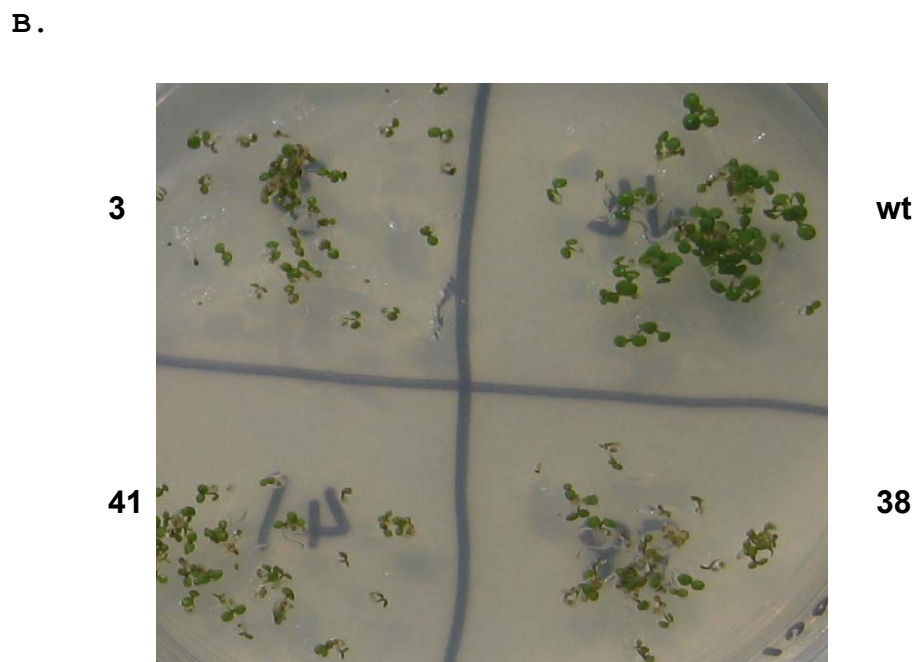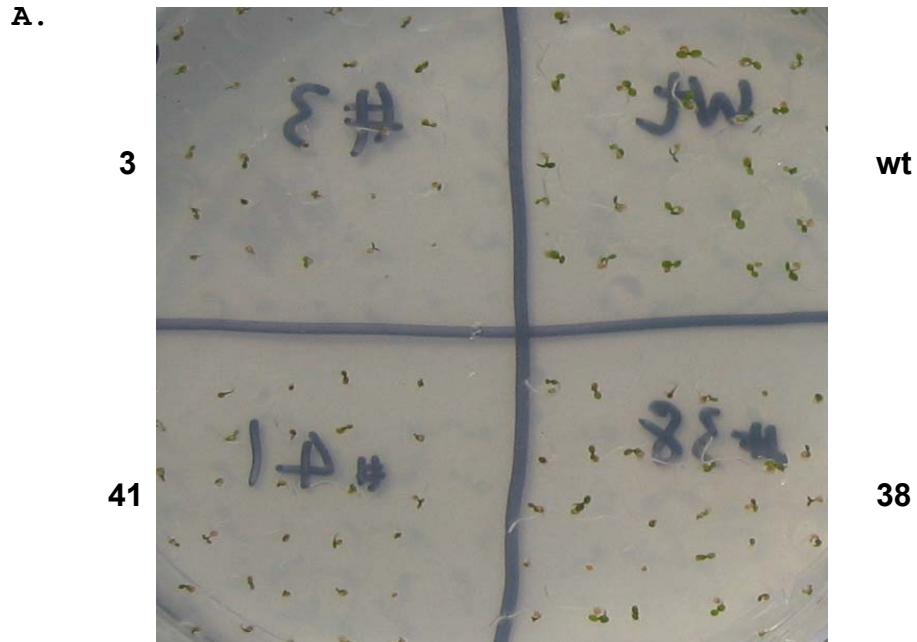
A.

3   **wt**

41   **38**

B.

3   **wt**

41   **38**

Figure 3.5. T-DNA insertion mutants showing reduced growth in the absence of sugar. Three homozygous Salk insertion lines (3,38, and 41) and wild-type (wt) seedlings were grown on water agar (panel A) or MS medium containing 1% sucrose (panel B) for a week. Note that several seedlings of line 38 grown on the water agar plate (panel A) developed extended cotyledons, which were also seen in wild-type seedlings. Similar sporadic growth was not seen amongst the line 3 or 41 plants.

A.
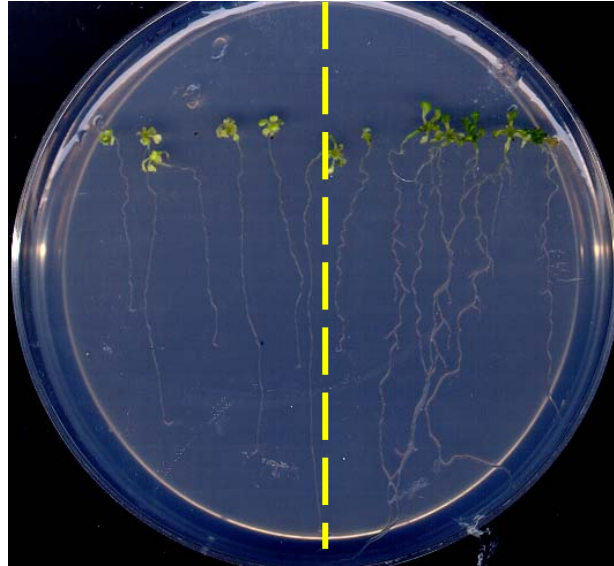
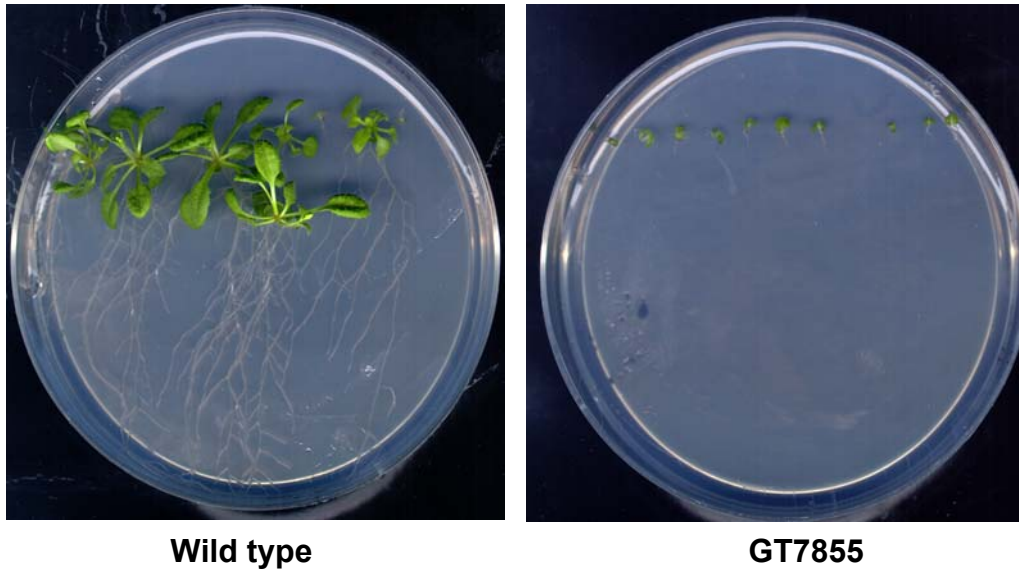
Wt   GT7855

B.

Wt   GT   Salk

Salk GT Wt

Figure 3.6. Growth alterations in the GT7855 mutant. The mutant showed altered root morphology and severe repression in soil, but not on MS medium containing sucrose. **A.** 14-day-old wild-type (Wt) and GT7855 mutant (GT7855) seedlings were vertically grown on MS media supplemented with 1% sucrose. **B.** 14-day-old wild-type (Wt), GT7855 mutant (GT), and Salk-031910 mutant (Salk) seedlings were grown on MS medium supplemented with 1% sucrose (left panel) or in soil (right panel).

A.

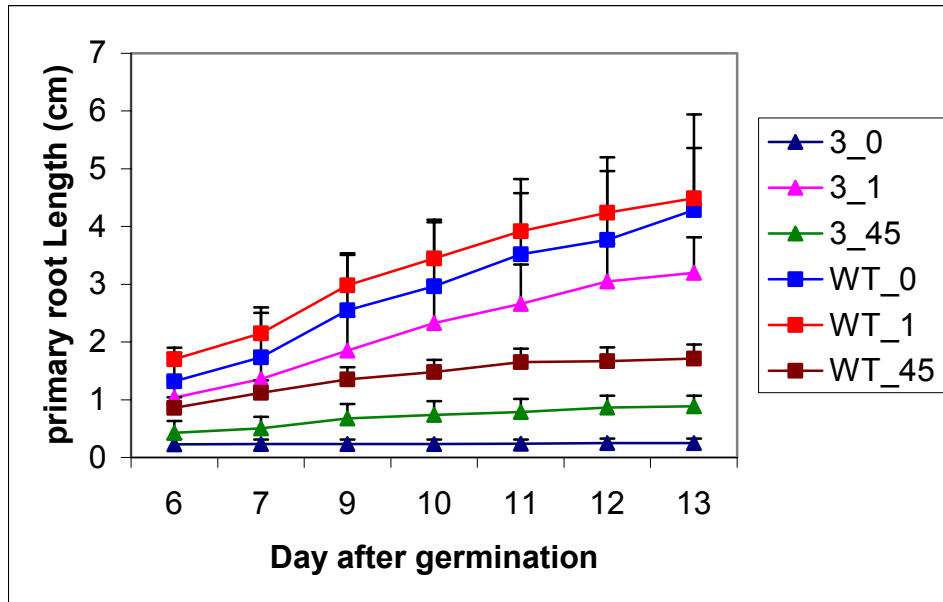**Wild type**   **GT7855**

B.

Figure 3.7. GT7855 insertion line grown on MS medium without sucrose. **A.** 14-day-old wild-type (left) and GT7855 mutant (right) seedlings grown on MS medium without sucrose. **B.** Root growth during the first two weeks on different concentrations of sucrose after germination. GT7855 mutant line 3 was used to compare with wild type. At least 8 seedlings were measured in each treatment. Individual replicates were averaged and bars show one standard deviation. 0: without sucrose; 1: 1% sucrose; 45: 4.5% sucrose.

A.

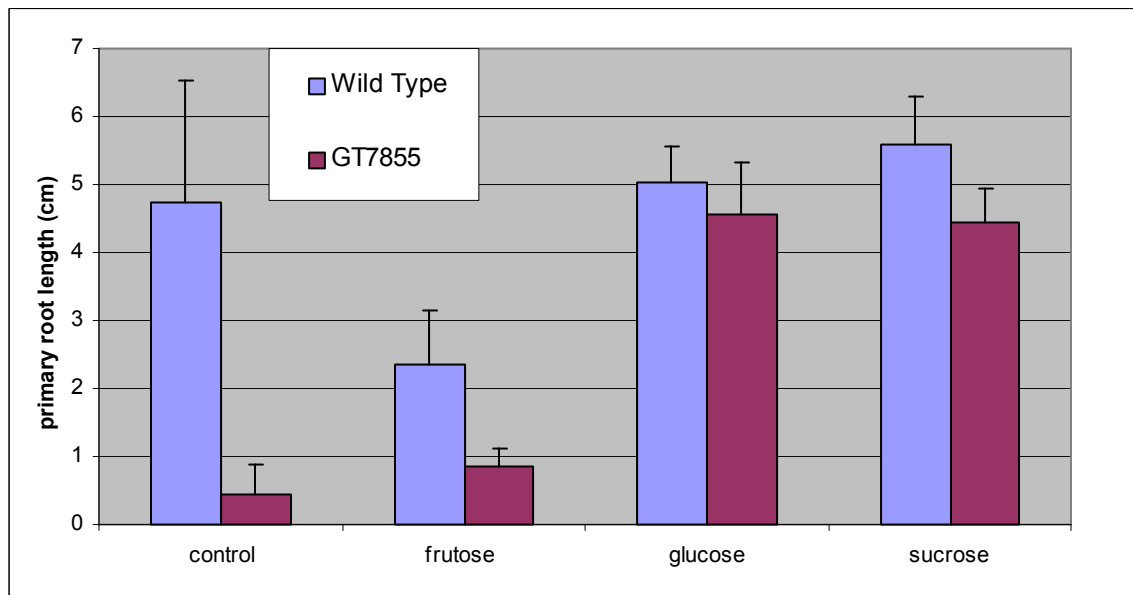| WT | GT7855 | GT7855
+ Sucrose |

B.

Figure 3.8. Exogenous sugar enables growth of the GT7855 insertion mutant in soil. **A.** 21-day-old wild-type (WT) and GT7855 mutant (GT7855) seedlings grown in soil. Some GT7855 mutant seedlings (shown as GT7855+sucrose) were irrigated with sucrose solution (1%) on day 14 and day 17 after germination. Picture was taken at day 22 after germination. GT7855 mutants, irrigated with water as a control, remained undeveloped. **B.** Seedlings vertically grown on MS medium supplemented with different sugars (1%) as indicated and MS medium only as control. At least 8 seedlings were measured in each treatment and error bars represent S.D.

Figure 3.9. Salt sensitivity in the gene-trap insertional mutant. The GT7855 mutant, but not Salk mutant, showed altered sensitivity to salt levels. Seedlings were grown on MS medium (Left) and MS containing 40mM sodium chloride (right). WT: wild-type seedlings.
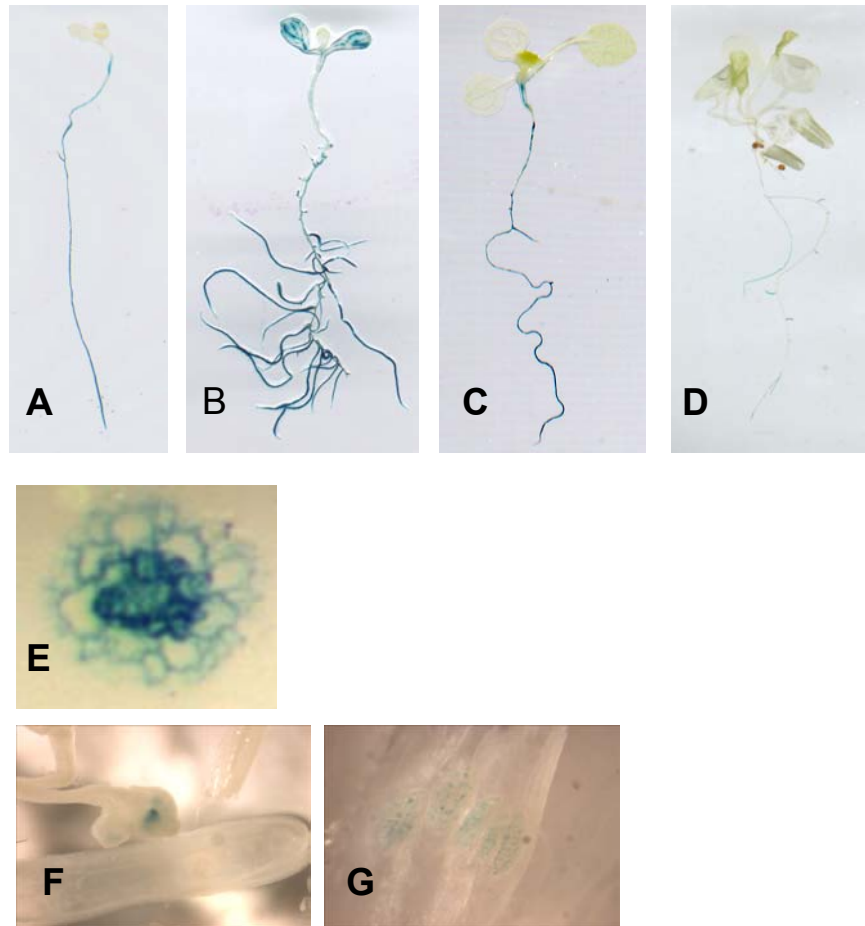
Figure 3.10. At2g30210 promoter expression patterns. GUS activity was detected in tissues of different aged plants. **A.** 5-day-old seedling on MS plate. **B.** 2-week-old in MS liquid culture. **C.** 2-week-old on MS plate. **D.** 5-week-old seedling on plates. **E.** Cross section taken from 1 cm behind the root tip of 5-week old seedling. **F.** Undeveloped flower stained for 3 days. **G.** A high magnification of dissected anthers.
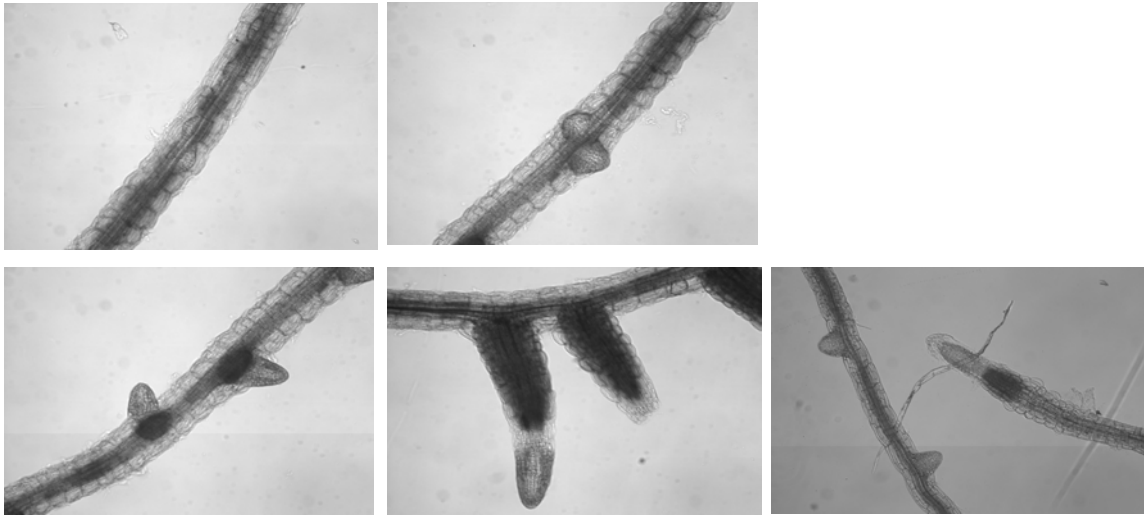
Figure 3.11. Developmental regulation of the At2g30210 promoter. Promoter activity of At2g30210 was detected in a series of lateral roots in a single seedling. GUS activity is shown as the darkened zones.
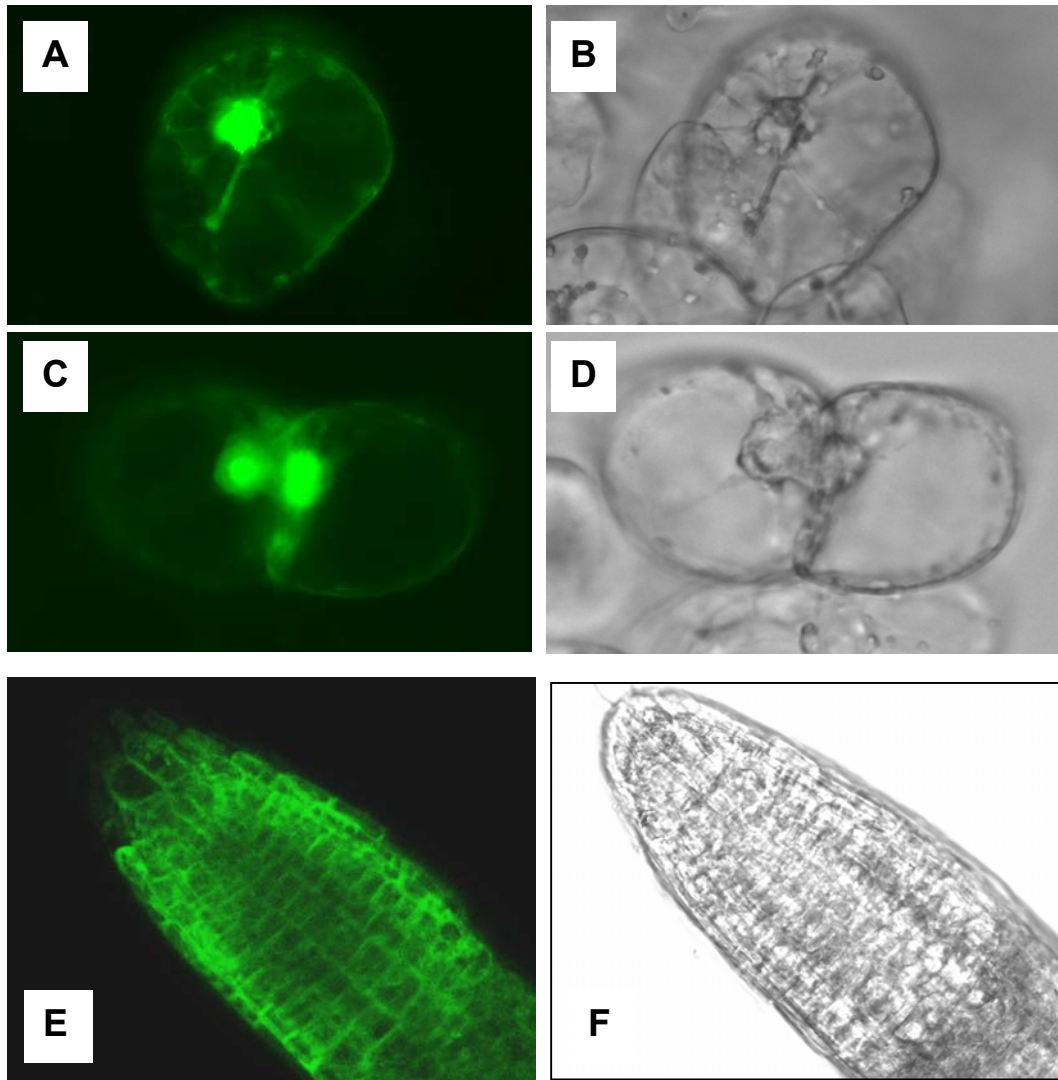
Figure 3.12. Subcellular localization of the At2g30210-EYFP chimeric protein in BY2 tobacco cells and transgenic *Arabidopsis*. BY2 cells were observed using a light microscope with UV illumination. Root tips from 5-day old seedlings were observed using confocal microscopy. A and B: At2g30210-EYFP signal (A) and bright field view (B) of BY2 cells transiently expressing At2g30210-EYFP. C and D: EYFP signal (C) and bright field view (D) of BY2 cells transiently expressing EYFP alone as a control. E and F: At2g30210-EYFP signals (E) and differential interference contrast image (F) of root tip of 5-day-old *Arabidopsis* seedling expressing At2g30210-EYFP. Note that EYFP fluorescence, shown in green, was localized to the cell periphery.

CHAPTER 4

ANALYSIS OF A LACCASE-LIKE MULTICOPPER OXIDASE (LMCO) IN

RESPONSE TO ABIOTIC CONDITIONS[1]

Abstract

The impact of abiotic stress on expression of three *Arabidopsis* LMCO genes was followed using quantitative PCR. The results showed that expression of all three LMCO genes was slightly up-regulated by excess copper. At2g30210 LMCO gene expression was not affected by changes in iron levels. However, the gene was up-regulated in seedlings grown on MS medium lacking sucrose, a response that was consistent with the phenotype previously observed for mutants of this gene. Although mutations in this gene increased the sensitivity of plants to salt stress, transcription of At2g30210 did not respond to salt concentration. These results are consistent with a possible function for the At2g30210 gene product in the endodermis.

Introduction

Laccase or laccase-like multicopper oxidase (LMCOs) were first discovered in plants over 100 years ago, but relatively little is known about the physiological function(s) of these enzymes *in vivo.* Wound healing through oxidative polymerization of alkylcatechols in the sap of lacquer trees is the only strongly supported physiological function advanced for plant LMCOs to date (Mayer and Staples 2002, McCaig et al. 2005). Although the Enzyme Commission definition for LMCOs (*p*-diphenol:O_2 oxidoreductase) is based on their ability to oxidize para-substituted diphenols, one of the unusual characteristics of LMCOs is their enzymatic activity against a broad range of organic and inorganic substrates (Sterjiades et al.1993, Dean and Eriksson 1994, Hoopes and Dean 2001). This unusual characteristic of LMCOs makes it difficult to identify their precise physiological function(s) *in vivo*. Earlier studies have focused on their ability to oxidize phenolic compounds, especially lignin precursors, from which they have been proposed to be involved in lignin biosynthesis (Dean and Eriksson 1994, Mayer and Staples 2002). However, recent demonstration of ferroxidase activity in a LMCO from yellow-poplar (Hoopes and Dean 2004) was taken to suggest that some plant LMCOs might take part in iron metabolism in a fashion similar to the FET3p component of the high-affinity iron uptake system in yeast.

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Indeed, some LMCOs isolated from a variety of different organism have the ability to oxidize various metal ions, including Cu^{1+}, Mn^{2+}, and Fe^{2+}, implying the possible function of these enzymes in metal homeostasis (Brouwers et al. 1999, Roberts et al. 2002, Herbik et al. 2002, Stoj and Kosman 2003, Shi et al. 2003, Hoopes and Dean 2004, Harris et al. 2004). Furthermore, those LMCOs associated with metal oxidation have been shown to be regulated by their corresponding metals at either transcriptional and/or post-transcriptional level.

To investigate the possible involvement of plant LMCOs in iron metabolism, we monitored the expression of three plant LMCO genes in *Arabidopsis* seedlings treated with various metal ions. The *Arabidopsis* At2g30210 knockout mutant (GT7855), which requires exogenous sugar for proper development and has altered sensitivity to salt, was also analyzed in this study using quantitative PCR.

Materials and Methods

All samples were harvested from two-week-old seedlings of *Arabidopsis* (ecotype Columbia) grown vertically on semi-solid medium in Petri plates. Light was provided on a 16-h/8-h light/dark cycle. Standard medium was 1X Murashige and Skoog salts (Invitrogen, Carlsbad, CA), ph 5.7, containing 1% (w/v) sucrose. For metal treatments, media containing 100μM cupric

sulfate, 400µM ferrous sulfate, or no iron were prepared to compare with the 10µM Cu^{2+} and 100µM Fe^{2+} in standard MS medium. Seedlings were also germinated and grown on media containing three different concentrations of sucrose (0%, 1%, and 4.5%, w/v) or sodium chloride (0, 20mM, and 40mM), respectively. Each treatment and RNA extraction was replicated in two independent experiments. Total RNA was extracted from whole seedlings using Trizol reagent (Invitrogen) as described previously. RNA samples from each replicate were pooled to obtain a single RNA sample for cDNA preparation. Protocols for cDNA synthesis and qPCR were as described previously (Chapter 3). For qPCR measurements, in addition to the previously described qPCR primers for the At2g30210 gene, primer sets specific for two additional LMCO genes, At5g58910 (5'- AGAAACACCGTTGGAGTTCC-3' and 5'-GACGAATGCCATCTTTAATCC-3'), and At5g07130 (5'-GAGTTCTTGGTTCAAAGCAGAG-3' and 5'-ACATCTCAGACGCCTTTACC-3'), as well as the iron transporter gene, IRT1 (5'GTCTTGGCGGTTGTATCCTC-3' and 5'-GTAAACAGTTGATAGAGCGATCC-3'), were used to analyze gene expression in the different experiments.

Microarray data mentioned in this study were retrieved from the GENEVESTIGATOR server at https://www.genevestigator.ethz.ch (Zimmermann et al. 2004). Massively parallel signature sequencing (MPSS) data were retrieved from the Arabidopsis MPSS website (http://mpss.udel.edu/at/) (Meyers et al. 2004).

Results and Discussion

Effect of metal treatment on LMCOs

To examine the impact of metal stress on LMCO expression, two-week-old *Arabidopsis* seedlings grown on excess copper or iron, or in the absence of iron, were compared to seedlings grown on standard medium. An At2g30210 LMCO knockout mutant (GT7855) was also grown under the same conditions to compare with wild-type plants. No significant phenotypes were noted under any of these conditions, except more lateral roots in the mutant, as reported in our earlier study (Chapter 3).

In the presence of excess copper, all three LMCOs showed slight increases in transcription, although the At5g07130 response was not significantly different from controls (Figure 4.1). Copper is an essential micronutrient, serving as a cofactor for numerous enzymes, including LMCOs. However, being a redox-active transition metal, it is also cytotoxic through its propensity to generate reactive oxygen species (ROS) in aerobic cells (Singh et al. 2004). Elevated LMCO transcription seems to be a nearly universal response to increases in copper concentration as the same phenomenon was reported for LMCOs produced by a variety of other organisms (Kim et al. 2001, Herbik et al. 2002, Shi et al. 2003, Singh et al 2004). Shi et al. (2003) suggested that bacterial LMCOs provide a defense against copper toxicity through cuprous oxidase activity acting

in the extracellular space (Shi et al. 2003, Stoj and Kosman 2003). Biochemical studies have shown that some LMCOs catalyze the oxidation of Cu^{1+} to Cu^{2+}, thereby controlling the concentration of copper and maintaining the redox balance in plasma (Brouwers et al. 2000, Shi et al. 2003). It is unclear whether *Arabidopsis* LMCOs also possess the cuprous oxidase activity found in LMCOs from yeast and bacteria. However, it is plausible that increases in LMCO levels could play a role in accumulating and sequestering excess copper due to the unique structure of these enzymes that enables them to bind copper efficiently.

At2g30210 and its closest homologue, At5g07130, showed no changes in expression in response to either iron-excess or iron-deficient conditions. However, At5g58910 gene transcription was slightly elevated in response to iron deficiency. The expression of At5g58910 is, however, relatively low compared to the other two LMCO genes studied, an observation confirmed by available microarray and MPSS data. Thus, the observed change could not be classified as statistically significant. In contrast, the strong inductive response of the IRT gene to iron deficiency confirmed that the treatments were appropriate to test specific gene responses to iron levels.

In *Arabidopsis*, At2g30210 is the LMCO gene most strongly expressed in root tissues, according to microarray and MPSS

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data. We previously demonstrated that no ferroxidase activity is associated with the At2g30210 gene product (Chapter 2). The high-affinity iron uptake system in yeast is induced by iron deficiency, and its expression is relatively low under normal growth conditions (Askwith et al. 1996). Thus, these results together strongly suggest that the At2g30210 gene product is not involved in iron metabolism. That said, there are another 12 LMCO genes, not included in this study, that also express in root tissues with various intensities revealed by available microarray data (see Figure 1.5, Chapter 1). Further study to screen the entire LMCO family in *Arabidopsis* will be necessary to determine whether any of the other genes are regulated by iron. Those members occurring in the same phylogenetic group as the *Liriodendron* LMCO that was shown capable of oxidizing ferrous iron *in vitro* will be of particular interest (Hoopes et al. 2004, McCaig et al. 2005).

Effect of sugar treatment on At2g30210 gene expression

Because of the previously noted growth defect related to sugar availability in the At2g30210 mutant, GT7855 (see Chapter 3), we hypothesized that expression of this gene would respond to sugar levels. Without exogenous sucrose, At2g30210 gene expression in wild-type plants was significantly up-regulated compared to plants grown on medium containing 1% sucrose (Figure 4.2). The result was consistent with the phenotypic response

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observed in the At2g30210 gene knockout mutant (GT7855), which indicated that the gene might play a critical role in early root development. A similar response was not observed for the At5g58910 LMCO gene.

 Recently, Rogers et al. (2005) demonstrated a role for sugars in the regulation of lignin biosynthesis. Their study indicated that sugars are not merely a source of carbon skeletons for lignin precursors, but also function as a signal for increased lignin synthesis. Many of the lignin biosynthetic genes were up-regulated when seedlings were grown in the dark on medium supplemented with sucrose. However, LMCO genes were not mentioned in the report (Rogers et al. 2005). After retrieving Affymetrix data from a public database (Zimmermann et al. 2004), we noted that the At2g30210 gene was up-regulated 1.7- fold on the 8k chips, but only 1.12-fold (not significant) on the 22k chips, respectively, in the study by Rogers et al. (2005). Those results conflict with our data, which showed that the At2g30210 gene was up-regulated in the absence of sucrose. The different observations between experiments may be due in part to the different growth conditions and seedling ages. In the Rogers study, seeds were germinated and grown in the dark for 6 days, which stimulated elongation of hypocotyls, a tissue where the At2g3210 gene is expressed strongly. In contrast, seedlings in our study were grown for two weeks on a long-day cycle (a 16-

h/8-h light/dark cycle). Seedlings in our study produced less hypocotyl tissue, so the portion of tissues expressing the gene was likely less. These results suggest that in addition to sugar, At2g30210 gene expression may also be regulated by a circadian clock, as was shown for some lignin biosynthetic genes (Rogers et al. 2005).

It remains unclear exactly how sugar signaling links with lignification. Based on the proposed model (Rogers et al. 2005), starch turnover would create a pool of soluble sugar that is made available to sink tissues, like xylem or roots, for synthesis of carbon-rich compounds, such as lignin. Starch turnover would thereby create a pool of sugar that could signal for lignin biosynthesis to begin. It was also suggested that sucrose must be hydrolyzed to its component hexose monomers, particularly glucose, and that transport alone is not sufficient to stimulate lignin synthesis.

Interestingly, in addition to conserved copper-binding domains, the At2g30210 protein sequence was predicted to contain a glycosyl hydrolase family 1 domain near the C-terminus. It may be of interest to speculate on whether this LMCO could cleave sugar residues from monolignol glucosides, the transport form of lignin precursors. Release of these sugars could stimulate LMCO transcription at a time when potential phenoloxidase substrates (monolignols) are abundant.

Effect of salt treatment on At2g30210 gene expression

At2g30210 knockout mutants showed increased sensitivity to salt compared to wild-type plants. To evaluate the impact of salt on At2g3021 gene expression, two-week-old *Arabidopsis* seedlings grown on MS medium containing NaCl were compared to plants grown on standard medium. QPCR results showed that the At2g30210 gene was slightly, but not significantly, repressed by salt (Figure 4.3), which fits with available microarray data. A contrasting observation was reported for tomato, where a LMCO cDNA that was not detectable under control conditions, was strongly up-regulated in salt-treated or salt/ABA-treated seedlings (Wei et al. 2000). A full-length cDNA is not available for the gene. The partial sequence, when aligned with other plant LMCOs, showed strongest similarity to the *Acer* LMCO, which does not occur in the same phylogenetic group as the At2g30210 LMCO (McCaig et al. 2005).

These results together support the idea that plant LMCOs play multiple functions in plants. It is not surprising to see functions other than lignin biosynthesis associated with plant LMCOs based on their broad substrate ranges, numerous family members, and varied expression profiles in a single species.

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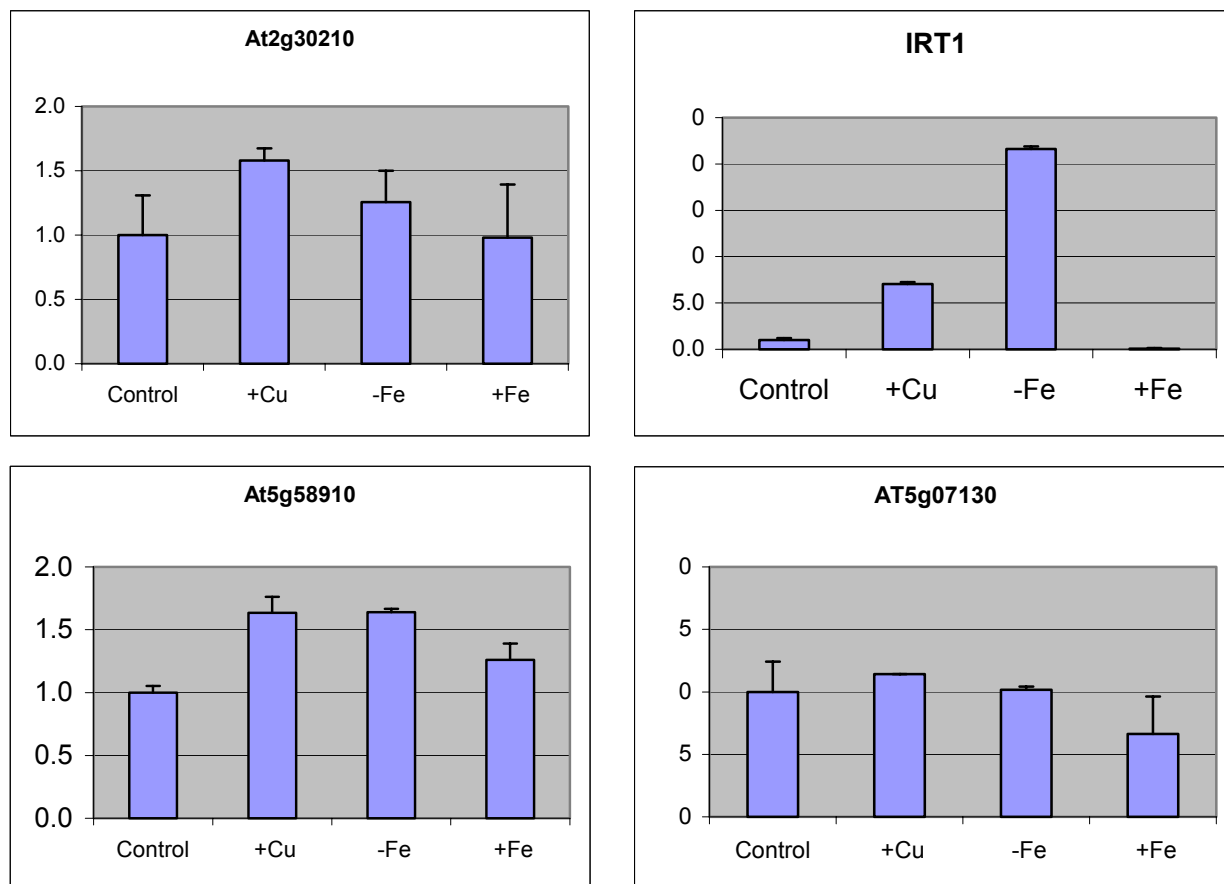
Figure 4.1. Responses of Arabidopsis LMCOs to metal treatment. Transcript levels of three LMCOs gene were analyzed in samples from plants grown in the presence of excess copper (+Cu), iron deficiency (-Fe), and excess iron (+Fe). IRT, an iron transporter gene, was used as a positive control for the iron response (Vert et al. 2002). Y-axis indicates the fold change in transcription compared to control. Error bars represent standard deviation (S.D.).
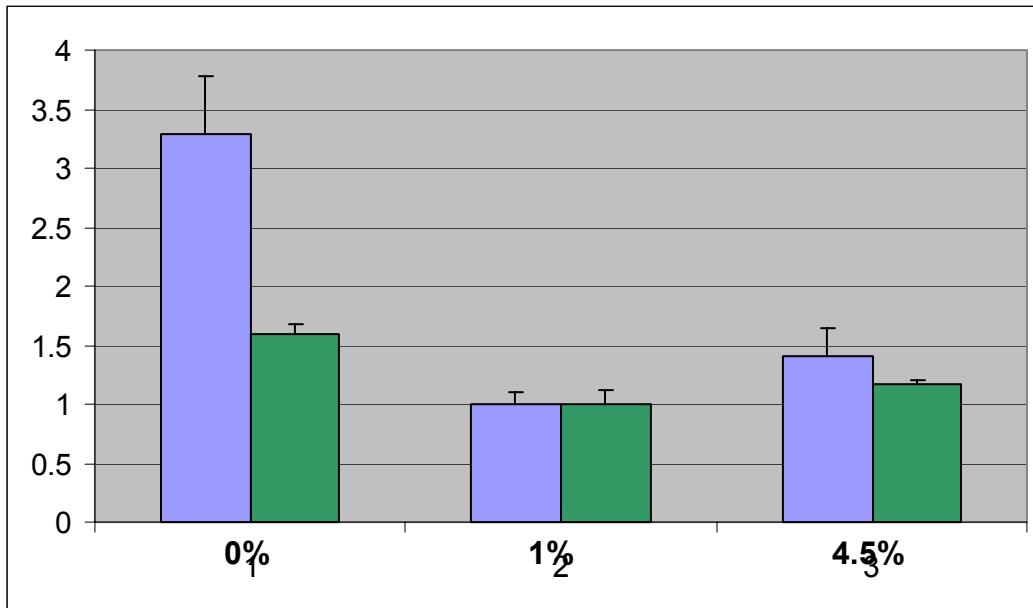
Figure 4.2. Responses of Arabidopsis LMCOs to sucrose. At2g30210 (blue) and At2g58910 (green) gene expression levels were analyzed in samples from plants grown in the presence of different sucrose concentrations in MS medium. Y-axis indicates the fold change of transcription compared to control (1% sucrose). Error bars represent S.D.
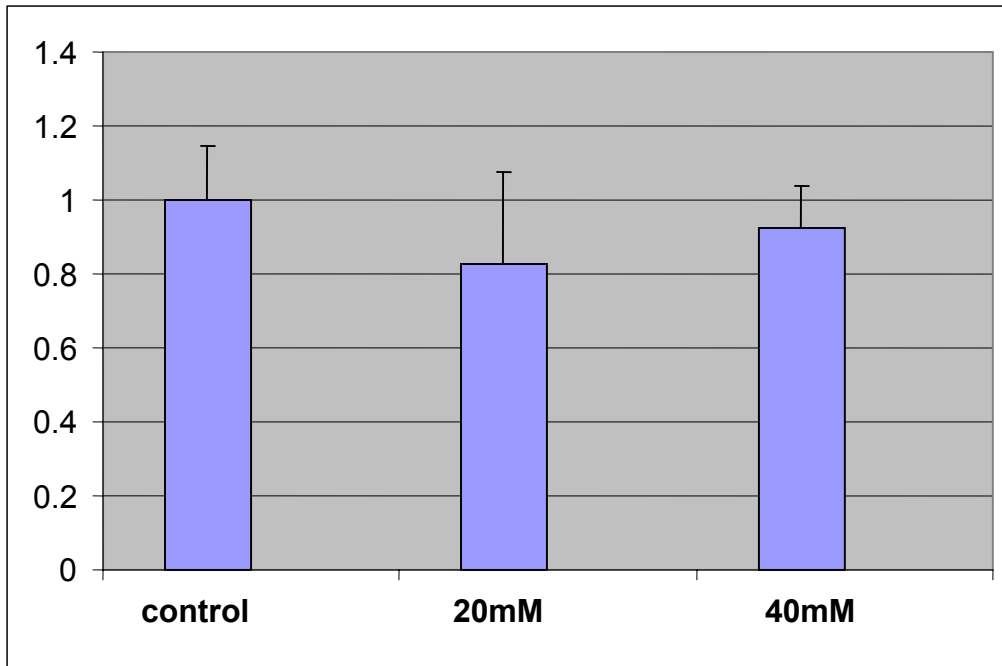
Figure 4.3. Responses of At2g30210 gene expression to salt. At2g30210 was analyzed from plants grown on different sodium chloride concentrations in MS medium. Y-axis indicates the fold change in transcription compared to control. Error bars represent S.D.

CHAPTER 5

Summary and Conclusion

Laccase or laccase-like Multicopper oxidases (LMCOs) are blue copper proteins that catalyze oxidation reactions coupled to the four-electron reduction of molecular oxygen to water. Because of their wide range of potential substrates and difficulty of purification, the physiological functions of the plant LMCOs, in particular, have been difficult to determine. Early studies of plant LMCOs were focused primarily on woody plants due to the abundance of the enzyme in certain species and industrial interests. However, well-founded physiological roles for these LMCOs were difficult to identify because of lack of basic genetic information and tools to manipulate genes in these species. Because of its completed genome sequence and simple techniques for manipulating genes, *Arabidopsis* provides a good system in which to study the physiological function of LMCOs. The work described in this dissertation was directed toward understanding of the physiological function of one particular *Arabidopsis* LMCO gene, At2g30210, by measuring its enzyme activities, patterns of tissue-specific expression, subcellular localization, effects of loss-of-function mutations, and regulation by external factors.

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The At2g30210 gene cDNA was cloned and used to produce the
encoded LMCO in a heterologous expression system. The predicted
amino acid sequence revealed that the At2g30210 protein
possesses the four conserved copper-binding domains that
characterize the multicopper oxidase, as well as a glycosyl
hydrolase family 1 domain near the C-terminus. Amino acid
sequence analyses showed that the At2g30210 protein is closest
to another *Arabidopsis* LMCO, At5g07130, with 73% identity.
Although we were unable to produce active At2g30210 enzyme using
E. coli, transgenic tobacco cells allowed us to recover
sufficient enzyme for biochemical analyses. Heterologously
expressed At2g30210 LMCO showed phenoloxidase activity, but no
ferroxidase activity. These results argue against our initial
hypothesis, that the At2g30210 gene product might be involved in
iron metabolism, which was based on its expression pattern,
primarily in roots, and the fact that other LMCOs having
ferroxidase activity function in iron homeostasis in a variety
of eukaryotes. Further analyses showed that transcript levels
from the At2g30210 gene were not regulated by external iron
availability, and that mutants exposed to different iron
conditions did not show significant phenotypic changes in gene
expression. Altogether the results strongly suggest that the
At2g30210 gene product is not involved in iron metabolism.

To determine the spatial expression pattern of At2g30210 transcripts, a promoter-reporter gene fusion was introduced into wild-type *Arabidopsis*. Histochemical staining for GUS activity revealed that At2g30210 transcripts were primarily expressed in developing endodermis of the root elongation zone and newly matured roots. The result suggested a new function for *Arabidopsis* LMCOs in modification of the endodermal walls. Indeed, endodermal walls of roots are characterized by the Casparian strip, a specialized cell wall structure containing suberin and lignin that functions to seal the perimeter of endodermal cells and, thereby, create an apoplastic transport barrier to water and solutes between the root cortex and stele (Sattlemacher 2001). Changes in structure or composition of the Casparian strip are known to impact hydraulic conductivity and ion flow (Schreiber et al. 2005).

The endodermis is one of the most important adaptations of terrestrial plants. In the absence or malfunction of an endodermis plants would not be able to regulate water uptake by the roots or maintain the water balance of the plant. Soil water enters the root through root hairs and then travels via the symplast (cell-to-cell) and/or apoplast (cell wall and inter-cellular space). However, the endodermis is impervious to water because of a suberized cell wall matrix called the Casparian strip. Therefore, to enter the stele, apoplastic water must

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enter the symplasm of the endodermal cells, from where it can

pass by plasmodesmata into the cells of the stele (Kramer and

Boyer 1995). Although the water movement is thought to be driven

by transpiration, root pressure also plays a role in the

transport of water in the xylem in some plants under certain

conditions. Root pressure is created by the osmotic potential of

xylem sap, which is, in turn, kept high by dissolved minerals

and sugars that have been actively transported into the apoplast

of the stele (Patrick 1997, Ma and Peterson 2001). One familiar

example of the importance of root pressure is seen in the sugar

maple where, in early spring, it hydrolyzes starches stored in

the roots to produce simple sugars. The increased levels of

sugar in these root tissues draws water through the endodermis

and into the xylem ducts where the continuous inflow forces sap

to rise up the ducts into the above ground portions of the tree

where it can be tapped (harvested) (Kimball 2004).

Plant growth and development requires transport of

photoassimilates (e.g. sucrose) from source tissues into

developmental sinks. The transport of sugars through the

differentiated phloem is explained well by Münch's pressure flow

hypothesis (Ross and Tyree 1980, Bret-Harte and Silk 1994). In

the growing root, the phloem transport system ends in roots at

the point where differentiation of protophloem sieve tubes is

initiated. This is the same region where the most rapid root

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growth occurs, proximal to the root cap (Esau, 1965). The
pathway for solute transport from the phloem is thought to be
apoplastic in mature stem (Hayes et al. 1985) and mature roots
(Wyse 1979). Thus, the endodermal cell walls with Casparian
strip as apoplastic barriers become critical to insure the
delivery of the photoassimilates to root tips for growth. A
loss-of-function At2g30210 mutant displayed phenotypic responses
to sugar availability and salt levels consistent with functional
alteration of the Casparian strip such that sugars destined for
the growing root tip diffuse from the stele before reaching the
zone of rapid growth at the root tip.

Suberin is a plant polymer containing a core of polyphenolic
material resembling lignin. Work done by Kolattukudy (1981)
showed that the inclusion of atypical phenolic subunits, such as
various hydroxycinnamic acids, distinguishes the "lignin" core
of suberin from true lignin (Bernards 2002). Although
peroxidases have been implicated most frequently in the
polymerization events related to suberization (Quiroga et al.
2000, Bernards and Razem 2001), no one has previously associated
LMCOs with suberizing cell walls. Our results are the first to
suggest a possible association between an *Arabidopsis* LMCO and
deposition of the lignin-like core associated with suberin.

Combining the results of biochemical analyses showing
phenoloxidase activity, cell-specific expression pattern in

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endodermis, subcellular localization to cell peripheries, and mutant analyses, we conclude that the At2g30210 gene is unlikely to be involved with iron-uptake, but is very possibly involved with early lignification of the root endodermis in the process giving rise to the Casparian strip, a structure of some significance to root function.

Further study will focus on uncovering addition details of At2g30210 function. Recognition of the glycosyl hydrolase family 1 domain in the At2g30210 protein C-terminus has stimulated new ideas on LMCO function. Rogers and Campbell (2004) proposed a model in which monolignols are transported from cytoplasm to the cell wall as beta-glucoside intermediates. Once the glucosides have been transported to the cell wall, they must be cleaved, before the resulting aglycones can be polymerized. Perhaps the novel glucosyl hydrolase domain found near the C-terminus of 8 *Arabidopsis* LMCOs functions to hydrolyze the monolignol glucosides. In addition to phenoloxidase active, such a glucosidase activity would fit well with the previously proposed role for these enzymes in the early stages of lignin polymerization. Further study to demonstrate such glucosidase activity will help to clarify this new puzzle.

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