Identification and Characterization of Genes Involved in Regulation of Ascorbate Metabolic Pathway(s) in *Arabidopsis thaliana*.

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ABSTRACT

Vitamin C (ascorbic acid, AsA), an important primary metabolite of plants, functions as an antioxidant, an enzyme cofactor, and a cell-signaling modulator in a wide array of crucial physiological processes including biosynthesis of the cell wall, secondary metabolites and phytohormones, stress resistance, photoprotection, cell division, senescence, and growth. To identify genes that may regulate vitamin C levels in plants, about 3000 activation-tagged Arabidopsis lines were treated with ozone, which is a power oxidizing agent. Two mutants were selected for identification of potential genes involved in the regulation of vitamin C synthesis. A putative F-box gene, *VCF1,* and a purple acid phosphatase, *AtPAP15,* were identified for further characterization.

Two homozygous SALK T-DNA knockouts in the open reading frame (ORF) of *VCF1* exhibited high tolerance to ozone when treated with 450 ppb for 3 hours and the AsA levels of these mutants were 2 to 3 fold higher than wild-type (wt) plants. Developmental studies, using RT-PCR, indicated that foliar expression of the *VCF1* gene increased with plant age from 1 to 5 weeks, whereas AsA decreased during this same period. The expression of *VCF1* was higher under a low-light condition in which AsA was reduced considerably. The AsA levels in two *VCF1* overexpressing lines were only 50 to 70% of wt plants. These results suggested that the putative F-box gene functions as a negative regulator of leaf ascorbate content.

Overexpression of *AtPAP15* with the CaMV 35S promoter resulted in up to 3-fold higher AsA levels than wt plants, where two independent SALK T-DNA insertion mutants in *AtPAP15* had 50% less AsA than wt plants. Enzyme activity of bacterially expressed GST:AtPAP15 was greatest with phytate as a substrate indicating that AtPAP15 is a phytase. Phytase catalyzes hydrolysis of phytate (*myo*-inositol hexa*kis*phosphate) to yield *myo*-inositol and free phosphate. Thus, *AtPAP15* may regulate AsA levels by controlling the input of *myo*-inositol into this branch of AsA biosynthesis in Arabidopsis. *AtPAP15* was expressed in all tested organs in wt plants and suggests that the enzyme may have functions other than phytate degradation during seed germination.

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AsA	ascorbate
BME	ß -mercaptoethanol
CaMV	cauliflower mosaic virus
DTT	dithiothreitol
DHAR	dehydroascorbate reductase
DNA	deoxyribonucleic acid
GalDH	L-galactose dehydrogenase
GLDH	L-galactono-1,4-lactone dehydrogenase
GLOase	L-gulono-γ-lactone oxidase
GME	GDP mannose- 3',5'-epimerase
GMP	GDP mannose pyrophosphorylase
GPP	L-galactose-1-phosphate phosphatase
GST	glutathione-S-transferase
GUS	ß- glucuronidase
IP6	inositol hexaphosphate
IPTG	isopropylthio-ß-galactoside
L-GUL	L-gulono-1,4-lactone
MI	*myo*-inositol
MIOX	*myo*-inositol oxygenase
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAP	purple acid phosphatase
ROS	reactive oxygen species
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TAIL-PCR	thermal asymmetric interlaced PCR
WT	wild type

CHAPTER I

Literature Review

I.1 INTRODUCTION

I.1.1 The importance and Function of Vitamin C in Humans

L-Ascorbic acid (vitamin C, AsA) is a multifunctional compound in both plants and animals. For humans, AsA is essential to prevent disease associated with connective tissue (e.g., scurvy) and improves cardiovascular and immune cell functions (Nobile and Woodhill, 1981). As one of the most ubiquitous key nutrients affecting human health, vitamin C was believed to be a kind of amine and essential for life (*vita,* in Latin). That is why it was named as vitamin C. The chemical name, ascorbic acid (AsA), comes from the term "anti-scurvy," referring to the disease caused by lack of vitamin C. Scurvy has been known since antiquity and was prevalent in North Europe in the middle ages. It was a major cause of death of the crusaders and the sailors on long voyages. In the 18th century Lind (1753), described in detail the symptoms of scurvy and cure by eating fresh fruit and vegetables. He also demonstrated that oranges and lemons that were known to contain higher vitamin C were more effective than cider.

Vitamin C prevents scurvy by acting as a co-factor for prolyl hydroxylase, which catalyzes hydroxylation of the proline-lysine repeats in the collagen molecule. Collagen is the most abundant protein in the human body and is a major component of teeth and gums. Low levels of vitamin C lead to the production of underhydroxylated collagen fibers with low melting temperatures. Therefore, symptoms of scurvy include bleeding of the gums and breakdown of other collagen-rich regions of the body encompassing all connective tissues. AsA also acts as a co-factor for dioxygenases in animals in regulating the synthesis of hydroxyproline-rich proteins (Arrigoni and De Tullio, 2002).

Vitamin C is an important anti-oxidant in humans. It has the ability to give up hydrogen and become oxidized or to accept hydrogen and become reduced. Considered to be one of the most potent reducing agents found in nature, vitamin C protects plants and animals from oxidative damage. AsA helps protect the fat-soluble vitamins A and E as well as fatty acids from oxidation.

In addition to oxidative protection, AsA also assists in the formation of important messengers in the brain. Vitamin C is required for the conversion of dopamine to norepinephrine, which is a hormone and neurotransmitter secreted by the adrenal medulla and the nerve endings of the sympathetic nervous system to cause vasoconstriction and increases in heart rate, blood pressure, and the sugar level of the blood (Levine *et al*.,

1996). Additionally, AsA is required for the conversion of tryptophan to serotonin, a neurotransmitter that induces vasoconstriction, stimulates smooth muscle contraction, and regulates cyclic body processes (Wagner and Devito, 1987).

Humans cannot produce vitamin C and must obtain it from food sources. A slogan also says "it is better to take vitamins in food rather than in pills". This is particularly true when applied to vitamin C because it interacts and is interrelated to several other nutrients. Recently, the success of metabolic engineering in several plants greatly improves AsA levels and indicates promising alternative dietary choices.

I.1.2 Role of AsA in Plants

Ascorbate is the most abundant water-soluble antioxidant in plant cells that is found in most subcellular compartments. AsA serves as the major antioxidant in plants. Reactive oxygen species (ROS), such as the superoxide radical, H_2O_2, and the hydroxyl radical, are produced during the normal metabolic processes of photosynthesis, photorespiration and plasma membrane-linked electron transport (Foyer *et al*., 1994), as well as through adverse environmental assaults such as drought, ozone exposure and pathogen attack. Since ROS are highly toxic substances that can damage proteins, membranes and DNA, and can also act as signaling molecules (Davletova *et al*., 2005), they must be rigidly controlled to prevent oxidization of proteins and damage to the structural and functional integrity of membranes (Smirnoff, 1996). AsA functions to help protect plants from oxidative damage caused by ozone and helps plants survive in abiotic high salt environments. It is known that high concentrations of AsA are found in chloroplasts, suggesting a significant involvement in protecting plants from photo-oxidative stress (Smirnoff and Pallanca, 1996)**.** Ascorbate is a major component of the Halliwell-Asada pathway (Halliwell and Foyer, 1976) that is involved in protection of photosystem II (PSII) from photoinhibition.

In addition to protecting plants as a radical scavenger, AsA functions as enzyme cofactor that is involved in a wide array of crucial physiological processes and plays essential roles in growth and development. Ascorbic acid-dependent dioxygenases are required to produce flavonoids and the hormones gibberellic acid, ethylene and abscisic acid (Davey *et al*., 2000; Smirnoff *et al*., 2001). Ascorbate is a cofactor for mono- and

dioxygenases, which contain iron or copper at the active site. The function of AsA is to maintain the transition metal ion centers of these enzymes in a reduced form. AsA influences mitosis and cell growth in plants (Noctor and Foyer, 1998; Arrigoni and de Tullio, 2000; Smirnoff and Wheeler, 2000). Ascorbate is involved in root elongation, cell vacuolarization and cell expansion (Smirnoff, 1996). Ascorbate also appears to play a critical role in the cell division and cell cycle (Citterio *et al*., 1994; DePinto *et al.*, 1999; Kerk and Feldman, 1995), cell growth and differentiation (Alcain *et al.,* 1994; Cordoba *et al*., 1994; DeGara *et al*., 1997; DeCabo *et al*., 1993). AsA also plays a role in senescence; low AsA promotes senescence, whereas high AsA delays senescence (Navabpour *et al*., 2003). In the AsA deficient mutant, *vtc1-1*, induction of some senescence-associated genes (*SAG*s) occurred prematurely (Barth *et al*., 2004).

In general, three main types of biological activities can be defined for AsA in plant metabolism: its function as an enzyme cofactor, as a radical scavenger, and as a donor/acceptor in electron transport either at the plasma membrane or in the chloroplasts (Davey *et al*., 2000, Lorence *et al*. book chapter in press). AsA can donate electrons to a wide range of substrates because AsA is only mildly electronegative. For example, AsA is involved in the regeneration of vitamin E by non-enzymatic antioxidant activity. Also it has been reported that AsA is a substrate for oxalate and tartrate biosynthesis in certain plant species (reviewed in Loewus, 1999).

I.1.3 Ascorbate Biosynthetic Pathways

The complete AsA pathway for animals has been known since 1950 (Figure I.1). It begins with glucose-6-phosphate and proceeds through an inversion of the carbon chain, in which the carbon at the C1 position of glucose appears at the C6 position in ascorbic acid (Jackel *et al.,* 1950). From glucose-6-P, the pathway continues through multiple intermediates including D-glucuronate and L-gulono-1,4-lactone, with an inversion occurring after the formation of glucuronate (Conklin, 1998). Results obtained using labeled glucose molecules have shown that this carbon inversion does not happen in the plant pathway (Loewus, 1963).

Ascorbate biosynthesis in plants occurs through a complex, interconnected network with mannose*, myo*-inositol and galacturonate as principle entry points. A

generally accepted non-inversion pathway for AsA in plants has been proposed by Wheeler *et al.* (1998, Fig. 2). It includes an oxidation step at the C1 position along with the formation of 1,4-lactone, oxidation at C2 and C3, and lastly an epimerization at the C5 carbon to form the L configuration of ascorbic acid found in living systems (Loewus, 1999). Although not all the steps have been proven to date, Wheeler's pathway is consistent with current observations. In this pathway, vital intermediates include D-mannose and L-galactose. Wheeler *et al.* (1998) have shown that the substrate for the enzyme L-galactono-1,4-lactone dehydrogenase (GLDH) is provided by the formation of L-galactono-1,4-lactone. Since this enzyme can also catalyze the formation of ascorbic acid from sorbosone, it is possible that this is the activity that Loewus (1990) purified in an attempt to prove the presence of sorbosone in the pathway (Conklin, 1998). Conklin and colleagues have demonstrated the importance of mannose as an intermediate in ascorbic acid biosynthesis (Conklin *et al.*, 1999). After identifying an ascorbic acid-deficient *A. thaliana* mutant (*vtc*1), they discovered that these plants had a mutation in a gene encoding GDP-mannose pyrophosphorylase. GDP-mannose pyrophosphorylase is required for the synthesis of GDP-mannose from mannose-1-phosphate (Conklin *et al.,* 1999, step 4, Fig. 2). When the *vtc*1 mutant plants were transformed with the wild type gene complementary to this region, the ascorbic acid levels increased from a low value (0.7μmol/gm FW) back to wild type levels (2.5-3.5 μmol/gm FW). Barber (1971) demonstrated that GDP-*D*-mannose-3,5-epimerase can produce L-galactose in plants (Fig. 2). This activity was later detected in peas (*Pisum sativum*) and *Arabidopsis* (Wheeler *et al.*,1998). Plant extracts were also able to produce L-galactono-1,4-lactone and later ascorbate from GDP-mannose when NAD^+ and cytochrome *c* were supplied (Wheeler *et al.*, 1998).

Although the AsA biosynthetic pathway proposed by Wheeler and colleagues is consistent with most available data, there is growing evidence indicating the existence of other pathways operating in plants that contribute to the AsA pool. Precursors that are not part of the Wheeler pathway have been shown to end in AsA production. Davey *et al.* (1999) demonstrated that molecules such as L-gulono-1,4-lactone (L-GUL), methyl-D-galacturonic acid and D-glucurono-1,4-lactone can also be precursors of AsA in *Arabidopsis*. A 7-fold increase in AsA has been produced in plants such as lettuce and

tobacco by transformation with the GLOase (L-Gulono-γ-lactone oxidase) gene obtained from rat (Fig. 1; Jain & Nessler, 2000). This is only possible if the precursor L-gulono-1,4-lactone is present to be converted into AsA or if GLOase can utilize a different substrate for AsA production.

An alternative AsA pathway has been found in strawberries. D-galacturonic acid reductase (GalUR, step 2, Figure II.2), a gene isolated from strawberries (*Frararia)* encoding this enzyme has been cloned and characterized. Overexpression in *A. thaliana* greatly increased the AsA level (Agius *et al*., 2003). GalUR catalyzed the enzymatic reduction of D-galacturonic acid to galactonic acid, which can then be converted into L-Gal by an aldono-lactonase to form AsA. Strawberry transformed with the antisense version of the gene contained significantly less AsA than the wt, indicating that this pathway makes a significant contribution to the general AsA pool.

Recently, a new AsA biosynthetic route has been proposed by the Nessler group, in which *myo*-inositol (MI) is used as the initial substrate (Lorence *et al*., 2004). The AsA level increased 2- to 3-fold by overexpressing the *miox4* gene in Arabidopsis. *Miox4* was primarily expressed in flowers in Arabidopsis, suggesting different AsA biosynthesis pathways might be operating in different organs or tissues type.

I.1.4 Regulation of the AsA Metabolism Network

It has been recognized that the AsA level in plants changes when environmental conditions change (Conklin and Barth, 2004). Environmental conditions that increase endogenous ROS, such as ozone, drought and high temperatures, also raise AsA levels (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000; Panchuk *et al*., 2002; Sircelj *et al*., 2005). AsA level increases under temperature stress (Koh, 2002), and during water deficit in photosynthetic plants (Hoekstra *et al*., 2001; Iturbe *et al*., 1998; Price and Henry, 1991). The pool size in leaves and chloroplasts increases during acclimation to high light intensity (Smirnoff, 2000). High light intensity increased AsA content in rosette leaves (Mishra *et al*., 1993; Grace and Logan, 1996; Smiroff and Pallanca, 1996; Gatzek *et al*., 2002; Tabata *et al*., 2002; Tamaoki *et al*., 2003). Biotic stresses such as infection with tobacco mosaic virus (Milo and Santilli, 1967), nematodes (Arrigoni *et al.*, 1979) and nitrogen-fixating bacteria (Dalton *et al.*, 1998) have been reported to increase AsA levels as well. AsA content of plants also can be increased through enhanced ascorbate

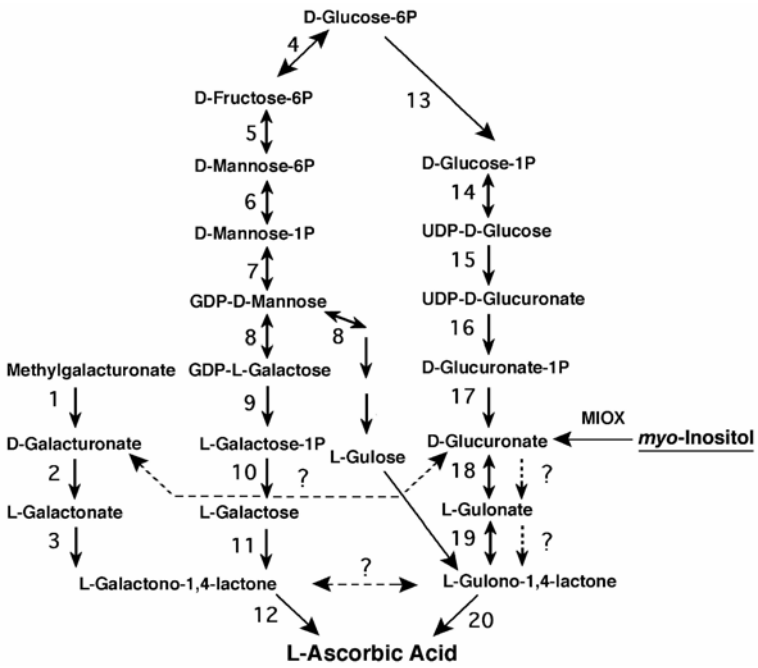

Figure I.1 AsA biosynthetic pathways. AsA biosynthetic network in plants (reactions 1–12), and animals (reactions 13–20). Four potential branch pathways operating in plants, the Mannose pathway, galacturonate pathway, the gulose pathway, and the MI pathway, are shown. Enzymes catalyzing the numbered reactions are: 1, methyl-esterase; 2, D-galacturonate reductase; 3, aldono-lactonase; 4, Glc-6-phosphate isomerase; 5, Man-6-phosphate isomerase; 6, phosphomannomutase; 7, GDP-Man pyrophosphorylase; 8, GDP-Man-3,5-epimerase; 9, phosphodiesterase; 10, sugar phosphatase; 11, L-Gal-1-dehydrogenase; 12, L-galactono-1,4-lactone (L-GalL) dehydrogenase; 13, phosphoglucomutase; 14, UDP-Glc pyrophosphorylase; 15, UDP-Glc dehydrogenase; 16, glucuronate-1-phosphate uridylyltransferase; 17, glucurono kinase; 18, glucuronate reductase; 19, aldono lactonase, and 20, guluno-1,4-lactone dehydrogenase. The reaction catalyzed by MIOX, and the possible pathway from MI to AsA (dashed arrows), are also shown. Question marks indicate enzymatic reactions that have not been demonstrated in plants. (Lorence *et al*., 2004)

recycling by overexpression of dehydroascorbate reductase (DHAR) in tobacco leaves and in maize leaves (Chen *et al.*, 2003). In addition, AsA levels also vary at different developmental stages. AsA content in leaf tissue is developmentally controlled, being highest in young leaves and declining with age (Conklin *et al.*, 1996). The AsA level in a pre-senescent leaf is only 30% of levels in an expanding leaf (Chen *et al.*, 2003). Dark-induced senescence caused a decrease in the ascorbate content in mitochondria (Jiménez

et al., 1998). The AsA biosynthesis appears to be controlled by both developmental triggers and environmental cues (Smirnoff and Wheeler, 2000). Ascorbate is implied to modulate plant defense transcripts and regulate genes that regulate development through hormone signaling (Pastori *et al*., 2003). However, little is known about molecular regulatory mechanisms.

Data regarding a feedback system in the Smirnoff-Wheeler pathway vary from different experiments. Since administration of L-Gal increased the levels of AsA synthesized dramatically, it was surmised that a negative feedback system was present in the Wheeler pathway upstream of GalD (Arrigoni and De Tullio, 2002). Results published more recently suggest that feedback inhibition may play a role at the terminal step. Tobacco cell lines fed L-Gal did not show a marked increase in AsA production over a 6-h period (Tabata *et al*., 2002).

Compared to the recent advances of knowledge in AsA biosynthesis pathways, there is little information concerning the regulation of AsA pools size in plants. No plant mutant completely devoid of AsA has ever been described, strongly suggesting that AsA is critical for plants to survive. To make AsA available, one of the strategies of plants is to synthesize this important molecule through multiple biosynthetic pathways. But no research about how these pathways are regulated and how AsA levels change in response to varying environmental conditions has been reported.

I.2 ACTIVATION TAGGING OF ARABIDOPSIS MUTANTS

A mutant screen, using ozone to promote foliar oxidative damage, has previously been successful in identifying several low AsA phenotypes in Arabidopsis plants derived from ethylmethane sulfonate (EMS) treated seeds (Conklin, 1998). The efficiency of discovering mutants through chemical mutagenesis is often low, however, since point mutations must occur at critical sites in the protein (or promoter) so that function (or gene expression) is substantially altered. T-DNA activation-tagged mutagenesis (Weigel *et al*., 2000) is a highly efficient alternative since insertion of the foreign DNA either activates (up or downstream of the ORF) or disables (in the ORF) gene expression at a high frequency. Activation tagging is a powerful tool for metabolic prospecting (Borevitz *et al.*, 2000). Activation tagging uses T-DNA vectors that contain four copies of an

enhancer element from the constitutively active cauliflower mosaic virus 35S (CaMV 35S) promoter. The vector pSKI015 was constructed by Igor Kardailsky in the Weigel lab (Weigel *et al.*, 2000) (Figure I.2).

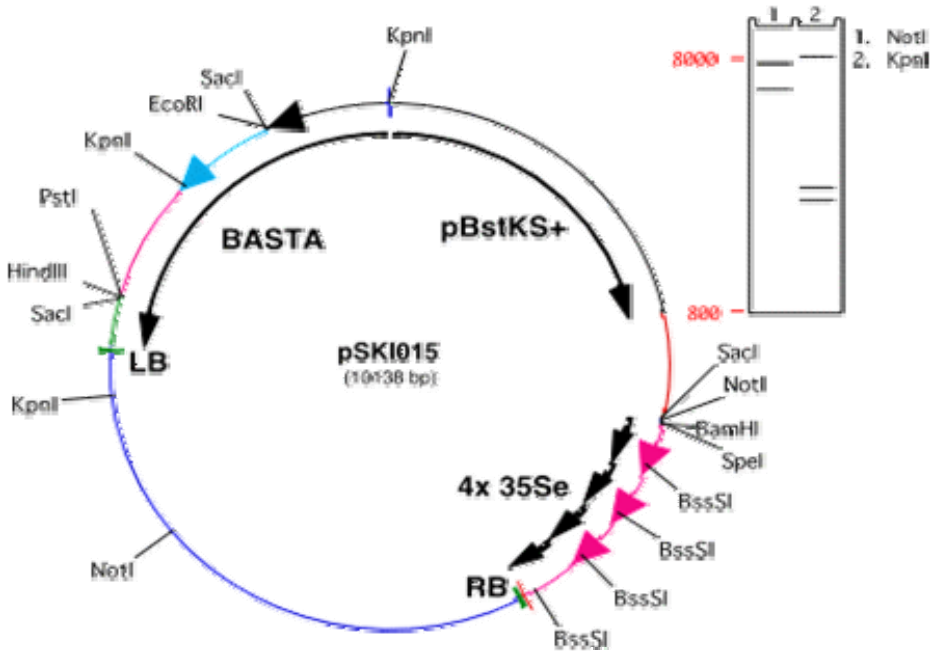

Figure I.2 Map of the activation-tagging vector pSKI015 (Weigel et al., 2000)

These enhancers can cause transcriptional activation of nearby genes, and because activated genes will be associated with a T-DNA insertion, this method has become known as activation tagging. The CaMV 35S enhancers lead primarily to an enhancement of the endogenous expression pattern rather than to constitutive ectopic expression (Weigel *et al.*, 2000). Analysis of a subset of mutants has shown that overexpressed genes were almost always found immediately adjacent to the inserted CaMV 35S enhancers, at distances ranging from 380 bp to 3.6 kb. Compared to T-DNA knockouts, activation can overcome the limitation of loss-of-function screens that rarely identify genes that act redundantly. In addition to activating genes in close proximity to the "activator" it is also possible that the T-DNA will disrupt genes by "landing" in their coding region or promoter. Thus one can screen activation tagged lines for both up- and down-regulated genes.

I.3 OBJECTIVES AND SIGNIFICANCE OF THE RESEARCH

The overall goal of this research was to identify and characterize novel genes encoding components of AsA biosynthetic and/or regulatory pathways.

Objective 1. The first specific objective of this research was to identify activation-tagged mutants that had phenotypes with altered AsA levels (either high or low) and identify activated genes utilizing TAIL-PCR and plasmid rescue.

Objective 2. The second specific objective was to clone the genes from a high and a low ascorbate activation-tagged mutant and characterize the phenotype of 1) T-DNA insertion knockouts and 2) overexpressors generated using the CaMV 35S constitutive promoter.

Objective 3. The third specific objective was to study the expression patterns of the novel genes (*VCF1* and *AtPAP15*) using RT-PCR and the GUS reporter gene.

Objective 4. The fourth specific objective was to characterize the products of the novel genes by examining the possible protein-protein interactions and identifying the substrate(s) of AtPAP15.

The significance of this research is to broaden the current understanding of AsA metabolism including AsA biosynthesis, degradation and recycling and/or AsA regulatory pathways.

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CHAPTER II

Negative Regulation of Leaf Ascorbate Levels in Arabidopsis by *VCF1*, a Novel F-box Gene

II.1 ABSTRACT

Ascorbate (AsA) biosynthesis in plants occurs through a complex, interconnected network with mannose, *myo*-inositol and galacturonate as principle entry points. Regulation within and among pathways in the network is largely uncharacterized. A novel gene, containing an F-box motif (*VCF1*, vitamin C F-box protein 1), was identified in *Arabidopsis thaliana* from an activation-tagged (AT), ozone-sensitive mutant that had 60% less leaf AsA than wild type (wt) plants. In contrast, two independent T-DNA knockout lines disrupting *VCF1* accumulated 2- to 3-fold greater foliar AsA and were more ozone-tolerant than wt plants. Constitutive expression of *VCF1* in the wt Arabidopsis Col-0 genetic background resulted in AsA levels similar to those in the AT mutant, whereas expression of *VCF1* in the knockouts reduced AsA content to wt levels. RT-PCR analysis of steady-state transcripts of genes involved in AsA biosynthesis showed that *VCF1* negatively affected the expression of GDP-mannose pyrophosphorylase, GDP-mannose-3',5'-epimerase, L-galactose-1-phosphate phosphatase, and L-galactose dehydrogenase, early and late enzymes of the mannose/galactose pathway to AsA. *VCF1* expression appears to be developmentally controlled, since, as leaf tissue aged, *VCF1* transcripts accumulated with a concomitant decrease in AsA. These findings provide the first evidence of an F-box-like gene that is involved in regulating the expression of multiple enzymes in a major plant biosynthetic pathway.

II.2 INTRODUCTION

L-Ascorbic acid (vitamin C, AsA) is a major antioxidant molecule in plants, an essential cofactor for several important metal-containing enzymes and is implicated in control of cell division and growth (Smirnoff and Wheeler, 2000; Davey *et al.*, 2000; Arrogoni and De Tullio, 2002). As an antioxidant, AsA protects plants from oxidative stress by decomposing reactive oxygen species (ROS) generated through normal oxygenic metabolism and adverse environmental assaults such as drought, pathogen attack and ozone exposure (Smirnoff, 1996; Noctor and Foyer, 1998; Conklin and Barth, 2004). Since ROS are highly toxic substances and can act as signaling molecules (Davletova *et al.*, 2005), they must be rigidly controlled to prevent oxidation of proteins and damage to the structural and functional integrity of membranes (Smirnoff, 1996). Although AsA

plays important critical roles in plant growth and development, a biosynthetic pathway for this versatile compound was not elucidated until 1998 (Wheeler *et al.*, 1998). Since that time, other biosynthetic routes have been discovered and a complex network for producing AsA is now recognized (Valpuesta and Botella, 2004). Entry points into the network are D-mannose (Wheeler *et al.*, 1998), D-galacturonate (Agius *et al.*, 2003) and *myo*-inositol (Lorence *et al.*, 2004). The mannose pathway branches at GDP-D-mannose where an epimerase can form either GDP-L-gulose or GDP-L-galactose (Wolucka and van Montagu, 2003). A degradation route for apoplastic AsA was recently proposed (Green and Fry, 2005). Since many genes encoding the proposed enzymes in the network have yet to be cloned and characterized, regulatory mechanisms, operating within the different synthetic and degradation routes to modulate AsA content, are just beginning to be described. Global control processes within the different pathways and crosstalk in the network are still largely unexplored.

Foliar AsA content can be regulated through gene expression by both developmental triggers and environmental cues (Smirnoff and Wheeler, 2000). Temperature stress (Koh, 2002) and high light intensity (Smirnoff, 2000) cause AsA levels to increase. The specific mechanisms eliciting these changes are not known, although low light levels decreased transcripts of GDP-mannose pyrophosphorylase and L-galactono-1,4-lactone dehydrogenase (GLDH) in tobacco leaves (Tabata *et al.*, 2002). In Arabidopsis and tobacco, AsA changes diurnally, being lowest at night and increasing throughout the day (Tamaoki *et al.*, 2003; Pignocchi *et al.*, 2003). This type of fluctuation is absent in other species such as potato (Imai *et al.*, 1999) and wheat leaf tissue (Bartoli *et al.*, 2005). Ascorbate content of foliar tissue can be increased through enhanced AsA recycling by over-expression of dehydroascorbate reductase (DHAR) (Chen *et al.*, 2003). Developmentally, foliar AsA is higher in young leaves than in pre-senescent leaves and the loss of AsA as tissue matures has been associated with decreases in DHAR activity in tobacco and corn (Chen *et al.*, 2003) and GLDH expression in Arabidopsis (Tamaoki *et al.*, 2003).

Within the mannose pathway, substrate availability and enzymatic activity have been shown to regulate AsA synthesis to some degree. Endogenous L-galactose content is rate limiting (Wheeler *et al.*, 1998), suggesting that control lies in production of this

substrate. Suppression of GDP-D-mannose pyrophosphorylase activity lowered AsA levels (Keller *et al.*, 1999), whereas down regulation of both L-galactose dehydrogenase (Gatzek *et al.*, 2002) and GLDH (Tabata *et al.*, 2002) had little affect on AsA content. The activity of the branch pathway enzyme, GDP-D-mannose-3',5'-epimerase, is partially inhibited or stimulated by several substrates, as well as the redox state of NADH/NADPH, making the epimerase a likely regulatory point (Wolucka and van Montagu, 2003). Detailed control of AsA biosynthesis in the *myo*-inositol and galacturonate pathways is not known, although high light has been reported to have a positive effect in the expression of the promoter of D-galacturonate reductase (Agius *et al.*, 2005).

In the present work, we demonstrate that expression of *VCF1*, a novel F-box motif gene isolated from an activation-tagged mutant with reduced AsA level, is correlated with changes in leaf AsA content. F-box proteins are involved in the ubiquitin E3 ligase/26S proteosome degradation pathway of proteins and control processes such as cell division, floral development, hormonal response, circadian rhythms and senescence in plants (del Pozo and Estelle, 2000; Vierstra, 2003; Moon *et al.*, 2004). We examined the AsA levels and ozone response of two T-DNA *VCF1* insertion mutants and transgenic plants over-expressing the *VCF1* ORF. The inverse relationship between *VCF1* expression and AsA levels suggests that the increase in transcripts of this F-box protein is part of a negative regulatory system that controls the AsA content of Arabidopsis and may contribute to ozone sensitivity as leaf tissues age. RT-PCR analysis of steady-state transcripts of genes involved in the AsA metabolic network demonstrated that *VCF1* is involved in the negative regulation of the expression of enzymes of the mannose/galactose pathway to AsA.

II.3 MATERIALS AND METHODS

II.3.1 Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used for comparison T-DNA insertion mutants. Growth conditions were 16-h days at 22°C and 8-h nights at 16°C, under 100 to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR illumination. The activation-tagged plants containing *pSKI015* (Basta resistance) were sprayed with 0.1% Basta before ozone treatment. The homozygous *vcf1-1* and *vcf1-2* mutant lines (mutant line *SALK_088186* and

SALK_113413) were identified as segregating lines in T_3 seeds provided by the SALK Institute for Genomic Analysis Laboratory. Homozygous lines were selected for the segregation of kanamycin resistance. PCR was used to distinguish heterozygous at the *vcf1* locus. Binary T-DNA vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). Arabidopsis plants were transformed by floral dip method (Clough and Bent, 1998). The Pro_{35S}*:VCF1* transgene was introduced into the wild-type Columbia ecotype or into *vcf1-1* and *vcf1-2* mutant plants. Seedlings were selected on Murashige and Skoog (Murashige and Skoog, 1962) plates containing 500 mg L^{-1} carbenicillin and 25 mg L^{-1} hygromycin. Both primary transformants and their progeny were used for RT-PCR and AsA assays.

II.3.2 Ozone Treatment and Ozone Sensitive Mutant Selection

Activation-tagged Arabidopsis plants were exposed to ozone at concentrations of 150 to 200 ppb for 4 h in continuously-stirred tank reactors (CSTRs) in a charcoal-air-filtered greenhouse. Ozone was generated from O_2 by U.V. discharge (Osmonics, Minnetonka, Minnesota) and delivered to the chambers by flow meters. Ozone concentrations in the chambers were monitored with a TECO U.V. O_3 analyzer (Thermo Electron, Hopkinton, Massachusetts) and regulated through the flow meters. Plants expressing foliar damage within 48 hours after ozone exposure were selected for analysis to identify lines with reduced AsA levels.

II.3.3 Leaf Tissue Ascorbate Measurement

Ascorbic acid content of leaves was measured by the AsA oxidase assay (Rao and Ormrod, 1995). About ~50 mg leaf tissue from one or more plants in three different pots were collected and frozen immediately in liquid N_2 and stored at -80C until utilized. Plant extracts were made from tissue frozen in liquid nitrogen, ground in 6% (w/v) metaphosphoric acid, and centrifuged at 15,000g for 15 min. Reduced AsA was determined by measuring the decrease in A_{265} (extinction coefficient of 14.3 mM) after addition of 1 unit of ascorbate oxidase (Sigma) to 1 mL of the reaction medium containing the plant extract and 100 mM potassium phosphate buffer (pH 6.9). Oxidized AsA was measured in a 1 mL reaction mixture plus 1 µL of 2 mM DTT (dithiothreitol) after incubating at room temperature for 15 min. Three different tissue samples from the

same genetic background were measured; ascorbate levels were determined as the mean and standard deviation was calculated.

II.3.4 Isolation of Genomic DNA and Southern-Blot Hybridization

Total Arabidopsis DNA was isolated using the Qiagen plant DNA extraction kit (Qiagen, Valencia, CA). Isolated DNA was digested with the restriction endonuclease *Eco*RI, separated onto a 0.8% (w/v) agarose gel, denatured with 0.5 M NaOH/1.5 M NaCl, and transferred by mass flow to a blotting membrane (Bio-Rad). The membrane was briefly neutralized in 0.5 M Tris- HCl, 1.5 M NaCl, pH 8.0, and DNA was immobilized by UV irradiation. The membrane was treated with a pre-hybridization solution (0.5 M sodium phosphate, pH 7.2, and 7% SDS) for 1 h at 65°C. Gene-specific ^{32}P-labeled probe, a 1576bp DNA fragment complementary to pSKI015 (base 1 to 1576), was obtained from pBluescript-KS+ plasmid (Stratagene, La Jolla, CA) by digestion with *Kpn*I and *Nco*I. The labeled probe was denatured by incubation at 65°C in the presence of 0.1 M NaOH for 10 min, and added directly to the prehybridization buffer. The probes were allowed to hybridize to their target sequences overnight at 65°C. Non-specific binding was removed by successive 10-min washes in: 2X SSC/0.1% SDS (w/v), 0.2% SSC/0.1% SDS (w/v), 0.1% SSC/0.1% SDS (w/v) (at room temperature), followed by 0.1% SSC/0.1% SDS (w/v) at 65°C. Hybridizing bands were visualized by exposure to radiographic film (Kodak, Rochester, NY).

II.3.5 Identification of Genomic DNA Flanking the Activation-Tagged T-DNA Insertion

Identification of the T-DNA insertion site in the *At23061* line in Arabidopsis was determined by thermal asymmetrical interlaced (TAIL)-PCR as described (Liu and Whittier, 1995). Genomic DNA was prepared with a DNA extraction kit (Qiagen). Two rounds of PCR amplifications were used to isolate DNA flanking the T-DNA insertion. 15 pmoles of the left border T-DNA primer L1 (5'-GACAACATTGTCGAGGC-AGCAGGA-3') was used with 150 pmol of the partially degenerate primer AD-2 (5'-NGTCGASWGANAWGAA-3') for the first PCR reaction. Conditions for the first reaction were as follows: (1) 95°C for 4 min; (2) 94°C for 15 s; (3) 65°C for 30 s; (4) 72°C for 1 min; (5) repeat five additional cycles of steps 2 through 4; (6) 94°C for 15 s;

(7) 25°C for 3 min; (8) ramp to 72°C over 3 min; (9) 72°C for 3 min; (10) 94°C for 15 s; (11) 65°C for 30 s; (12) 72°C for 1 min; (13) repeat one additional cycle of steps 10 through 13; (14) 94°C for 10 s; (15) 44°C for 1 min; (16) 72°C for 1 min; (17) repeat 14 additional cycles of steps 10 through 16; (18) 72°C for 3 min; and (19) 4°C. DNA produced in the first PCR reaction was diluted 1:50, and 1 µL of this dilution was used for the second round of PCR. 15 pmol of the left border nested primer L2 (5'-TGGACGTGAATGTA GACACGTCGA-3') was used with 15 pmol of AD-2 for amplification. The PCR cycle conditions were as follows: (1) 95°C for 5 min; (2) 94°C for 15 s; (3) 61°C for 30 s; (4) 72°C for 1 min; (5) repeat one additional cycle of steps 2 through 4; (6) 94°C for 15 s; (7) 44°C for 1 min; (8) 72°C for 1 min; (9) repeat 17 additional cycles of steps 2 through 8; (10) 72°C for 4 min; and (11) 4°C until needed. The resulting PCR products were sequenced with the L3 primer (5'-TTGGTAATTACTCTTTCTTTTCCTCC-3').

II.3.6 Constructs

For the over-expression of *VCF1*, an *Nco*I-*Xba*I fragment containing the CaMV 35S promoter and an *Xba*I-*Hind*III fragment containing the *VCF1* transcription terminator were excised from and cloned in the binary vector pCAMBIA1300. The *VCF1* coding region was amplified from wild type genomic DNA with primers FB-F (5'-CCCATGGGCTGATTGGTCTA-3') and FB-R (5'-CCTCTAGATCAGAGAAAGC -3') under the following conditions: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 50 s, 50°C for 50 s, and 72°C for 1 min using Taq Polymerase (Promega, Madison, WI) under conditions specified by the manufacturer. The amplified fragment was cloned into the pGEM-T Easy vector (Promega) sequenced to verify its integrity, and sub-cloned into pRTL2 (Rastrepo *et al.*, 1990) to create the Pro_{35S}:*VCF1* construct.

II.3.7 mRNA Detection by Reverse Transcriptase–Mediated PCR

For expression analysis, approximately 100 mg of leaves from 4-week old plants were harvested and frozen immediately in liquid nitrogen. Total RNA was extracted with the RNeasy plant mini kit (Qiagen). Crude RNA preparations were treated with 10 units of RNase-free DNase I (Promega) and further purified according to the RNeasy plant mini kit protocol. Each RNA sample was run in a PCR reaction with *Taq* DNA polymerase

(Promega) to assess any genomic DNA contamination. No PCR products detected were regarded as DNA-free-RNA. RNA samples were quantitated by spectrophotometry, and the integrity confirmed using agarose gel electrophoresis. For reverse transcriptase–mediated PCR studies, cDNA was synthesized from 1.5 µg of DNA-free RNA template using an oligo(dT) primer and Superscript Reverse Transcriptase (Ambion, Austin, TX). One-tenth volume of each cDNA was used as a template for PCR amplification. PCR reactions were conducted using the following thermal profile: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, with a 10-min terminal extension step at 72°C. To determine whether comparable amounts of RNA had been used, the ß -ATPase gene was used as a loading control (Riechers and Timko, 1999). Specific primers were designed to amplify ORFs involved in the AsA metabolic network, based on their published sequences: GDP-mannose pyrophosphorylase (GMP, *At2g39770*, Conklin *et al.*, 1999); GDP-mannose-3',5'-epimerase (GME, *At5g28840*, Wolucka and van Montagu, 2003); L-galactose-1-phosphate phosphatas*e (GPP, At3g02870; Laing et al., 2004); L-galactose dehydrogenase (GalDH, At4g33670*; Gatzek *et al.*, 2002), and vitamin C F-box protein 1 (*VCF1*, *At1g65770*, this work). A specific set of primers was designed to amplify the conserved domain among all four myo-inositol oxygenases present in the Arabidopsis genome (MIOX, *At1g14520*, *At2g19800*, *At4g26260* and *At5g56640*, Lorence *et al.*, 2004). The gene-specific primers used for RT-PCR were as follows: VCF1, VCF1-F (5'-CAGGGCTGGCTTATCAAATC-3') and VCF1-R (5'-GAGCCAAAACCGAGAAACA-3'); GMP, GMP -F (5'-ATGGCAGCAC-TCATTCTTGTTGG-3'), GMP-R (5'-GAGCTC-ACATCACTATCTCTGGC-3'); GME GME-F (5'-CCCATGGGAACTACCAATGGAA-CAG-3') and GME-R (5'-GGATCC-TCACTCTTTTCCATCAGCC-3'); GPP, GPP-F (5'-CCCATGGCGGACAATGATTCTC-3') GPP-R (5'-CGGATCCTCATGCCCCTGTAA-GCCGC-3'); GalDH, GalDH-F (5'-CTCTTCAGAAACTGAAAC-3'), GalDH-R (5'-GTTTCAGTTTCTGAAGAG-3'); MIOX, MIOX-F (5'-TCTAGACTCGAGATGAGCA-TTTGGGAATGTTG-3'), MIOX-R (5'-ATCGATGGTACCGGAAATGTTTCACCAAC-TACAGC-3'), β-ATPase, ATP-F (5'-GTATATGGTCAAATGAATGAGCC-3'), ATP-R (5'-GATCGACAGCAGGATA-GATACC-3'). PCR products were detected on 1.0% agarose gels containing 0.5 µg/ml ethidium bromide. Kinetic studies of each gene were

performed to provide a linear range of amplification before semi-quantitative analysis (data not shown). PCR products obtained under the linear range were analyzed and visualized. Each RT-PCR experiment was repeated three times and representative gels are presented in Figures.

II.3.8 Reporter Gene Construct and GUS Activity Staining

The putative promoter region (1 kb upstream of the start codon) of *VCF1* was cloned from wt genomic DNA with primers FBPH-F (5'-CCAAGCTTCCCACAACACAA-3') and FBPN-R (5'-CCCATGGCATGGTTTCCTT-3') and fused with GUS in the binary vector pCAMBIA1301. The fusion construct was transferred into *Agrobacterium tumefaciens* GV3101 and transformed into plants by the floral dip method as described above. Young seedlings or an entire leaf were incubated in staining solution: 50 mM $NaPO_4$, pH 7.0, 1% Triton X-100, 0.1% X-Gluc (bromochloroindoyl-β-glucuronide) by vacuum infiltrating the tissue for 5 min. Tissues were incubated at 37°C overnight. When visualizing results, chlorophyll was removed with 95% ethanol (rinsed several times) followed by a final rinse in 70% ethanol before viewing and photographing.

II.4 RESULTS

II.4.1 Identification of an Activation-Tagged Mutant with Reduced Ascorbate Leaf Content

Mutant selection was conducted on lines developed using the *pSKI015* activation-tagged (AT) vector, seeds were obtained from the the Arabidopsis Biological Resource Center (ABRC). This vector contains four repeats of the CaMV 35S enhancer and introduction of this T-DNA into the genome can cause increased gene expression near the site of integration in an orientation-independent manner (Weigel *et al*., 2000). An ozone screen was used to select ozone sensitive plants from four-week-old AT lines, and low AsA mutants were selected for further study (see Methods). We identified *AT23061*, as a low AsA phenotype, from ~5,000 independent T1 lines. This AT mutant grows somewhat slower than wt plants (Figure II.1A) and is sensitive to ozone (Figure II.1B). Total foliar AsA in *AT23061* (1.0 µmol g-1 FW) was about 60% less than that in wt plants (2.6 µmol g-1FW) at three weeks of age (Figure II.1C). A Southern blot, using the pBluescript-KS plasmid as a probe, showed that the mutant contained two T-DNA insertions (Figure

II.1D). RT-PCR analysis indicated that the expression of an ozone sensitive F-box-motif gene in *AT23061* (see identification procedure below) had been increased compared to wt plants (Figure II.1E).

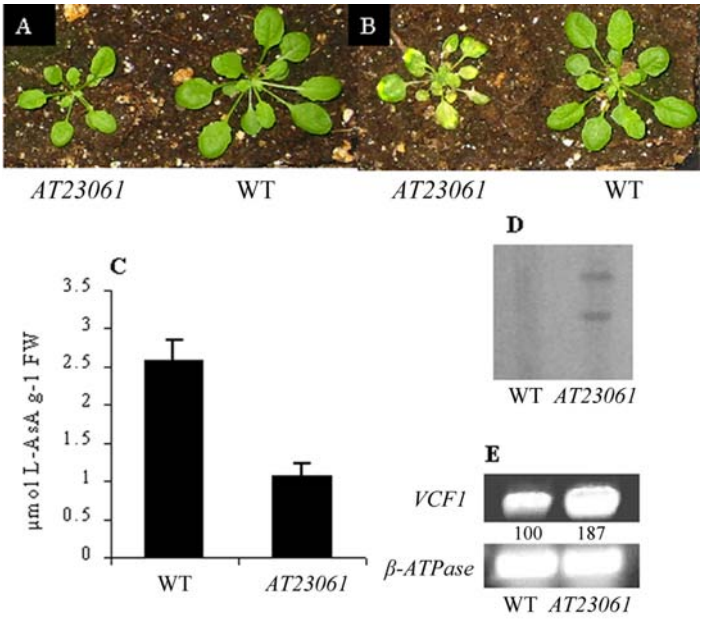

Figure II.1 Phenotype of the activation-tagged (AT) mutant AT23061 (ecotype Columbia), sensitivity to ozone, foliar AsA levels and expression of VCF1 compare to wt plants. **(A)** *AT23061* at 3 weeks of age is smaller than wt plants, but otherwise morphologically similar. **(B)** Leaf injury on *AT23061* after ozone treatment at 200 ppb for 4 h was considerably more severe than on wt plants. **(C)** Foliar total AsA (oxidized + reduced) in *AT23061* was 60% less than in Col-0. Tissue was sampled from the second rosette leaf of 3-week-old plants. Bars represent the mean and one standard deviation, n=3. **(D)** DNA gel blot analysis of the *pSKI015* plasmid indicated two insertions in *AT23061*. Total DNA from *AT23061*(4 µg) was digested with *Eco*RI and hybridized with the whole KS plasmid, digested with *Eco*RI, as the probe. **(E)** Expression analysis by RT-PCR of *VCF1* in *AT23061*. The *VCF1* signal was first normalized to *the β-ATPase* signal and then compared with the wt. Relative *VCF1* intensities are shown under each lane as the percentage of signal relative to that in wt.

II.4.2 Identification of VCF1 by TAIL-PCR and Analysis of Its Gene Structure

To identify AT insertion positions and possible genes responsible for the mutant AT phenotype, the sites of the two T-DNA insertions within the *AT23061* genome were determined by thermal asymmetrical interlaced (TAIL)-PCR (Liu and Whittier, 1995). Two sequences were obtained from the TAIL-PCR analysis. One T-DNA insertion was located in chromosome 4 in an intergenic region **(**Figure II.2A). The second insertion was situated in chromosome 1 and 400 bp upstream of *At1g65770*, a sequence containing an F-box domain (Figure II.2A). The gene, *At1g65770*, was named *VCF1* (vitamin C F-box protein 1) and has an uninterrupted ORF of 1083 bp encoding a novel 360-amino acid protein (Figure II.2B). Domain search analyses, using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/SMART), (SMART, Schultz *et al*., 1998, Letunic *et al*., 2004) and Pfam (http://www.sanger.ac.uk/Software/Pfam/), (Bateman *et al*., 2004) databases, indicated that VCF1 contains an F-box domain situated at amino acids 4 to 50. The F-box motif location, near the amino terminus, is typical for this class of proteins and VCF1 shows sequence homology to several known F-box proteins in humans (SKP2, and FBX4) and Arabidopsis (ORE9, SLEEPY1, FKF1, UFO, and SON1) (Figure II.2C). Many F-box proteins contain functional domains in their C-terminal regions, such as Leu-rich, WD-40, Armadillo, or Kelch repeats, which are implicated in protein–protein interactions and believed to play a role in substrate recognition (Patton *et al*., 1998; Gagne *et al*., 2002). Our search indicated that VCF1 contains a common plant Domain of Unknown Function (DUF295) (MMEVKSLGDKAFVIATDTCFSVLAHEF-YGCLENAIYFTDDT) from amino acids 285 to 332, suggesting that VCF1 may be different from other known F-box proteins in Arabidopsis. However, several Arabidopsis proteins, whose function is not yet described, contain both the F-box and DUF295 motifs indicating that VCF1-type proteins are not unique.

II.4.3 Insertion Mutants, *vcf1-1* and *vcf1-2*, have Higher Foliar Ascorbate and Ozone-Tolerance

The SALK 'T-DNA Express' Arabidopsis gene mapping tool (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to identify two insertional mutant lines *SALK_081886*, designated as *vcf1-1*, and *SALK_113413,* designated as *vcf1-2*, which contained an insertion in the ORF of *At1g65770*. PCR analysis, using T-DNA internal and 3'/5' primer

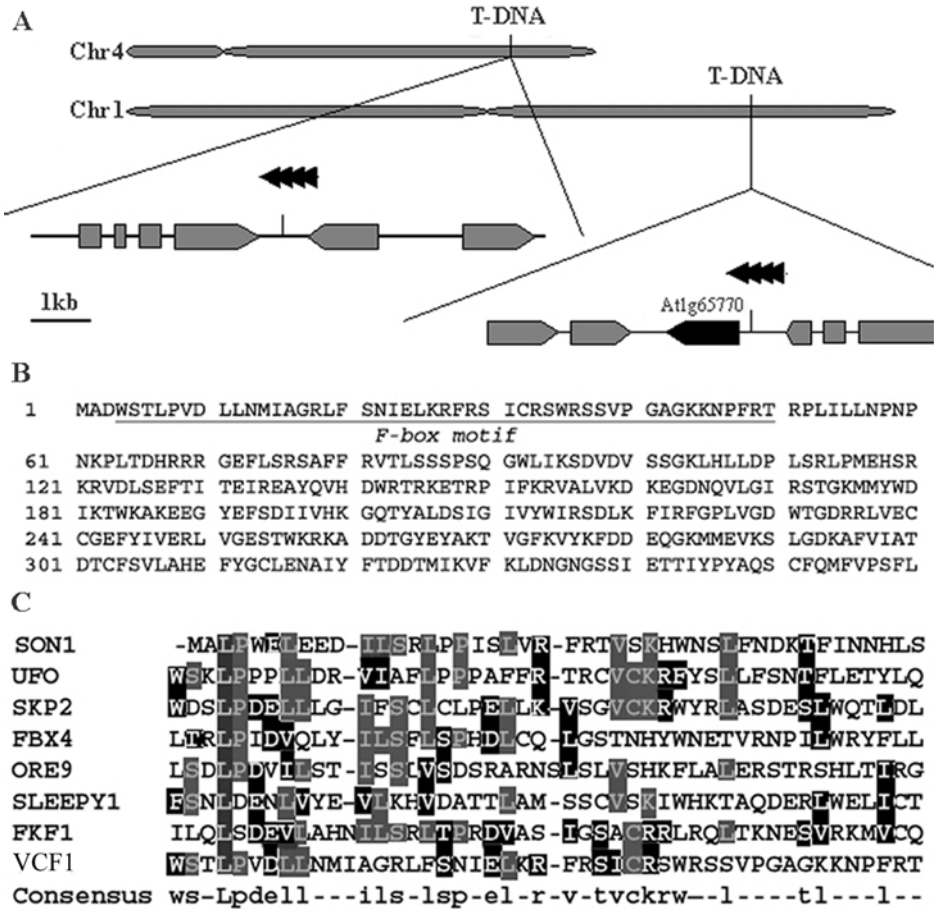

Figure II.2 Integration positions of the pSK1015 plasmid in the activation-tagged mutant AT23061, the primary structure of VCF1, and sequence homology of the VCF1 F-box motif with known F-box proteins. **(A)** In *AT23061*, T-DNA insertions were identified in chromosome 4 and 1 by TAIL-PCR. The insertion in chr 4 was in an intergenic region, whereas the insertion in chr 1 was about 400 bp upstream of *At1g65770*. **(B)** The primary structure of *At1g65770* indicated an F-box motif near the amino terminus of the protein. **(C)** Sequence homology of VCF1 with human (SKP2 and FBX4) and Arabidopsis (ORE9, SLEEPY1, FKF1, UFO, and SON1) F-box proteins. The consensus sequence was generated using ClustalW on the aligned sequences.

pairs of *VCF1*, confirmed that the two mutants have insertions in different regions of the *VCF1* coding sequence (data not shown). These mutants had increased resistance to ozone, displaying less foliar injury than wt plants when exposed to 450 ppb for 4 h **(**Figure II.3A). The mutant *vcf1-1* appeared to be somewhat more ozone tolerant than *vcf1-2* and this may be a function of a slightly higher foliar AsA content in *vcf1-1*. Total AsA levels in *vcf1-1*(5.3 µmol g-1 FW) and *vcf1-2* (4.9 µmol g-1 FW) were approximately 2-fold higher than in control plants (2.6 µmol g-1 FW) (Figure II.3B). RT-PCR of *VCF1* showed that there was no gene expression in the two T-DNA insertion lines. Thus, the absence of *VCF1* expression in the *vcf1*mutants led to increased levels of AsA, which suggests that *VCF1* may function to inhibit the synthesis or promote the degradation of AsA.

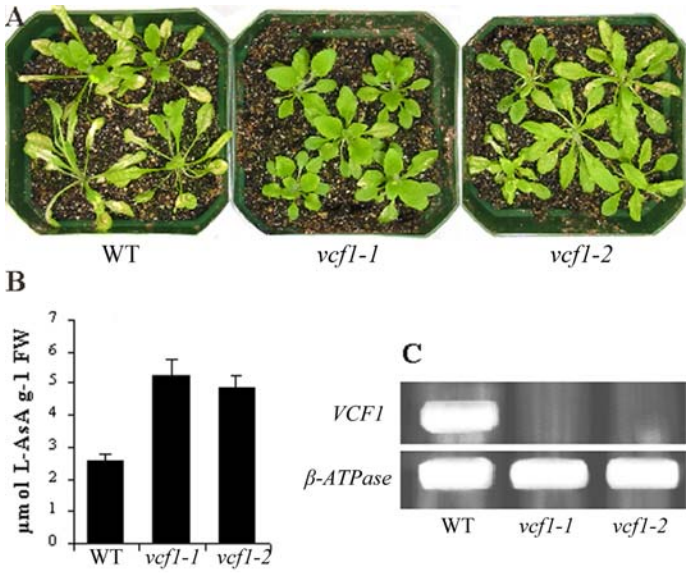

Figure II.3 Ozone responses, foliar AsA levels and expression of *VCF1* in vcf1-1 and vcf1-2 compared to wt. **(A)** Leaf injury in wt plants at three weeks of age in *vcf1-1* and *vcf1-2* after ozone exposure at 450 ppb for 4 h. (**B**) Foliar total AsA was about 2-fold higher in *vcf1-1* and *vcf1-2* than in wt. Tissue was sampled from the second rosette leaf of three-week old plants. Bars represent mean and one standard deviation, n = 3. (**C**) RT-PCR analysis of the insertion mutants to examine gene expression of *VCF1* in *vcf1-1*and *vcf1-2* plants, *β-ATPase* was used as a loading control.

II.4.4 Overexpression of *VCF1* in Wild Type Plants Reduces Ascorbate Leaf Content

The *VCF1* open reading frame was cloned and placed under control of the CaMV 35S promoter to characterize the effect of constitutive expression of the F-box gene in the Col-0 ecotype background used in generating the AT collection. A reduction in AsA levels in Pro$_{35S}$:*VCF1* transformants, similar to that found in the activated mutant, would provide additional support that the AT insertion's activation of the *VCF1* gene formed the basis for the low AsA phenotype *AT23061*. Three independent transgenic lines, Pro$_{35S}$:*VCF1-A*, *-B* and *-C* had foliar AsA levels 30 to 45% less than wt plants (Figure II.4A) and RT-PCR analysis of *VCF1* transcripts indicated a 2-fold increase in gene expression in the constitutively-expressing mutants (Figure II.4B). The reduction in AsA in the Pro$_{35S}$:*VCF1* lines was less than that observed in the AT mutant. Further evidence demonstrating that *VCF1* is involved in the negative control of foliar AsA was provided by the reversion of high AsA levels to lower, wt levels in the *vcf1-1* insertion mutant transformed with the Pro$_{35S}$:*VCF1* construct. In three independent transformed lines, the mean AsA level of 5.2 µmol g-1 FW in *vcf1-1* was reduced to less than 2.5 µmol g-1 FW (Figure II.4C).

II.4.5 VCF1 is Involved in the Negative Regulation of the Expression of Genes Encoding Enzymes of the Mannose/Galactose Pathway to Ascorbate

Since AsA pool homeostasis is determined by many factors such as degradation, transport, and utilization, in addition to synthesis (Smirnoff *et al.*, 2001), we conducted semi-quantitative RT-PCR experiments to address the relationship between *VCF1* and AsA metabolism. Steady-state transcripts of GDP-mannose pyrophosphorylase (*GMP*, Conklin *et al*., 1999), GDP-mannose-3',5'-epimerase (*GME*, Wolucka and van Montagu, 2003), L-galactose-1-phosphate phosphatase (*GPP*, Laing, *et al*., 2005), and L-galactose dehydrogenase (*GalDH*, Gatzek *et al*., 2002), early and late enzymes of the mannose/galactose pathway to AsA, were upregulated in the *vcf1-1* insertional mutant, but down-regulated in the AT mutant and in the *VCF1* over-expresser when compared to wt plants (Figure II.5). *VCF1* seems to be involved in negative regulation that specifically affects genes/enzymes of the Smirnoff/Wheeler pathway (Wheeler *et al*., 1998). There is no apparent effect on the expression of *myo*-inositol oxygenase (*MIOX*, Lorence *et al*., 2004), the first enzyme of the *myo*-inositol pathway to AsA.

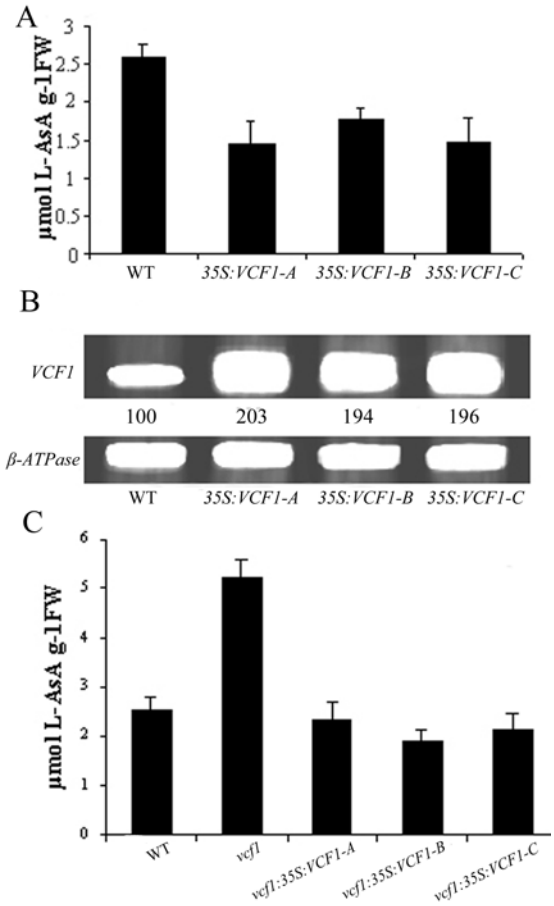

Figure II.4 AsA levels and expression levels of VCF1 in ectopic expression of VCF1 in the wt Col-0 genetic background and vcf1-1 plants. **(A)** AsA levels in three mutant lines, independently transformed with Pro$_{35S}$:*VCF1*, compared to the AT mutant *AT23061*. Tissue was sampled from the second rosette leaf of three-week old plants. Bars represent mean and one standard deviation, n = 3. **(B)** RT-PCR analysis of Pro$_{35S}$:*VCF1* mutants to examine *VCF1* expression. Relative gene expression is shown under each lane as the percentage of signal relative to that in wt. (**C**) AsA levels of Pro$_{35S}$:*VCF1* in *vcf1-1* mutants. Tissue sampled from the second rosette leaves. Bars represent the mean and one standard deviation, n=3.

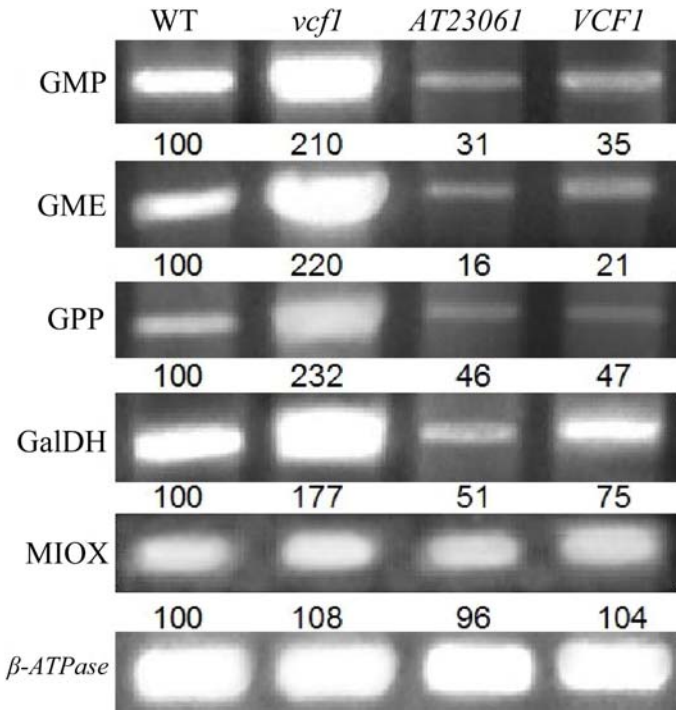

Figure II.5 VCF1 effects on the expression of genes of the mannose/galactose biosynthetic pathway to AsA. Steady-state level of GDP mannose pyrophosphorylase (*GMP*), GDP mannose- 3',5'-epimerase (*GME*), L-galactose-1-phosphate phosphatase (*GPP*), L-galactose dehydrogenase (*GalDH*), and *myo*-inositol oxygenase (*MIOX*) transcripts in wild type (WT), *vcf-1* insertional mutant, *AT-23061* activated-tagged mutant, and *VCF1* overexpresser (*VCF1*) are shown. *ß-ATPase* was used as control. Relative gene expression is shown under each lane as the percentage of signal relative to that in wt.

II.4.6 Expression of VCF1 is Developmentally and Light controlled

Both RT-PCR and promoter-reporter gene fusion studies were used to examine the expression patterns of *VCF1*. RT-PCR analysis of transcript levels in leaves was compared to AsA content during development. AsA was highest in one week-old plants (5.4 µmol g^{-1} FW), and progressively decreased with age to 1.5 µmol g^{-1} FW in five-week-old plants (Figure II.6A). Concurrent with the decrease in AsA, expression of *VCF1* increased over time, being minimally detectable in week-old seedlings and reaching a maximum, 4-fold greater, in five-week-old plants (Figure II.6B). This inverse correlation between AsA content and *VCF1* gene expression further supports the idea that *VCF1* is involved in the negative regulation of ascorbate content.

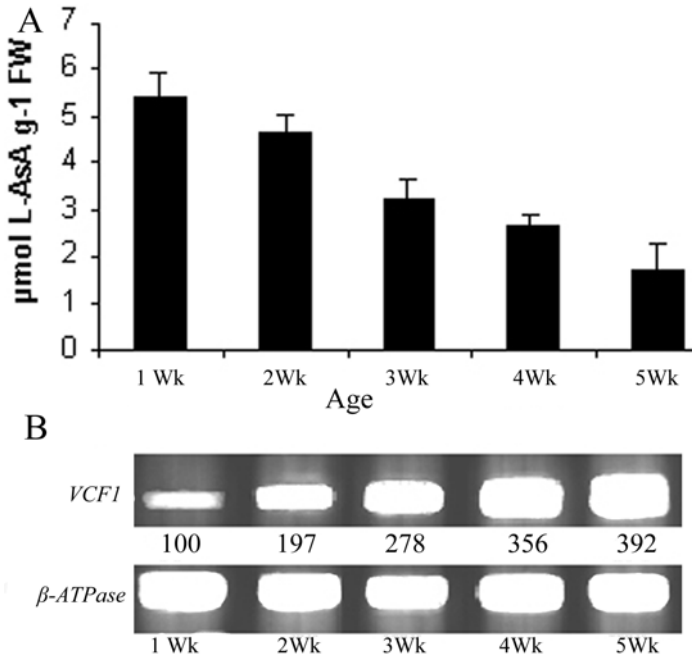

Figure III.6 Correlation of AsA content with VCF1 expression in Arabidopsis leaves at different developmental ages. (A) Total leaf AsA content in wt plants at 1 week old to 5 week old leaf age. Tissue sampled from the rosette leaves. Bars represent the mean and one standard deviation, n=3. (B) RT-PCR expression analysis of *VCF1* in wt plants indicated an increase in transcript with leaf age. Signal intensity calculated as in Fig. 1.

To examine the overall expression of *VCF1* in more detail, we fused the *β-glucuronidase* (*GUS*) reporter gene to a 1 kb segment of the *VCF1* promoter and transformed Col-0 Arabidopsis with the fusion construct. At least six independently transformed lines containing the construct were examined histochemically for GUS expression. A homozygous line was used to compare the expression under low light condition (~ 50 $\mu E \cdot m^{-2} \cdot s^{-1}$) and high light condition (~200 $\mu E \cdot m^{-2} \cdot s^{-1}$, at different developmental stages (1, 2 and 3-week-old, respectively). GUS activity was evident in the low-light condition at all ages (Figure II.7 A, C, E); much less staining was present in the same age seedlings under the high-light condition (Figure II.7 B, D, F). No GUS activity was apparent in the first true leaf as it emerged in 2-week-old and 3-week-old plants, nor was GUS expression obvious in the roots or hypocotyls at this time. In 12-day-old seedlings under a normal light condition (100 to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$), a progression of staining was apparent

dependent on leaf age (Figure II.7G). The oldest leaves stained most intensely, especially at the leaf tips and margins. Color intensity decreased steadily with leaf juvenility and no GUS activity was detectable in the apical meristem. This GUS expression pattern, driven by the *VCF1* promoter, confirmed the developmental and light control of *VCF1* expression, and additionally, demonstrated a tissue-specific regulation of the F-box gene.

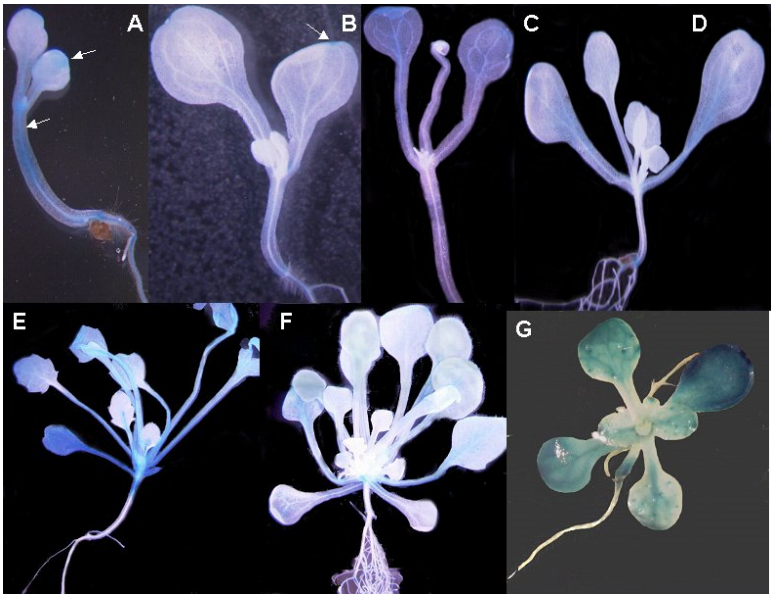

Figure II.7 Expression analysis of VCF1 using the GUS reporter gene. (A) In 1-week-old seedlings, *VCF1* expression was most evident in the apical region of the cotyledons and in the hypocotyls under low-light condition (~50 $\mu E \cdot m^{-2} \cdot s^{-1}$). (B) *VCF1* was slight expressed in the apical region of the cotyledons of 1-week-old seedlings grown under high-light condition (~200 $\mu E \cdot m^{-2} \cdot s^{-1}$). (C) In 2-week-old seedlings grown under low-light, *VCF1* expression was evident in the cotyledons and less in the newly emerging leaf. (D) *VCF1* expression in 2-week-old seedlings under high-light condition was confined primary to cotyledons and was not evident in young leaves. (E) In 3-week-old seedlings grown under low-light condition, *VCF1* was intensively expressed in older leaves and was less expressed in young leaves. (F) In 3-week-old seedlings under high light, *VCF1* was expressed minimally in older leaves and was much diminished in younger leaves. No expression was detected in roots and hypocotyls. (G) In 2-week-old seedlings grown under normal light conditions (100 to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$), *VCF1* expression was greatest in the older leaves and was less in younger leaves, no staining was evident in the apical meristem.

II.5 DISCUSSION

A mutant screen, using ozone to promote foliar oxidative damage, has previously been successful in identifying several low AsA phenotypes in Arabidopsis plants derived from EMS-treated seeds (Conklin, 1998). T-DNA activation-tagged mutagenesis (Weigel *et al*., 2000) is a highly efficient alternative method since insertion of the foreign DNA either activates (up or downstream of the ORF) or disables (in the ORF) gene expression at a high frequency. We screened ~5,000 activation-tagged lines of Arabidopsis and identified twelve mutants with either higher (>150%) or lower (<40%) AsA compared to Col-0. One of these mutants (*AT23061*), which displayed ozone-induced foliar lesions, had leaf AsA levels about 60% less than wt plants. TAIL-PCR analysis indicated a T-DNA insertion in the promoter region of *At1g65770*, a gene with unknown function. Further *in silico* analysis demonstrated that the *At1g65770* gene product, named VCF1, contains an F-box domain near its amino terminus, suggesting that *At1g65770* encodes a novel F-box protein that is associated with the negative control of leaf AsA content. The VCF1 F-box domain has high homology with several other F-box proteins in both plants and animals including ORE9 and UFO that are known to form SCF (Skp1-Cullin-F-box) E3 ligases (Woo *et al*. 2001; Moon *et al*., 2004; Ni *et al*., 2004). We propose that VCF1 can interact in an SCF complex to target degradation of a protein that controls foliar AsA levels.

F-box proteins are involved in a universal regulatory strategy that is common to both plants and animals. These proteins are part of the SCF-ubiquitin-E3 ligase complex that is involved in recognition of both the E2 protein, containing activated ubiquitin, and the substrate targeted for ubiquitination (Bai *et al*., 1996; Xiao and Jang, 2000). Target specificity of F-box proteins is conferred by their C-terminal end that usually contains a recognizable protein–protein interaction region in the form of Leu-rich, WD-40, Armadillo (Arm) or Kelch repeats (Craig and Tyers, 1999; Andrade *et al*., 2001; Gagne *et al*., 2002). The C-terminal region of VCF1 includes an identifiable plant protein domain (DUF295), but its function is unknown. This domain may be involved in target recognition and binding, however, the absence of a recognizable protein-protein interaction region suggests that VCF1 is unique among known Arabidopsis F-box proteins. Target protein interaction with the SCF complex requires some type of recognition signal. Most commonly, a kinase phosphorylates the F-box target, which

facilitates binding; however, proline hydroxylation is required for degradation of the hypoxia-inducible factor, which is present in numerous eukaryotes from yeasts to mammals (Ivan *et al*., 2002). In plants, domain II of Aux/IAA proteins undergo a single hydroxylation, however, this modification does not affect the auxin-regulated interaction of Aux/IAA targets with SCFTIR1 (Kepinski and Leyser, 2004). The possibility that the VCF1 target may undergo hydroxylation is intriguing since prolyl hydroxylase, which catalyzes this reaction, requires AsA as a co-factor to maintain an iron atom within the reaction center in the proper oxidation state. The typical Km of prolyl hydroxylase for AsA is around 0.35 mM (Majamaa *et al.*, 1986), whereas the plant cytosol contains 5 to 10 mM AsA, suggesting that VCF1 depletion of AsA has an inconsequential effect on proline hydroxylation.

In Arabidopsis, over 700 F-box proteins are predicted based on genomic analysis. The known F-box proteins have been found to regulate a diverse range of cellular functions, which include auxin responses by TIR1 (Ruegger *et al*., 1998), floral development by UFO (Samach *et al*., 1999), leaf senescence by ORE1 (Woo *et al*., 2001), circadian rhythms by ZTL1 and FKF1 (Nelson *et al.*, 2000; Somers *et al*., 2000), wound- and jasmonate-regulated gene expression by COI1 (Xie *et al*., 1998), modulation of systemic acquired resistance by SON1 (Kim and Delaney, 2002), and regulation of AsA synthesis by VCF1 (this study). The large number of F-box proteins encoded in the Arabidopsis genome suggests considerable diversification in the function of this gene family in plants.

Our data demonstrate that *VCF1* regulates the expression of several genes in the mannose/galactose pathway for AsA biosynthesis. This is the first evidence of an F-box gene regulating a major metabolic pathway in plants. Transcriptional control appears to be specific to one portion of the AsA biosynthetic network since *myo*-inositol oxygenase expression was unaffected by *VCF1* transcript levels. It is interesting to note that mannose/galactose pathway genes are single copy genes in Arabidopsis and *VCF1*, therefore, can regulate metabolite flux to AsA efficiently without potential compensatory effects of gene family members. We propose a model for VCF1 function wherein the F-box protein forms a SCF E3 ubiquitin ligase. VCF1 binds a protein, VCA (Vitamin C activator), that increases expression of genes in the mannose/galactose AsA biosynthetic

pathway. In the absence of VCF1, transcription occurs and AsA production is high. When VCF1 is present, the VCA is targeted, ubiquinated and degraded by the 26S proteosome resulting in depletion of foliar AsA. Demonstration of VCF1 binding to an ASK protein, identification of the VCF1 target, and evidence that the target protein interacts with promoters of the biosynthetic genes will provide confirmation of the proposed model.

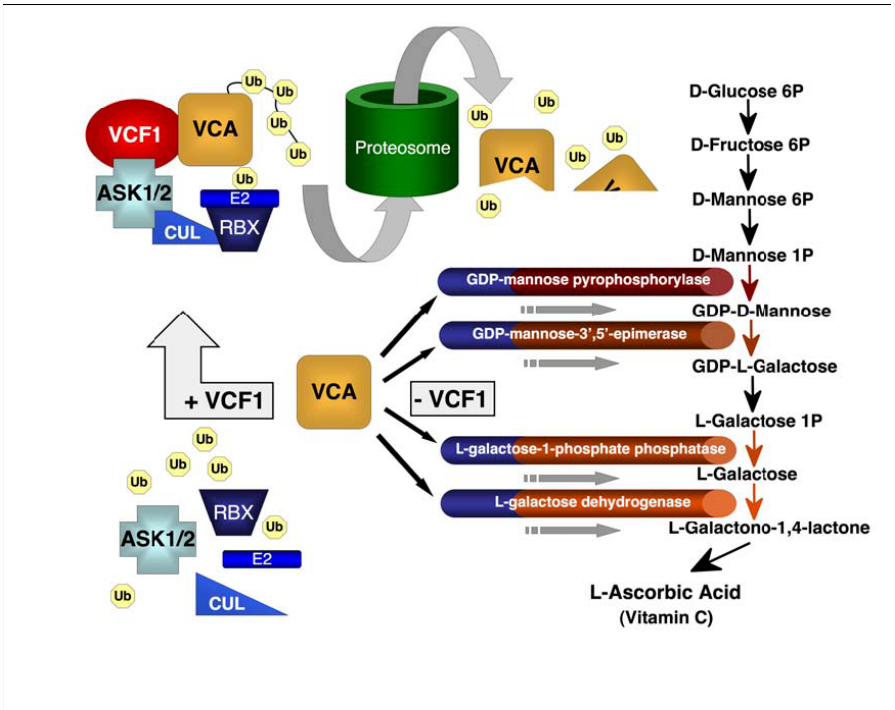

Figure II.8 Model for VCF1 (vitamin C F-box protein 1) regulation of the mannose/galactose pathway for AsA biosynthesis in Arabidopsis. In the presence of the VCF1 protein (+VCF1) a hypothetical activation factor for AsA biosynthetic genes, VCA, is bound to the SCF complex, ubiquinated and degraded by the proteosome. Destruction of VCA results in a reduction in AsA pathway enzyme gene transcription and a subsequent decline in AsA levels. In the absence of VCF1 (-VCF1) VCA accumulates and increases transcription of AsA enzyme pathway genes, which raises AsA levels. Components of the SCF complex are designated: ASK1/2=Arabidopsis SKP 1 and 2 homologs, CUL1=cullin, E2=ubiquitin conjugating enzyme, RBX1=RING-H2 finger protein, and Ub=ubiquitin.

Because *VCF1* mutants demonstrated increased levels of AsA, it was of interest to determine whether the loss of VCF1 would result in greater tolerance to ozone. Ascorbate is an integral weapon in the defense against ROS generated by oxidative stress and foliar application of AsA has been known as an ozone protectant for decades (Freebairn, 1960). More recently, several studies have demonstrated a correlation between total foliar AsA levels and ozone tolerance, the classic example being the *vtc1-1* Arabidopsis mutant (see review, Conklin and Barth, 2004). Also, over-expression in tobacco of the AsA recycling enzyme, dehydroascorbate reductase, increased foliar AsA and conferred greater ozone tolerance (Chen and Gallie, 2005). Ascorbate in the apoplastic space, where ROS are initially generated, has been considered the primary defense against ozone incursion. However, in the two white clover clones, NC-S and NC-R, neither the amount of extracellular or symplastic AsA, nor its oxidation state, correlated with ozone tolerance indicating that factors other than AsA can influence oxidant sensitivity in some species (D'Haese *et al*., 2005). Ozone treatment revealed that the two insertion mutants, *vcf1-1* and *vcf1-2*, are indeed more tolerant to this oxidative stress compared to wt plants. Furthermore, in wt plants, ozone injury increased with leaf age while AsA levels decreased. These results support previous studies showing the relationship between high foliar AsA and ozone tolerance. The mechanism of AsA protection against ozone has not been clearly demonstrated and may involve signaling, redox status, cell wall structure or other metabolic processes in addition to scavenging of ROS.

AsA also plays a role in senescence; low AsA promotes senescence, whereas high AsA delays senescence (Navabpour *et al*., 2003). In the AsA deficient mutant, *vtc1-1*, induction of some senescence-associated genes (*SAG*s) occurred prematurely (Barth *et al*., 2004). Furthermore, in the presence of exogenous AsA, expression levels of *SAG13* and *PR1* decreased to wt levels. The expression pattern of *VCF1* in wt plants demonstrated that this gene had highest expression in older leaf tissue, which also had lowest AsA. VCF1 may therefore function in controlling leaf senescence by depleting AsA levels and expression studies of *SAGs* in *VCF1*plants could provide support for this hypothesis.

We suggest that VCF1 functions as a component of an SCF ubiquitin ligase complex and interacts specifically with a substrate protein, thus controlling its quantity and stability through ubiquitination. The F-box protein is involved in negative regulation

of the mannose/galactose AsA pathway and, in *VCF1* knockout mutants, the VCF1 substrate accumulates, promoting AsA biosynthesis. The target protein may be a transcriptional activator since most presently identified F-box targets belong to the activator/repressor class of proteins. Although VCF1 has the necessary signature-domains to be classified as an F-box protein, further research is necessary to verify that it indeed forms an SCF complex and binds a specific substrate. Of primary interest is the identification of the VCF1 target and how this protein functions to control AsA levels in leaves and other plant tissues.

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Chapter III

An Arabidopsis Purple Acid Phosphatase with Phytase Activity also Influences Foliar Ascorbate Content

III.1 ABSTRACT

Ascorbate (ascorbate acid, AsA) biosynthesis in plants consists of a complex network with entry points at mannose, galacturonate and *myo*-inositol. Although the AsA level in plants can be controlled by developmental and environmental cues, very little is known about mechanisms involved in ascorbate regulation. An Arabidopsis mutant, *AT23040*, was isolated from a population of activation tagged Arabidopisis mutants. *AT23040* showed a pleiotropic phenotype including resistance to ozone, rapid growth and 2- to 3-fold higher AsA in leaves than for wild-type (wt) plants. The phenotype was caused by activation of the *AtPAP15* gene that encodes a purple acid phosphatase. *AtPAP15* was expressed in all tested organs in wt plants and may have multiple functions. Overexpression of *AtPAP15* with the CaMV 35S promoter increased foliar AsA up to 3-fold. Two independent SALK T-DNA insertion mutants in *AtPAP15* had 50% less AsA than wt plants. Enzyme activity of bacterially expressed GST:AtPAP15 was greatest with phytate as a substrate indicating that the AtPAP15 is a phytase. Phytase catalyzes hydrolysis of phytate (*myo*-inositol hexa*kis*phosphate) to yield *myo*-inositol and free phosphate. Thus, *AtPAP15* may regulate AsA levels by controlling the input of *myo*-inositol into this branch of AsA biosynthesis in Arabidopsis.

III.2 INTRODUCTION

As the most abundant antioxidant in plant tissues, ascorbate (ascorbic acid, AsA) protects cells and organelles from oxidative damage by decomposing reactive oxygen species (ROS) (Noctor and Foyer, 1998). Ascorbate is important in various aspects of development and metabolism due to its ability to interact directly with ROS, which play a key role in cellular signaling and defense. Apart from a major function in maintaining redox homeostasis in cells, AsA also serves as a co-factor for several important enzymes (Conklin and Barth, 2004). Consequently, ascorbate plays a critical role in many different processes within the plant cell including hormone and cell-wall biosynthesis, stress resistant, photoprotection and cell growth (Smirnoff and Wheeler, 2000). It is also reported to be involved in controlling flowering time and the onset of senescence (Pavet *et al.*, 2005, Barth *et al*. 2006).

To achieve these multiple functions, an important strategy for higher plants is the ability to synthesize AsA through multiple pathways utilizing a variety of precursor

substrates. Entry points for the biosynthesis of AsA that have been characterized include mannose (Wheeler *et al*., 1998), *myo*-inositol (Lorence *et al*., 2004), D-galacturonate (Agius *et a*l., 2003) and L-gulose (Woluka and van Montagu, 2003). The function of these pathways in different plant tissues and developmental stages is not known. However, the presence of such a biosynthetic network may be important for plants to survive in a constantly fluctuating environment. Compared to the characterization of the enzymatic steps in the AsA biosynthesis network, relatively little is known about how these pathways are regulated and how AsA biosynthesis may be controlled by the availability of precursor substrates. Knowledge of AsA biosynthesis and its regulation would advance the techniques of metabolic engineering of this important antioxidant.

Activation tagging (AT), utilizing four copies of an enhancer from the CaMV 35S promoter, is a technique useful in plant functional genetics to create mutants in which the T-DNA is introduced into the genome at random sites (Hayashi *et al*., 1992; Weigel *et al*., 2000). Analysis of gain-of-function mutants created through AT can provide insight to a gene's function and such mutants have played a pivotal role in dissecting cytokinin (Kakimoto, 1996), brassinosteroid (Neff *et al*., 1999), and light (Nakazawa *et al*., 2001) signaling. To further understand the role and regulation of AsA in oxidative stress tolerance, we utilized an ozone screen of AT Arabidopsis lines (Weigel, *et al*., 2000) to select gain-of-function mutants. The screen selects for high AsA mutants, since there is a positive correlation between total foliar AsA and ozone tolerance (Conklin *et al*. 1996). From approximately 4000 AT lines, an ozone-tolerant phenotype, with elevated leaf AsA, was isolated. Here we show that the high AsA level in the mutant was caused by activated expression of *AtPAP15*, a gene belonging to the major group of Arabidopsis purple acid phosphatases. We have expressed AtPAP15 as a GST fusion protein in bacteria and characterized its activity against a variety of substrates.

III.3 MATERIALs AND Methods

III.3.1 Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used for comparison with mutant plants. Growth conditions were 16-h days at 22°C and 8-h nights at 16°C, under 100 to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination. The activation-tagged Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) were grown and were sprayed with

0.1% Basta before ozone treatment. The homozygous *SALK_004788* and *SALK_05899* mutants were in the Columbia ecotype and identified as segregating lines in T_3 seeds provided by the SALK Institute for Genomic Analysis Laboratory. Homozygous lines were selected for the segregation of kanamycin resistance. PCR was used to distinguish heterozygous at the *atpap15* locus. Binary T-DNA vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). Arabidopsis plants were transformed by floral dip method (Clough and Bent, 1998). The Pro_{35S}*:AtPAP15* transgene was introduced into the wild-type Arabidopsis Columbia ecotype plants. Seedlings were selected on Murashige and Skoog (1962) plates containing 500 mg L^{-1} carbenecillin and 25 mg L^{-1} hygromycin. Both primary transformants and their progeny were used for RT-PCR and AsA assays.

III.3.2 Ozone Treatment and Ozone Tolerant Mutant Selection

Activation-tagged Arabidopsis plants were exposed to ozone at concentrations of 450 to 500 ppb for 4 h in continuously-stirred tank reactors (CSTRs) in a charcoal-air-filtered greenhouse. Ozone was generated from O_2 by U.V. discharge (Osmonics, Minnetonka, Minnesota) and delivered to the chambers by flow meters. Ozone concentrations in the chambers were monitored with a TECO U.V. O_3 analyzer (Thermo Electron, Hopkinton, Massachusetts) and regulated through the flow meters. Plants expressing less foliar damage compared to wt were selected for analysis to identify lines with increased AsA levels.

III.3.3 Leaf Tissue Ascorbate Measurement

Ascorbic acid content of leaves was measured by the AsA oxidase assay (Rao and Ormrod, 1995). About ~50 mg leaf tissue from one or more plants in three different pots were collected and frozen immediately in liquid N_2 and stored at -80C until utilized. Plant extracts were made from tissue frozen in liquid nitrogen, ground in 6% (w/v) metaphosphoric acid, and centrifuged at 15,000g for 15 min. Reduced AsA was determined by measuring the decrease in A_{265} (extinction coefficient of 14.3 mM) after addition of 1 unit of ascorbate oxidase (Sigma) to 1 mL of the reaction medium containing the plant extract and 100 mM potassium phosphate buffer (pH 6.9). Oxidized AsA was measured in a 1 mL reaction mixture plus 1 µL of 2 mM DTT (dithiothreitol)

after incubating at room temperature for 15 min. Three different tissue samples from the same genetic background were measured; ascorbate levels were determined as the mean and standard deviation was calculated.

III.3.4 DNA Preparation and Plasmid Rescue

Genomic DNA from the activation tagged mutant was prepared with a DNA extraction kit (Qiagen, Valencia, CA). For plasmid rescue, 3-5 µg genomic DNA was digested overnight with *Eco*RI (Promega, Madison, WI) in a 50 µL reaction. After phenol-chloroform extraction, digested DNA was self-ligated overnight at 4 °C with T4 DNA ligase in a total volume of 150 µL. Ligated DNA was precipitated with ethanol and was transformed into electroporation-competent *E.coli max DH10B* cells (Invitrogen, Carlsbad, CA) by electroporation. Plasmid DNA extracted from Kanamycin-resistant clones was sequenced with the primer SKI015: GCAAGAACGGAATGCGCG.

III.3.5 Constructs

For the over-expression of *AtAPAP15*, an *Nco*I-*Xba*I fragment containing the CaMV 35S promoter and an *Xba*I-*Hind*III fragment containing the *AtPAP15* transcription terminator were excised from and cloned in the binary vector pCAMBIA1300 (http://www.cambia.org.au). The *AtPAP15* coding region was amplified from a cDNA library (ABRC) with primers AtPAP15-F (5'- CCCATGGATGACGTTTCTACTACTT-3') and AtPAP15-R (5'- CCTCTAGATTAGC-AATGGTTAACAAGG-3') under the following conditions: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 50 s, 50°C for 50 s, and 72°C for 1 min using Taq Polymerase (Promega). The amplified fragment was cloned into the pGEM-T Easy vector (Promega) sequenced to verify its integrity, and sub-cloned into pRTL2 (Rastrepo *et al*., 1990) to create the Pro_{35S}::*AtPAP15* construct.

III.3.6 Expression and Purification of GST: AtPAP15 Fusion Protein

The coding region cDNA of *AtPAP15* was amplified with primers PAP15pGEX-F (5'-CGAATTCCATGACGTTTCTACTACTT-3') and PAP15PGEX-R (5'-GCGGCCG-CTTAGCAATGGTTAACA -3') and cloned into the bacteria expression vector pGEX-4T-3 (Pharmacia) through *Eco*R1 and *Not*1. The construct was transformed into *E. coli*

cells (strain BL21) by heat shock. Expression of GST:AtPAP15 was induced with 1mM isopropylthio-ß-galactoside (IPTG). Cells were pelleted by centrifugation at 4°C, and resuspended in buffer PBS (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), followed by mild sonication. Triton X-100 was added to a final concentration of 1% then mixed gently at room temperature for 30 min. The supernatant was collected by centrifuging the crude extract at 10,000 x g for 5 minutes at 4°C. Lysate from induced expression of GST:AtPAP15 fusion protein in *E.coli* was applied to glutathione- agarose prepacked columns (Sigma). Protein bound to the column was eluted with 10 mM reduced glutathione in 50 mM PBS buffer pH 7.5, and buffer exchanged with 50 mM Tris-HCl pH 7.0 using a 10 kDa molecular weight cutoff centrifugal filter device (Millipore, Billerica, MA).

III.3.7 Acid Phosphatase Assays

Inorganic phosphate released by acid phosphatase activity was measured by a modification of the ammonium molybdate assay (Heinonen and Lahti, 1981). Partially-purified, recombinant GST:AtPAP15 was incubated with 0.4 mM substrate of either phytic acid salt (Sigma), *p*NPP (*p*-Nitrophenyl Phosphate), ATP, or G-6-P in a 100 µL reaction volume of 100mM NaOAc, pH 4.5, at 37ºC for 30 minutes. One mL of freshly prepared AAM solution (2:1:1 acetone: 10mM ammonium molybdate : 5N H_2S0_4), and 100 µl of 1M citric acid were then added to the reaction, vortexed, and assayed at 355 nm spectrophotometrically. A standard curve was generated for quantification of Pi utilizing a 50 ppm stock of NaH_2PO_4) in 50 mM Tris HCl buffer pH 7.5 to generate standard concentrations from 1 to 10 nmol of Pi. The AAM/citric acid assay was used as described to measure Pi. Identification of the optimal pH for enzyme activity used phytic acid as the substrate and purified GST:AtPAP15 in 100 mM NaOAc buffer at pH 3.0 to 7.0 in 0.5 pH increments. The AAM/citric acid assay was used to measure free Pi.

III.3.8 Gene Expression by Reverse Transcriptase–Mediated PCR

For expression analysis, approximately 100 mg tissues of leaves, stem, roots, flower and cotyledon were harvested and frozen immediately in liquid nitrogen. Total RNA was extracted with the RNeasy plant mini kit (Qiagen). Crude RNA preparations were treated with 10 units of RNase-free DNase I (Promega) and further purified according to the

RNeasy plant mini kit protocol. Each RNA sample were run in a PCR reaction with *Taq* DNA polymerase (Promega) to assess any genomic DNA contamination. No PCR products detected were regarded as DNA-free-RNA. RNA samples were quantitated by spectrophotometry, and the integrity confirmed using agarose gel electrophoresis. For reverse transcriptase–mediated-PCR studies, cDNA was synthesized from 1.5 µg of DNA-free RNA template using an oligo(dT) primer and Superscript Reverse Transcriptase (Ambion, Austin, TX). One-tenth volume of each cDNA was used as a template for PCR amplification. PCR reactions were conducted using the following thermal profile: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, with a 10-min terminal extension step at 72°C. To determine whether comparable amounts of RNA had been used, *β-ATPase*, ATP-F (5'-GTATATGGTCAAATGAATGAGCC-3'), ATP-R (5'-GATCGACAGCAGGATAGAT-ACC-3'), or *actin7* primers Actin7-F (5'-GGTGAGGAT-ATTCAGCCACTTGTCTA-3') and Actin7-R (5'-TGTGAGATCCCGACCCGCAAGAT-C-3'), were used as a loading control (Riechers and Timko, 1999). PCR products were detected on 1.0% agarose gels containing 0.5 µg/ml ethidium bromide. Kinetic studies of each gene were performed to provide a linear range of amplification before semi-quantitative analysis (data not shown). PCR products obtained under the linear range were analyzed and visualized. Each RT-PCR experiment was repeated three times and representative gels are presented in Figures.

III.4 RESULTS

III.4.1 Identification of an Activation-tagged Mutant with High Foliar Ascorbate

Arabidopsis mutant lines used for screening were developed with the *pSKI015* AT vector (Weigel *et al*., 2000). Introduction of the T-DNA into the genome can cause increased gene expression near the site of integration in an orientation-independent manner. A high concentration ozone screen of four-week-old plants identified an oxidant-tolerant phenotype (*AT23040*) from ~4000 independent T_1 lines. Under normal growth conditions, the total foliar ascorbate (5.81 µmol gFW^{-1}) in *AT23040* was more than 2 times that in wild-type plants (2.50 µmol gFW^{-1}). A southern blot, using the SK plasmid as a probe, indicated that *AT23040* contained a single T-DNA insert (data not shown).

III.4.2 Identification of *AtPAP15* by Plasmid Rescue and Analysis of Gene Structure

To identify the AT insertion position and the gene responsible for the mutant phenotype, the site of the T-DNA insertion was determine by plasmid rescue (Weigel *et al.*, 2000). The T-DNA was located in chromosome 3 in the promoter region of *At3g07130* (Figure III.1A), a putative purple acid phosphatase, *AtPAP15* (Zhu *et al*., 2005). *AtPAP15* contains seven exons (Figure III.1A) and encodes a 533 amino acids protein with a calculated M.W. of 60.4 kDa. *AtPAP15* contains eight possible glycosylation sites and a probable N-terminal signal sequence of 27 amino acids targeting the protein to the cellular endomembrane system. Sequence alignment with two other *AtPAP*s and a *Glycine max PAP* indicated high homology of five conserved domains that are involved in the coordinate binding of metal ions, FeIII-MnII/ZnII, at the reaction center of the enzyme (Figure III.1B). Sequence homology of *AtPAP15* with the *GmPHY*, which is a phytase (Hegeman and Grabau, 2001), was 75%, suggesting that the Arabidopsis gene may be able to catalyze hydrolysis of phosphate from phytate and generate free *myo*-inositol.

III.4.3 Insertion Mutants Have Decreased Foliar Ascorbate, whereas Pro_{35S}:*AtPAP15* Mutants Have Increased Foliar Ascorbate

Two T-DNA insertion mutants (*SALK_004877* and *059899*) were obtained from the SALK T-DNA collection (ABRC, Ohio State University, Columbus). In the former mutant, the insertion was located in the promoter region of *At3g07130* and in the latter mutant, in exon 2 (Figure III.1A). Total foliar AsA in homozygous lines of the two insertion mutants was about 40% lower (1.35 and 1.68 µmol gFW^{-1}) than in wt plants (2.50 µmol gFW^{-1} (Figure III.2A). This difference contrasts with the greater than 2-fold increase in foliar AsA in the AT mutant (Figure III.2A.). Relative RT-PCR indicated slight (*SALK_004877*) to no (*SALK_059899*) gene expression in the knockout mutants and greater than 2-fold expression in the AT mutant (Figure III.2B). The trivial RT-PCR signal in *SALK_004887* may be due to the T-DNA insertion location in the promoter region that did not completely inactivate transcription.

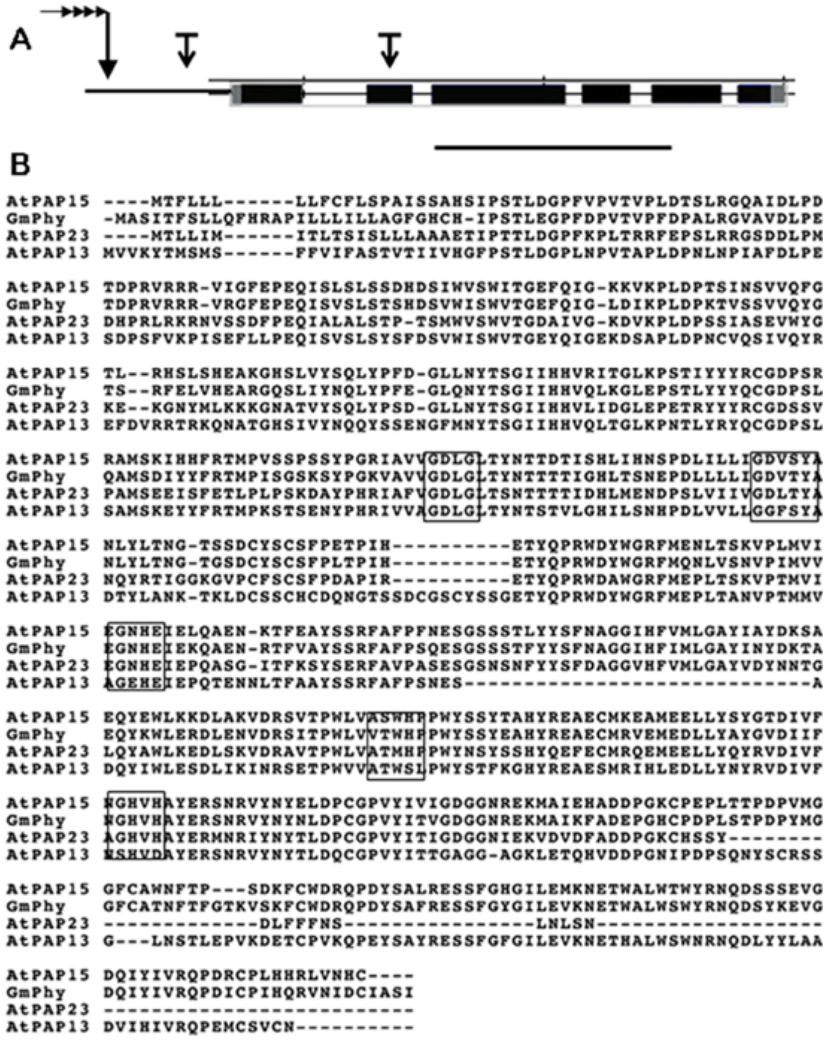


Figure III.1 Structure of the AtPAP15 gene. (A) Black boxes indicate exons, gray boxes indicate non-coding regions. The long arrow, with four small arrowheads, indicates the activation-tagged pSK1015 insertion site, short arrows with bars indicate the T-DNA insertion sites in the SALK mutants. Bar = 1000 bp. (B) Alignment of the amino acid sequences of *AtPAP15*, *GmPHY* (Hegeman and Grabau, 2001), *AtPAP13* and *AtPAP23* (Li *et a*l., 2002). Boxes show the consensus motifs that coordinately bind the metal ions Fe-Mn/Zn. The alignment was made with CLUSTALW.

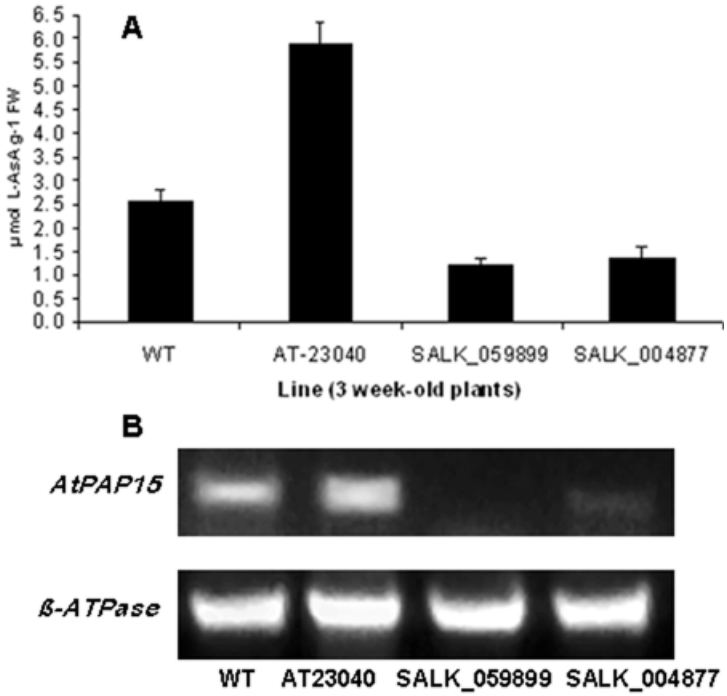
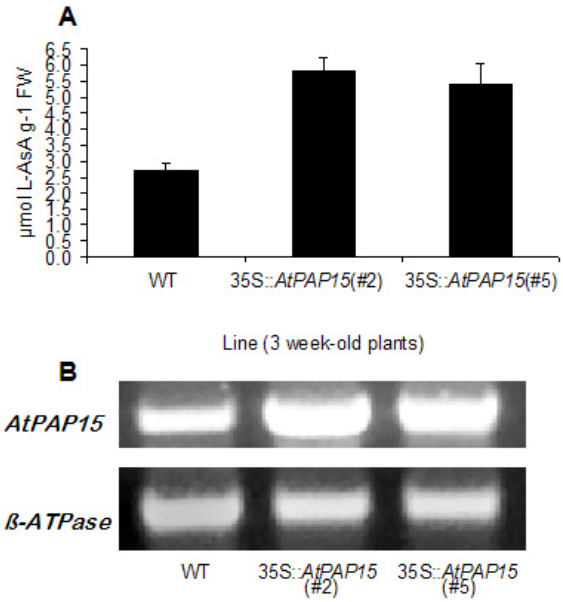


Figure III.2 The AsA and AtPAP15 expression level in activation-tagged mutant and two T-DNA insertion mutants. (A) Total foliar AsA in wild-type (wt), the activation-tagged mutant (*AT23040*) and two independent insertion mutants (*SALK_059899* and *SALK_004877*). Bars represent the mean (n = 3) and one standard deviation. (B) Relative RT-PCR of *AtPAP15* expression in wt and mutant lines. Expression of *β-ATPase* was used as control. PCR cycles were 28 for *AtPAP15* and 25 for *β-ATPase*. RT-PCR products were detected by staining with ethidium bromide.

To demonstrate that *AtPAP15* was responsible for increased gene expression which leads to increased foliar AsA, homozygous Pro_{35S}*:AtPAP15* mutant lines were developed. Two lines 35S::*AtPAP15*(#2) and 35S::*AtPAP15*(#5) had leaf AsA levels more than 2X higher compared to wt plants (Figure III.3A). Relative RT-PCR indicated that *AtPAP15* gene expression was also more that 2X higher in the Pro_{35S}*:AtPAP15* mutants (Figure III.3B). These observed changes in AsA in the various mutants indicate that expression of *AtPAP15* functions in some manner to positively influence foliar AsA.



FigureIII.3 Foliar AsA levels in Pro35S:AtPAP15 homozygous lines. (A) Total AsA foliar levels in wt and two *Pro$_{3SS}$:AtPAP15* lines, 35S::*AtPAP15*(#2) and 35S::*AtPAP15*(#5). (B) Relative transcript abundance of *AtPAP15* in two over-expressing lines determined by RT-PCR. *β-ATPase* was used as the RT-PCR loading control. PCR cycles were 28 for *AtPAP15* and 25 for *β-ATPase*. RT-PCR products were detected by staining with ethidium bromide.

III.4.4 AtPAP15 is a Phytase

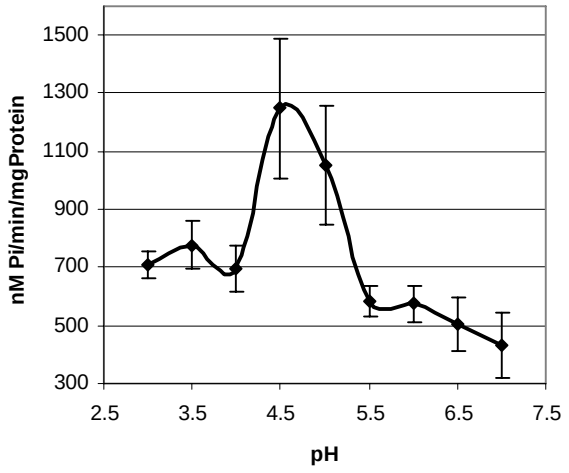
To determine the function of AtPAP15, the clone was expressed as a GST fusion protein in a bacterial system and partially purified by GST affinity chromatography. Analysis by SDS-PAGE, of the 10 mM glutathione eluant from the affinity column revealed the presence of several proteins with the most prominent band at 26 kDA, comprising about 70% of the total protein, tentatively identified as bacterial GST (data not shown). Although bacterial systems do not glycosylate proteins, and most PAPs possess several glycosylation sites, the effect of added carbohydrate chains on enzyme activity has not been extensively examined. Arabidopsis PAP23 has been expressed in *E. coli* as a GST:fusion protein and demonstrated phosphatase activity against a number of substrates (Zhu *et al*., 2005). GST:AtPAP15 activity was tested against the standard phosphatase substrate *p*-nitrophenyl phosphate (*p*NPP), as well as phytate, ATP and glu-6-P (Table III.1). Phosphate hydrolysis was high with *p*NPP and phytate, low with ATP and

negligible with glu-6-P. Cleavage of the GST moiety from the fusion protein increased enzyme activity against phytate by more than 2 times.

Substrate	Enzyme Activity (nM Pi/min/mgP)
pNPP	546 ± 40
ATP	52 ± 18
Glu-6-P	12 ± 4
phytate (fusion)	711 ± 112
phytate (cleaved)	1714 ± 317

Table III.1 Phosphatase activity of recombinant AtPAP15 against various substrates. Initial activity was determined using the GST fusion protein. Activity was then characterized after cleavage of the GST protein. Values represent the mean (n = 4) and one standard deviation.

Acid phosphatases typically have pH optima in the 4.5 to 5.5 range and analysis of AtPAP15 activity with phytate indicated maximum hydrolysis of phosphate at pH 4.5 with activity decreasing rapidly on either side of the pH optimum (Figure III.4).



FigureIII.4 Phytase activity of recombinant At PAP15 is pH dependant. Mean enzyme activity (n = 3) between pH 3.0 and 7.0 of recombinant protein cleaved from GST using phytate as substrate Bars represent ± one standard deviation.

III.4.5 AtPAP15 is Universally Expressed and Stimulates Shoot Growth

Analysis of *AtPAP15* transcription by RT-PCR demonstrated that the gene was expressed in leaves, cotyledons, stems, flowers and roots (Figure III.5). Minor differences in *AtPAP15* transcription occurred in all tissues except roots, which showed the highest gene expression levels.

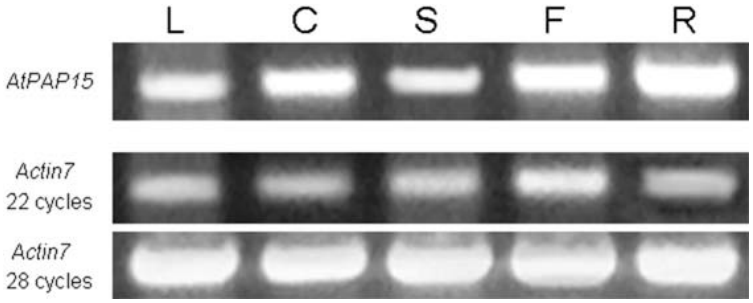


Figure III.5 Expression of AtPAP15 in leaf (L), cotyledon (C), stem (S), flower (F) and root (R) of wt Arabidopsis plants determined by RT-PCR. *AtPAP15* was measured after 28 PCR cycles. RT-PCR products were detected by staining with ethidium bromide.

Previous studies demonstrated a correlative relationship between foliar AsA levels and shoot growth. The *vtc1-1* mutant (low AsA) grows more slowly than wt plants (Conklin and Last, 1996; Velijovic-Jovanvoic *et al*., 2001), however, this phenotype is restored as wt when glulono-1,4-lactone oxidase (GLOase) is constitutively expressed in the mutant (Radizo *et al*., 2003). In studies in our laboratory, over-expression of GLOase and *myo*-inositol oxygenase in wt plants increased plant growth significantly under controlled-chamber conditions (data not shown). Growth rates of *AtPAP15* over-expression and knockout mutants were compared with wt plants to assess the effect of phytase activity on the accumulation of shoot biomass. In young seedlings (11 days after planting, DAP), shoot dry mass of Pro_{35S}*:AtPAP15* was 2.67X greater than wt plants (Figure III.6). As plants matured, 22 and 32 DAP, the over-expressing mutant had shoot biomass 1.97X and 1.70X that of wt, respectively. In contrast, the knockout mutant had shoot biomass about 0.75X of wt at all growth stages. The effect of phytase over-expression was most evident in young seedlings and may reflect the high phytate level in seeds and germinating seedlings compared to more mature plants.

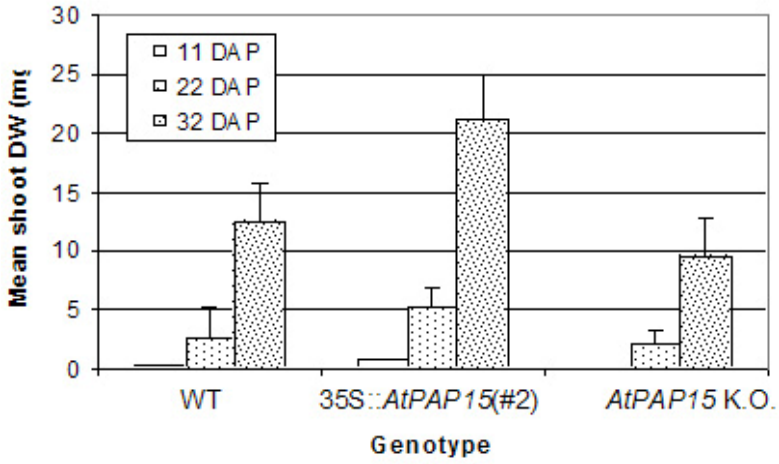


Figure III. 6 Mean shoot dry weights in wild-type (wt), a homozygous *AtPAP15* over-expressing line 35S::*AtPAP15*(#2) and a SALK T-DNA knockout line (*AtPAP15* K.O.). Data were collected at 11 to 32 days after planting (DAP). Bars represent the mean (n = 3) and one standard deviation.

III.5 DISCUSSION

In this work, we have identified an activation-tagged mutant with elevated AsA level and characterized a purple acid phosphatase (PAP), *AtPAP15* that exhibits phytase activity. Purple acid phosphatases are a family of enzymes that contain a dinuclear center in their active site (Doi *et al*. 1988; Que and true, 1990; Vincent *et al.* 1991). PAPs have been shown *in vitro* to non-specifically catalyze the hydrolysis of phosphate from numerous acid esters and anhydrides at a pH range of 4 to 7 (Klabunde *et al*. 1996). However, the *in vivo* functions of PAPs are not well defined. PAPs are suggested to act as multifunctional proteins and may play an important role in plant growth and development under both normal and stressed conditions (Zhu *et al*. 2005). Several PAPs are regulated by phosphorous starvation (Haran *et al*., 2000) and secreted outside the root cells (Bozzo *et al*., 2002) implying that they are important in phosphate acquisition. The expression of two PAPs, isolated from cultured tobacco cells, increased during cell wall regeneration in protoplasts and may have some role in the production of cellulose microfibrils (Kaida *et al*., 2003). *AtPAP23*, which shares the highest amino acid sequence similarity with *AtPAP15* (62%) in *Arabidopsis* is found predominantly expressed in flower, but its potential function is unknown (Zhu *et al*. 2005). *AtPAP17*, a type 5 acid phosphatase, displays peroxidase activity and has been suggested to be involved in ROS metabolism

during senescence (Pozo *et al*. 1999). A PAP from kidney bean has been thought to play an antioxidant role to prevent formation of oxygen radicals in the seeds (Klabunde *et al*. 1995). In this study, we have shown that plants with higher expression of *AtPAP15* have higher AsA and are more tolerant to ozone. The function of *AtPAP15* has not been studied before, but based on its amino acid sequence similarity with a soybean phytase *GmPHY* (75 %), *AtPAP15* is judged to act as phytase. In the present study, we have cloned *AtPAP15*, expressed a functional GST:AtPAP15 in *E. coli* and demonstrated phytase activity of the recombinant protein. AtPAP15 was most active against phytate and then pNPP and had minimal activity against ATP, which is comparable to the hydrolytic activity of GmPHY. The 4.5 pH optimum of AtPAP15 was also similar to the pH optimum of 4.5 to 5.0 for GmPHY (Hegeman and Grabau, 2001).

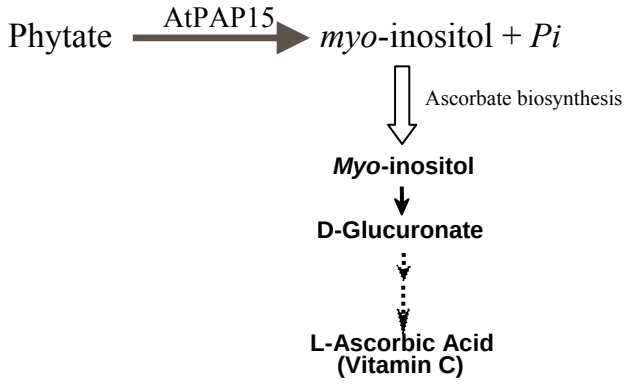
The expression pattern of *AtPAP15* established that gene transcripts were present in all tissue types, with the highest in the roots, which is in agreement with a previous study characterizing the expression of 28 *AtPAPs* in various organs (Zhu *et al*., 2005). The high expression in roots indicates that *AtPAP15* may have a role in recycling of phosphate from the phosphate ester pool. *AtPAP15* transcripts were not substantially higher in cotyledons compared to other shoot tissues. This suggests that the enzyme may not be critical in phytate hydrolysis of seed stores as may be case with *GmPHY*, which was most highly expressed in cotyledons (Hegeman and Grabau, 2001). Although phytate is found in large quantities in Arabidopsis seeds (16.2 ± 2.3 mg/g in dry seeds), it is also present in other plant tissues and organs including leaves, pollen, roots, and tubers (Raboy, 2003). Foliar phytate levels (5 to 30 μg/g FW) are much lower than in seeds and a positive correlation between the amount of phytate and free Pi in leaves is known (Bentsink *et al*. 2003). Phytate is synthesized from glucose-6-phosphate through an initial conversion to Ins(3)P_1 by Ins(3)P_1 synthase (MIPS) (Shukla *et al*. 2004) or from *myo*-inositol catalyzed by a *myo*-inositol kinase (Shi *et al*. 2005). Phytic acid and inositol derivatives are important to plant signaling functions. Phytate is known to act in regulating stomatal closure in response to ABA (Lemtiri-Chlieh et al., 2000). High transcript levels of MIPS in photosynthetic tissue (Keller *et al*. 1998) indicate that phytate might be actively synthesized in leaves. The presence of relatively high Pi levels, high expression of phytate biosynthesis genes and low accumulation of phytate in leaves

suggests that AtPAP15 could be involved in a constant hydrolysis of phytate and meanwhile, release *myo*-inositol and free phosphate. Although *AtPAP15* is highly expressed in root tissue, some of the phytate synthesis genes are not (Keller *et al*. 1998) thus AtPAP15 may function mainly as phosphate accumulator in roots.

High AsA and *AtPAP15* transcript levels in the activation-tagged mutant point toward a possible role of the phytate in AsA synthesis. Low AsA in *AtPAP15* knockouts and high AsA in Pro_{35S}*:PAP15* mutants imply *AtPAP15* might be important in maintaining the AsA pool. Phytate sequentially hydrolyzes phosphate moieties from phytate to produce free *myo*-inositol and P. Thus *AtPAP15* could function to increase AsA through additional supply of the initial substrate. The product of phytase, *myo*-inositol (MI) is an important precursor of AsA biosynthesis (Lorence *et al*., 2004). As the most abundant *myo*-inositol phosphate in plant cell, phytate is largely accumulated in vacuoles (Mitsuhashi *et al*. 2005). AtPAP15 might be one of the large number of hydrolytic enzymes found in the vacuole. Localization of AtPAP15 to vacuole is suggested by its pH optimum of 4.5, and is supported by the cellular localization PSORT prediction (Nakai and Horton, 1999).

The relationship between *AtPAP15* expression, elevated AsA levels and increased growth may involve a shared biosynthesis pathway of AsA and glucuronate-derived cell wall polysaccharides. In the *myo*-inositol oxygenase pathway (MIOP) to uronosyl and pentose subunits of pectin and hemicelluloses, MI is converted to glucuronate and then phosphorylated by as yet an uncharacterized kinase (Loweus and Murthy, 2000). In the AsA synthesis pathway, glucuronate is reduced to gulonate by as yet an uncharacterized glucuronate reductase (Lorence *et al*., 2004). Both overexpression of *myo*-inositol oxygenase (MIOX) (Lorence *et al*., 2004) or glucono-1, 4-lactone oxidase (Radizo *et al*., 2003) increased foliar AsA in Arabidopsis. Over-expression of MIOX also increased the incorporation of labeled MI into cell matrix polysaccharides (Kanter *et al*., 2005). *AtPAP15* could therefore function to increase both AsA and cell wall synthesis by supplying additional MI from phytate. This could be most important in young seedlings, where growth differences among our mutants were most noticable, and phytate reserves are known to be highest. In this research, we have reported a novel phytase, *AtPAP15*, which may increase the availability of free MI for ascorbate (Fig. II.7) and cell wall

biosynthesis and provide support for a possible additional AsA synthesis pathway (phytate to *myo*-inositol to AsA)



FigureIII.7 Possible mechanism of AtPAP15 involved in ascorbate biosynthesis

It is likely that an intricate regulatory system exists to control AsA levels in the various cellular compartments in response to developmental cues and to environmental conditions. In this complex AsA network, different pathways might subject to different regulatory mechanisms in order to survive in a constantly fluctuating environment.

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Chapter IV

Conclusions and Future Directions

As a ubiquitous antioxidant, AsA is important for plants to survive under fluctuating environmental conditions; not only protecting plants from stress-generated ROS, but also functioning in many physiology processes. Studies of AsA biosynthesis and regulation in plants are is very challenging because of the autotrophic nature of plants and their ability to response rapidly to a changing environment. Recently, several biosynthesis pathways in plants have been described and great progress has been made in characterizing specific enzymatic steps. Most genes encoding enzymes in these pathways have been cloned and characterized. In this present study, the regulation of AsA foliar levels was of considerable interest, since this aspect of AsA synthesis/recycling/degradation has been least studied and not many molecular mechanisms have been revealed. The AsA levels in plants change in response to several environment cues suggesting that regulatory mechanisms are likely to be complex, and may have cross-talk between different signals. Understanding the regulation of plant AsA levels as well as its relation to a varying environment has been a major goal of this research. To achieve this goal, activation-tagged Arabidopsis mutants were screened for ROS (ozone) tolerance or sensitivity and then assayed for foliar AsA content.

Analysis of two activation-tagged mutants, and the identification of a putative F-box gene, *VCF1* and a purple acid phosphatase gene, *AtPAP15*, led to the idea that plants may have multiple mechanisms to regulate AsA levels. In the present study, VCF1 may function in protein degradation and AtPAP15 may regulate the availability of AsA precursor substrates. RT-PCR and GUS data showed that the expression of *VCF1* is regulated by development stage and light intensity. Results of *in vivo* and *in vitro* experiments of enzyme activity have confirmed that AtPAP15 has phytase activity. Taken together, results of this study have provided significant new information on how AsA levels might be regulated. Such information will give new insights into the complex regulation of this essential molecule in plants.

Further studies including AsA response to different environmental cues, how signals are transduced, and protein-protein interactions will provide a better picture of AsA regulation in plants. Although I have shown that *VCF1* is involved in AsA regulation,

much more work is needed to elucidate the biochemical and genetic mechanisms. Firstly is to demonstrate that VCF1 binds to an ASK protein *in vitro*, and thereby has the capability to form an SCF complex. Other key experiments are to identify the protein(s) that bind to VCF1 using a pull-down assay and to characterize this target protein's function in controlling genes in biosynthesis/recycling/degradation pathways. Further studies of the *VCF1* promoter are also needed to characterize the developmental and light dependent control of expression in leaf tissue. In the future, the following experiments need to be done for *AtPAP15*: 1) Determine the phosphatase activity with other substrates such as IP3, IP4 and/or some of AsA biosynthetic pathway substrates and 2) Determine the role of phytase in leaf tissue.

VITA

The author, Wenyan Zhang was born in 1975 in a remote village in Jiangxi province of China. Although no electric power and no modern hospital facilities available, villagers survived well mainly relying on plants for energy, food and medicine sources. After living in mountains for 16 years, Zhang family moved to Guangdong province, the south coast area of China. She entered South China Agricultural University in 1993 and earned a Bachelor degree in plant science in 1997. She continued to pursue a Master degree in Plant Genetics and Breeding in the same university. After graduating in 2000, she worked as a research associate in Xinhui Botany Institute. In 2002, she began pursuing Doctoral of Philosophy degree in Plant Physiology with specialty in Molecular Cell Biology and Biotechnology at Virginia Polytechnic Institute and State University.

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