AMR1, an Arabidopsis Gene That Cooperatively and Negatively Regulates the Mannose/\(L\)-Galactose Ascorbic Acid Biosynthetic Pathway\(^1\[OA\])

Wenyuan Zhang, Argelia Lorence\(^2\), Hope A. Gruszewski, Boris I. Chevone, and Craig L. Nessler*

Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Ascorbic acid (AsA) biosynthesis in plants occurs through a complex, interconnected network with mannose (Man), myoinositol, and galacturonic acid as principal entry points. Regulation within and between pathways in the network is largely uncharacterized. A gene that regulates the Man/\(L\)-galactose (\(L\)-Gal) AsA pathway, AMR1 (for ascorbic acid mannose pathway regulator 1), was identified in an activation-tagged Arabidopsis (Arabidopsis thaliana) ozone-sensitive mutant that had 60% less leaf AsA than wild-type plants. In contrast, two independent T-DNA knockout lines disrupting AMR1 accumulated 2- to 3-fold greater foliar AsA and were more ozone tolerant than wild-type controls. Real-time reverse transcription-polymerase chain reaction analysis of steady-state transcripts of genes involved in AsA biosynthesis showed that AMR1 negatively affected the expression of GDP-Man pyrophosphorylase, GDP-\(L\)-Gal phosphorylase, \(L\)-Gal-1-phosphate phosphatase, GDP-Man-3',5'-epimerase, \(L\)-Gal dehydrogenase, and \(L\)-galactono-1,4-lactone dehydrogenase, early and late enzymes of the Man/\(L\)-Gal pathway to AsA. AMR1 expression appears to be developmentally and environmentally controlled. As leaves aged, AMR1 transcripts accumulated with a concomitant decrease in AsA. AMR1 transcripts also decreased with increased light intensity. Thus, AMR1 appears to play an important role in modulating AsA levels in Arabidopsis by regulating the expression of major pathway genes in response to developmental and environmental cues.

\(L\)-Ascorbic acid (AsA; vitamin C) is a major antioxidant molecule in plants, an essential cofactor for several important metal-containing enzymes, and is implicated in the control of cell division and growth (Davey et al., 2000; Smirnoff and Wheeler, 2000; Arrigoni and De Tullio, 2002; De Tullio and Arrigoni, 2004). As a major antioxidant, AsA protects plants from oxidative stress by decomposing reactive oxygen species (ROS) generated through normal oxygenic metabolism and adverse environmental assaults (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). To achieve these multiple functions, the ability to tightly control AsA levels through biosynthesis and degradation pathways would be an advantageous strategy for higher plants. A complex network for producing AsA through several biosynthetic routes has been discovered. Entry points into the network are \(\delta\)-Man (Wheeler et al., 1998), \(\delta\)-galacturonate (Agius et al., 2003), and myoinositol (Lorence et al., 2004). All genes of the major pathway, the Man pathway, have been characterized, including the GDP-\(\delta\)-Man pyrophosphorylase (Conklin et al., 1999), the GDP-Man-3',5'-epimerase (Wolucka and Van Montagu, 2003), \(L\)-Gal dehydrogenase (GalDH; Gatzek et al., 2002), \(L\)-galactono-1,4-lactone dehydrogenase (Imai et al., 1998), the \(L\)-Gal-1-P phosphatase (GPP; Laing et al., 2004), and, more recently, the GDP-L-Gal phosphorylase (also known as VTC2 and its close homolog VTC5) that converts GDP-L-Gal to GDP-L-Gal-1-P (Dowdle et al., 2007; Linster et al., 2007, 2008) and a \(L\)-Gal guanylyltransferase that also converts GDP-L-Gal to GDP-L-Gal-1-P (Laing et al., 2007). In support of the importance of the Man/\(L\)-Gal pathway, a double mutant (VTC2/VTC5) showed growth arrest immediately upon germination and the cotyledons subsequently were bleached, while normal growth was restored by supplementation with ascorbate or \(L\)-Gal (Dowdle et al., 2007). While the regulatory mechanisms, operating within the different synthetic, recycling, and degradation routes to modulate AsA content, are just beginning to be described (for review, see Wolucka and Van Montagu, 2007; Linster and Clarke, 2008), global control processes within the different pathways and cross talk in the network are still largely unexplored. Knowledge of AsA
biosynthesis and its regulation would advance approaches toward the metabolic engineering of this important antioxidant.

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; Bartoli et al., 2006) cause AsA levels to increase. Additionally, a degradation route for apoplastic AsA has been proposed (Green and Fry, 2005). The specific mechanisms eliciting these changes are not known in detail, although low light levels decreased transcripts of GDP-Man pyrophosphorylase (GMP) and l-galactono-1,4-lactone dehydrogenase (GLDH) in tobacco (Nicotiana tabacum) leaves (Tabata et al., 2002), while high light caused increased expression of VTC2 in Arabidopsis (Arabidopsis thaliana; Dowdle et al., 2007; Yabuta et al., 2007; Müller-Moulé 2008). In Arabidopsis and tobacco, AsA changes diurnally, being lowest at night and increasing throughout the day (Pignocchi et al., 2003; Tamaoki et al., 2003), although this type of fluctuation is absent in other species such as potato (Solanum tuberosum; Imai et al., 1999) and wheat (Triticum aestivum; Bartoli et al., 2005).

Ascorbate content of foliar tissue can be increased through enhanced AsA recycling by overexpression of dehydroascorbate reductase (DHAR; Chen et al., 2003). Developmentally, foliar AsA is higher in young leaves than in presenescent leaves, and the loss of AsA as tissue matures has been associated with decreases in DHAR activity in tobacco and corn (Zea mays; Chen et al., 2003) and GLDH expression in Arabidopsis (Tamaoki et al., 2003).

Within the Man/l-Gal pathway, substrate availability and enzymatic activity have been shown to regulate AsA synthesis to some degree. Endogenous l-Gal content is rate limiting (Wheeler et al., 1998), suggesting that control lies in production of this substrate. Suppression of GDP-d-Man pyrophosphorylase activity lowered AsA levels (Keller et al., 1999), whereas down-regulation of both GalDH (Gatzek et al., 2002) and GLDH (Tabata et al., 2002) had little effect on AsA content, although the last caused a strong reduction in leaf and fruit size in tomato (Solanum lycopersicum), mainly as a consequence of reduced cell expansion (Alhagdow et al., 2007). Ascorbate supplementation decreased VTC2 expression in Arabidopsis, suggesting feedback inhibition by AsA at the transcriptional level (Dowdle et al., 2007). The activity of the branch pathway enzyme, GDP-d-Man-3’-5’-epimerase, is partially inhibited or stimulated by several substrates, as is 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 myoinositol 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). We recently characterized a purple acid phosphatase with phytase activity (AtPAP15; At3g07130) that may modulate AsA by contributing to the input of free myoinositol into this branch of the AsA biosynthetic network in Arabidopsis (Zhang et al., 2008).

In this work, we show that expression of AMR1 (for ascorbic acid mannose pathway regulator 1), a gene isolated from an activation-tagged (AT) Arabidopsis mutant, is inversely correlated with changes in leaf AsA content. Furthermore, this decrease in AsA appears to result from a coordinated reduction in the expression of genes encoding enzymes in the Man/l-Gal AsA metabolic network, suggesting that AMR1 serves as a negative regulator of this major AsA pathway in plants.

RESULTS

Identification of an AT Mutant with Reduced Leaf AsA

Mutant selection was conducted on lines developed using the pSKI015 AT vector. This vector contains four repeats of the Pro-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 for low and high AsA (see “Materials and Methods”). We identified AT23061 as a low AsA phenotype from approximately 5,000 independent T1 lines. As illustrated in Figure 1A, line AT23061 was consistently observed to be smaller in size compared with wild-type controls of the same age growing under similar conditions and was found to be sensitive to ozone (Fig. 1B).

Total foliar AsA in AT23061 (1.0 μmol g⁻¹ fresh weight) was about 60% less than that in wild-type plants (2.6 μmol g⁻¹ fresh weight) at 3 weeks of age (Fig. 1C). A Southern blot, using the Stratagene pBluescript KS− (KS) plasmid as a probe, showed that the mutant contained two T-DNA insertions (Fig. 1D). Reverse transcription (RT)-PCR analysis (Fig. 1E) indicated that the increased expression of a gene of unknown function was responsible for conferring ozone sensitivity in AT23061 compared with wild-type plants (see identification procedure below).

Identification of AMR1 by Thermal Asymmetrical Interlaced-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 (Fig. 2A). The second insertion was situated in chromosome 1 and 400 bp upstream of At1g65770 (Fig. 2A). At1g65770 was designated AMR1 and has an uninterrupted open reading frame (ORF) of 1,083 bp encoding a novel 360-amino acid protein (Fig. 2B). Domain search
analyses, using SMART (Schultz et al., 1998; Letunic et al., 2004) and Pfam (Bateman et al., 2004) databases, indicated that AMR1 contains an F-box domain situated at amino acids 4 to 50. The F-box motif location, near the N terminus, is typical for this class of proteins, and AMR1 shows sequence homology to several known F-box proteins in humans (SKP2 and FBX4) and Arabidopsis (ORE9, SLEEPY1, FKF1, UFO, and SON1; Fig. 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 AMR1 contains a common plant domain of unknown function (MMEVKSLGDKAPVI\-ATDTCFSVLAHEFYGCLENAYFTDDDT) from amino acids 285 to 332 (DUF295), suggesting that AMR1 may belong to a separate class of proteins with F-box domains. However, several Arabidopsis proteins, whose functions are not yet described, also contain both the F-box and DUF295 motifs, indicating that AMR1-type proteins are not unique.

Insertion Mutants amr1-1 and amr1-2 Have Higher Foliar AsA 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 mutants: lines SALK_081886 (designated as amr1-1) and SALK_113413 (designated as amr1-2), which contained insertions in the predicted At1g65770 ORF. PCR analysis, using T-DNA internal and 3'/5' primer pairs of AMR1, confirmed that the two mutants have insertions in different regions of the AMR1 coding sequence (data not shown) and that each has only one T-DNA insertion. The phenotype of these two knockout lines was very similar when grown in an environmental control chamber, and both had increased resistance to ozone, displaying less foliar injury than wild-type plants when exposed to 450 nL L\(^{-1}\) for 4 h (Fig. 3A). The mutant amr1-1 appeared to be somewhat more ozone tolerant than amr1-2, and this may be a function of a slightly higher foliar AsA content in amr1-1. Total AsA

![Figure 1](image1.png)

Figure 1. The phenotype of the AT mutant AT23061 (ecotype Columbia) has increased sensitivity to ozone, higher foliar AsA, and higher expression of AMR1 than wild-type (WT) plants. A, AT23061 at 3 weeks of age is smaller than wild-type plants but otherwise morphologically similar. B, Leaf injury on AT23061 after ozone treatment at 200 nL L\(^{-1}\) for 4 h was considerably more severe than on wild-type 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 means ± SE (n = 3). FW, Fresh weight. D, DNA gel-blot analysis of the pSK1015 plasmid indicated two insertions in AT23061. Total DNA from AT23061 (4 μg) was digested with EcoRI and hybridized with the whole KS plasmid, digested with EcoRI, as the probe. E, Expression analysis by RT-PCR of AMR1 in AT23061. The AMR1 signal was first normalized to the ΔATPase signal and then compared with the wild-type signal. Relative AMR1 intensities are shown under each lane as the percentage of signal relative to that in the wild type.
no detectable expression of FW, Fresh weight. C, RT-PCR analysis of the insertion mutants indicated rosette leaves of 3-week-old plants. Bars represent means and

Enzymes of the Man/L-Gal AsA Pathway

AMR1 Is a Negative Regulator of Genes Encoding Enzymes of the Man/L-Gal AsA Pathway

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 real-time RT-PCR experiments to address the relationship between AMR1 and AsA metabolism. Steady-state transcripts of GMP (Conklin et al., 1999), VTC2 (Dowdle et al., 2007; Linster et al., 2007), GDP-Man-3′,5′-epimerase (GME; Wolucka and Van Montagu, 2003), GPP (Laing et al., 2004), GalDH (Gatzek et al., 2002), and GLDH (Wheeler et al., 1998), early and late enzymes of the Man/L-Gal pathway to AsA, were up-regulated in the amr1-1 insertion mutant but down-regulated in the AT mutant when compared with wild-type plants (Fig. 4). Therefore, AMR1 seems to be a negative regulator that specifically affects genes/enzymes of the Man/L-Gal pathway (Wheeler et al., 1998). Additionally, we did not observe any effect on the expression of myo-inositol oxygenase (MIOX4; Lorence et al., 2004), the first enzyme of the myo-inositol pathway to AsA.

Expression of AMR1 Is Developmentally and Light Controlled

Both RT-PCR and promoter-reporter gene fusion studies were used to examine the expression patterns of AMR1. RT-PCR analysis of AMR1 transcript levels in wild-type leaves was compared with AsA content during development. AsA was highest in 1-week-old plants (5.4 μmol g⁻¹ fresh weight) and progressively decreased with age to 1.5 μmol g⁻¹ fresh weight in 5-week-old plants (Fig. 5A). Concurrent with the decrease in AsA, expression of AMR1 increased over time, being minimally detectable in 1-week-old seedlings and reaching a maximum, 4-fold greater, in 5-week-old plants (Fig. 5B). This inverse relationship between AsA content and AMR1 gene expression during development further supports the idea that AMR1 is involved in the negative regulation of AsA content.

To examine the spatial expression of AMR1, we fused the GUS reporter gene to a 1-kb segment of the AMR1 promoter and transformed ecotype Columbia (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 conditions (approximately 50 μE m⁻² s⁻¹) and that under high-light conditions (approximately 200 μE m⁻² s⁻¹) at different developmental stages (1, 2, and 3 weeks old). GUS activity was evident in the low-light conditions at all ages (Fig. 6, A, C, and E), although much less staining

Figure 3. Phenotypes of amr1-1 and amr1-2, two T-DNA knockout lines in AMR1, demonstrated increased ozone tolerance and higher foliar AsA levels. A, Leaf injury in wild-type (WT) plants at 3 weeks of age was more severe than in amr1-1 or amr1-2 after ozone exposure at 450 μL L⁻¹ for 4 h. B, Foliar total AsA was about 2-fold higher in amr1-1 and amr1-2 than in the wild type. Tissue was sampled from the second rosette leaves of 3-week-old plants. Bars represent means ± SD (n = 3). FW, Fresh weight. C, RT-PCR analysis of the insertion mutants indicated no detectable expression of AMR1 in amr1-1 and amr1-2 plants.

Figure 4. Expression levels by real-time PCR of genes of the Man/L-Gal biosynthetic pathway to AsA and the MIOX4 gene in the wild type (WT), the amr1 insertion mutant, and the AMR1 AT mutant AT23061. Tested genes are as follows: GMP, VTC2, GPP, VTC5, GME, GalDH, GLDH, and MIOX4. Bars represent means ± SD (n = 3). Asterisks denote results that are significantly different from the wild-type findings (** P < 0.005, * P < 0.05). The statistical significance was evaluated by t test.
was observed in the same-age seedlings under the high-light conditions (Fig. 6, B, D, and 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-d-old seedlings under moderate light (100–150 μE m⁻² s⁻¹), differential staining was apparent depending on leaf age (Fig. 6G). 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 AMR1 promoter, confirmed the developmental and light control of AMR1 expression.

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 ethyl methanesulfonate-treated seeds (Conklin, 1998). T-DNA activation tagging (Weigel et al., 2000) is a highly efficient mutagenesis method, since insertion of the foreign DNA either activates (upstream or downstream of the ORF) or disables (in the ORF) gene expression at a high frequency. We screened approximately 5,000 AT lines and identified 12 mutants with either higher (>150%) or lower (<40%) AsA compared with Col-0. One of these mutants (AT23061), which displayed ozone-induced foliar lesions, had leaf AsA levels about 60% lower than wild-type plants. TAIL-PCR analysis localized a T-DNA insertion in the promoter region of At1g65770, a gene of unknown function. Further in silico analysis demonstrated that the At1g65770 gene product, named AMR1, contains an F-box domain near its N terminus, suggesting that At1g65770 may encode an F-box protein that is associated with the negative regulation of leaf AsA content. The AMR1 F-box domain has high homology with several other F-box proteins in both plants and animals, including ORF9 and UFO, which are known to form SCF (for Skp1-Cullin-F-box) E3 ligases (Woo et al., 2001; Moon et al., 2004; Ni et al., 2004).

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., 2004).
Selective protein degradation by the ubiquitin-proteosome pathway is a powerful regulatory mechanism in a wide variety of cellular processes, including floral development, circadian clock, lateral root formation, light signaling, pollen recognition, and response to plant growth regulators (del Pozo and Estelle, 2000; Lechner et al., 2006). In several of these regulation processes, F-box proteins are identified as negative regulators, such as EBF1 and EBF2, regulators involved in negative regulation in ethylene signaling (Potuschak et al., 2003). Additional examples of negative regulators are SCON2, Fbx13, and FBX2. SCON2 acts within the Neurospora crassa sulfur regulatory network as a negative regulator (Kumar and Piaietta, 1998), while Fbx13 controls the circadian clock by mediating the degradation of CRY proteins that establish a negative feedback loop (Busino et al., 2007). FBX2 was identified as a negative regulator in inorganic phosphate starvation responses (Chen et al., 2008), and in this study, we found AMR1 to be involved in negative regulation of AsA biosynthesis.

Target specificity of F-box proteins is conferred by their C-terminal ends, which usually contain a recognizable protein-protein interaction region in the form of Leu-rich, WD-40, Armadillo, or Kelch repeats (Craig and Tyers, 1999; Andrade et al., 2001; Gagne et al., 2006). The C-terminal region of AMR1 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 AMR1 is unique among known Arabidopsis F-box proteins.

Our data show that AMR1 is involved in modulating the expression of several genes in the Man/L-Gal pathway for AsA biosynthesis. The transcript level of GME was affected the most by AMR1. Although photosynthetic electron transport is suggested to play an important role in the regulation of the transcript levels of genes in the Man/L-Gal pathway, GME was not one of them (Yabuta et al., 2007). GME, a highly conserved enzyme among both dicotyledons and monocotyledons, also forms GPD-L-gulose and L-gulose is a possible precursor of vitamin C biosynthesis in plants. In addition, the detailed biochemical characterization of the GME protein (i.e., regulation redox state of NADH/NADPH) indicates that regulation of this enzyme is relevant for both the Man/L-Gal and gulose pathways (Wolucka and Van Montagu, 2003, 2007; Major et al., 2005; Watanabe et al., 2006). In addition, GME is regulated by jasmonates (Wolucka et al., 2005).

Our experiments also indicate significant increases in transcript levels of VTC2 in the amr1 mutant. It has been recently proposed that VTC2 might be a dual-function protein, with both enzymatic and regulatory functions, as fusion VTC2:fluorescent proteins have been localized in both the cytosol and the nucleus (Müller-Moulé, 2008). The transcripts of GMP, GPP, and GLDH were also affected by AMR1. It was recently demonstrated that the transcript levels of GMP, GPP, VTC2, and GLDH are elevated specifically in response to photosynthetic electron transport (Yabuta et al., 2007). The expression of GMP is also induced by jasmonates (Sasaki-Sekimoto et al., 2005). Transcript levels of GLDH in young rosette leaves of Arabidopsis increase about 2-fold during the day (Tamaoki et al., 2003). The conversion to ascorbate by L-galactono-1,4-lactone is also controlled by photosynthetic electron transport, although not at the transcriptional level (Yabuta et al., 2008). Transcriptional regulation appears to be specific to one portion of the AsA biosynthetic network, since myoinositol oxygenase expression was unaffected by AMR1 transcript levels. It is interesting that most Man/L-Gal pathway genes are single-copy genes in Arabidopsis and that AMR1, therefore, can regulate metabolite flux to AsA efficiently without potential compensatory effects of multiple gene family members.

Because amr1 mutants demonstrated increased levels of AsA, it was of interest to determine whether the loss of AMR1 would result in greater tolerance to ozone. AsA 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, a now classic example being the vtc1-1 Arabidopsis mutant (for review, see Conklin and Barth, 2004). Also, overexpression in tobacco of the AsA-recycling enzyme DHAR increased foliar AsA and conferred increased ozone tolerance (Chen and Gallie, 2005).

AsA in the apoplastic space, where ROS are initially generated, has been considered the primary defense against ozone incursion. However, in two white clover (Trifolium repens) clones, NC-S and NC-R, neither the amount of extracellular or symplastic AsA nor its oxidation state correlated with ozone tolerance, indicating that additional ROS-neutralizing strategies exist in some species (D’Haese et al., 2005). Ozone treatment revealed that the two Arabidopsis insertion mutants, amr1-1 and amr1-2, are indeed more tolerant to this oxidative stress compared with wild-type plants. These results support previous studies showing the relationship between high foliar AsA and ozone tolerance (Conklin et al., 1997; Maddison et al., 2002; Pasqualini et al., 2002; Conklin and Barth, 2004).

Biosynthesis of jasmonates is required for ozone tolerance, and jasmonate signaling regulates the biosynthesis of AsA by activating the expression of GMP and VTC2 (Sasaki-Sekimoto et al., 2005). The mechanism of AsA protection against ozone has not been clearly demonstrated and may involve jasmonate signaling, redox status, modification of 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 senes-
ence (Navabpour et al., 2004). In the AsA-deficient mutant vtc1-1, induction of some senescence-associated genes (SAGs) occurred prematurely (Barth et al., 2004). Furthermore, in the presence of exogenous AsA, expression levels of SAG13 and PRI decreased to wild-type levels. The expression pattern of AMR1 in wild-type plants demonstrated that this gene had highest expression in older leaf tissue, which also had lowest AsA. AMR1, therefore, may function in controlling leaf senescence by depleting AsA levels, and expression studies of SAGs in AMR1 plants could provide support for this hypothesis.

We hypothesize that AMR1 may function as a component of an SCF ubiquitin ligase complex and interact specifically with a positive regulatory protein controlling its quantity and stability through ubiquitination. Additional work is necessary to identify the various components of this complex, especially the target of AMR1, and to explore how the interaction between the target protein and the ubiquitination pathway is involved in the coordinated regulation of several genes in the Man/L-Gal pathway in response to developmental and environmental cues. Such future studies should lead to a better understanding of the regulation of this important molecule.

**MATERIALS AND METHODS**

**Materials and Growth Conditions**

Arabidopsis (Arabidopsis italiana) 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 μmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation. The AT plants containing pSK8105 (Basta resistance) were screened with 0.1% Basta before ozone treatment. The homozygous amr1-1 and amr1-2 mutant lines (lines SALK_088186 and SALK_113413) were identified as segregating lines in T3 seeds provided by the Arabidopsis Biological Resource Center (Alonso et al., 2003). Homozygous lines were developed by kanamycin selection and confirmed by PCR (the specific gene cannot be amplified in a homozygous line due to the T-DNA insertion).

**Ozone Fumigation Screen of AT Mutants**

Four-week-old AT plants were exposed to ozone at concentrations of 150 to 200 nL L$^{-2}$ s$^{-1}$ for 4 h in a continuously stirred tank reactor. Ozone was generated from oxygen by UV discharge (Osmonics) and delivered to the chambers by flow meters. Ozone concentrations in the chambers were monitored with a ozone analyzer (Thermo Electron) and regulated through the flow meters. Damaged plants were selected for analysis to identify lines with reduced AsA levels. Selected mutants were treated with ozone three times to confirm the ozone response.

**Leaf Tissue AsA Measurement**

AsA content of leaves was measured as the AsA oxidase assay (Rao and Ormrod, 1995). Plant extracts were made from tissue frozen in liquid nitrogen, ground in 6% (w/v) meta-phosphoric acid, and centrifuged at 15,000g for 15 min. Reduced AsA was determined by measuring the decrease in A$_{340}$ (extinction coefficient of 14.3 cm$^{-1}$ μmol$^{-1}$) after the addition of 1 unit of AsA oxidase (Sigma) to 1 mL of the reaction mixture containing the plant extract in 100 mM potassium phosphate buffer (pH 6.9). Oxidized AsA was measured by the increase in A$_{340}$ after addition of 1 μL of 0.2 mM dithiothreitol and incubating at room temperature for 15 min. Total AsA is the sum of reduced AsA and oxidized AsA.

**Isolation of Genomic DNA and Southern-Blot Hybridization**

Total Arabidopsis DNA was isolated using the Qagen plant DNA extraction kit. Isolated DNA was digested with the restriction endonuclease EcoRI, loaded 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 and 1.5 M NaCl, pH 8.0, and DNA was immobilized by UV irradiation. The membrane was treated with a prehybridization solution (0.5 M sodium phosphate, pH 7.2, and 7% SDS) for 1 h at 65°C. Gene-specific 32P-labeled KS plasmid, which was cut by EcoRI, 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 probe was allowed to hybridize to its target sequence overnight at 65°C. Nonspecific binding was removed by successive 10-min washes in 2× SSC/0.1% SDS (w/v), 0.2% SSC/0.1% SDS (w/v), and 0.1% SSC/0.1% SDS (w/v; all 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).

**Identification of Genomic DNA Flanking the AT T-DNA**

Identification of the genomic DNA insertion site in the AT23061 lines in Arabidopsis was determined by TAIL-PCR as described (Liu and Whitticker, 1995). Genomic DNA was prepared with a DNA extraction kit (Qagen). Two rounds of PCR amplification were used to isolate the DNA flanking the T-DNA insertion. A total of 15 pmol of the left border T-DNA primer L1 (5'-GAC-AACATTGTGGAGCCACGAGA-3') was used with 150 pmol of the partially degenerate primer AD-2 (5'-NGTGCAGSWSGNNAWGAA-3') for the first PCR. 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) 72°C for 3 min; (8) 94°C for 15 s; (9) 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 12; (18) 72°C for 3 min; and (19) 4°C until needed. DNA produced in the first PCR was diluted 1:50, and 1 μL of this dilution was used for the second round of PCR. A total of 15 pmol of the left border nested primer L2 (5'-TCGACCTGAAATGTAGACGACG)-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) 65°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' -TGGTGAATTTACTTCTTTCTTC-3').

**Gene Expression by RT-PCR and Real-Time PCR**

For expression analysis, approximately 100 mg of fresh leaf tissues was harvested and frozen immediately in liquid nitrogen. Total RNA was extracted with the RNAeasy Plant Mini Kit (Qagen). Crude RNA preparations were treated with 10 units of RNase-free DNase I (Promega) and further purified according to the RNAeasy Plant Mini Kit protocol. For RT-PCR studies, cDNA was synthesized from 1.5 μg of DNA-free RNA template using an oligo(dT) primer and SuperScript Reverse Transcriptase (Ambion). One-tenth volume of each cDNA was used as a template for PCR amplification. To determine whether comparable amounts of RNA had been used, β-ATPase or Actin2 primers were used as control (Kinoshi et al., 1992). PCRs were conducted using the following thermal profile: denaturation at 94°C for 4 min; followed by 25 to 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. The number of PCR cycles for the transcripts investigated was determined by testing between 18 and 30 to the linear range. PCR products were detected by 1% agarose gels infiltrated with ethidium bromide. Real-time PCR was performed on an optical 96-well plate with an ABI PRISM 7500 real-time PCR system (Applied Biosystem). Each reaction contained 12.5 μL of 2× SYBR Green Master Mix reagent (Thermo Fisher Scientific), 1.0 μL of cDNA samples, and 200 μM gene-specific primer in a final volume of 25 μL. The thermal cycle used was as follows: 95°C for 15 min, followed by 40 cycles of 94°C for 10 s, 58°C for 10 s, and 72°C for 10 s. Gene-specific primer pairs were designed with Beacon Designer 7.0 (Premier BioSoft International); for AMRI (AT1g65770), 5'-TTCACCAGAAGGCGAACA- TACC-3' and 5'-CACAACATTCCGCAAGTCTCC-3'; for GMP (AT2g3770), 5'-TTGGTGACAGGAAACCAGCTAC-3' and 5'-TGCCACCCCGATGATACGTG-3';
for VTC2 (At4g28650), 5'-CAATGTTGACGCTAGAATGAGTGG-3' and 5'-TCT ACGCGGTCTGAAATGTGGTCAAG-3'; for GATA (At1g29870), 5'-ACATAGGCC TGAACATCGATGTAATGCC-3' and 5'-AGCCACAACTGCTATTGGATCTT-3'; for VTC5 (At5g55120), 5'-AATGTTGACGCTAGAATGAGTGG-3' and 5'-AGGTTGTCGATGTCTGATGTCG-3'; for GALD (At4g35370), 5'-GGTGGTCGCTGATGTAATGCTG-3' and 5'-GACCAGAACTTCTCTTCTTC-3'; for GLDH (At3g7930), 5'-CAGGATGTGCTGCTATT TACTT-3' and 5'-GACCAGATTTCAACACTTCT-3'; for MOX4 (At4g26260), 5'-ATGAGTCTTGTGATGAGTGG-3' and 5'-TGTCAGAGAGGTTAATCGA-3'; for Actin2 (At3g17870) used as an internal control, 5'-GTA TCCGTCAGCACTTGAACT-3' and 5'-CTGTGCTGAATGCTGACTG-3'. Each PCR was performed in triplicate, and the experiments were repeated three times. Specificity was verified at the end of each PCR run using ABI Prism dissociation curve analysis software and also separating the PCR products by electrophoresis on a 1% agarose gel. Relative expression levels were first normalized to Actin2 expression and then to the respective wild-type controls with the 2ΔΔCt method (Livak and Schmittgen, 2001).

**Gene Reporter Construct and Gus Activity Staining.** The promoter (1 kb upstream of the start codon) of AMRII was cloned from wild-type genomic DNA with primers FBPI-R (5'-CAGCGCGCTATCCACA-3') and FBPN-R (5'-CCATCGCGCATGGTTTCCT-3') and fused with Gus in the binary vector pCAMBIA1301. The fusion construct was transferred into Agrobacterium tumefaciens C58C1 and transformed into plants by the floral dip method (Clough and Bent, 1998). Young seedlings or an entire leaf were incubated in staining solution (50 mM NaPO4, pH 7.0, 1% Triton X-100, 500 μg/ml 5-bromo-4-chloro-3-indolyl-b-glucuronide) by vacuum infiltrating the floral dip method (Clough and Bent, 1998). Young seedlings or an entire leaf were incubated in staining solution (50 mM NaPO4, pH 7.0, 1% Triton X-100, 500 μg/ml 5-bromo-4-chloro-3-indolyl-b-glucuronide) by vacuum infiltrating the floral dip method (Clough and Bent, 1998). When visualizing GUS activity, the fluorescence was observed in the dark.

**Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_105250.**

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**LITERATURE CITED**


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