Introduction

The importance and function of vitamin C

Considered to be one of the most potent reducing agents found in nature, vitamin C protects plants and animals from oxidative damage. The chemical name, ascorbic acid (L-AsA), fittingly comes from the term "anti-scurvy," referring to the disease caused by lack of this vitamin in humans. L-AsA has important roles in the production of collagen and also in neutralizing pollutants in the body. Collagen is the most abundant protein in the human body and is a major component of teeth and gums. It is formed through the activity of prolyl hydroxylase which hydroxylates the proline-lysine repeats in the collagen molecule. L-AsA is considered to be a co-substrate for this enzyme. 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. The production of collagen is only one example of many where L-AsA is required as a cofactor (or co-substrate as in the case of collagen production) for molecules such as ascorbic acid-dependent dioxygenases. In plants, these dioxygenases operate to produce secondary products such as flavonoids and important hormones such as gibberellic acid, ethylene and abscisic acid (Davey et al., 2000; Smirnoff et al., 2001). L-AsA-dependent dioxygenases also operate in animals performing functions such as regulating the synthesis of hydroxyproline-rich proteins (Arrigoni and De Tullio, 2002).

Vitamin C is an important anti-oxidant in humans. It was originally thought to have developed after animals moved to land in response to oxygen's ability to act as an oxidizing agent but recent studies have found ascorbic acid in aquatic organisms (Moreau and Dabrowski, 1998; Arrigoni and De Tullio, 2002). L-AsA helps protect the fat-soluble vitamins A and E as well as protecting fatty acids from oxidation. In addition to oxidative protection, L-AsA also assists in the formation of important messengers in the brain. It has been shown that vitamin C is needed 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., 1985). Additionally, L-AsA causes the conversion of tryptophan to serotonin, a

neurotransmitter that induces vasoconstriction, stimulates smooth muscle contraction, and regulates cyclic body processes (Wagner and Devito, 1987).

L-AsA also serves as an antioxidant in plant systems. It functions to help protect plants from oxidative damage caused by ozone and helps plants survive in high salt environments. It is known that high concentrations of L-AsA are found in chloroplasts, suggesting a significant involvement in protecting plants from photo-oxidative stress (Smirnoff and Pallanca, 1996). There is also evidence that ascorbic acid is used in the production of cell wall components and cell cycle regulation (Arrigoni and De Tullio, 2002).

A number of studies indicate that L-AsA may affect transcription of genes in both animals and plants. Transcription of type IV collagenase is downregulated by low levels of ascorbic acid in cultured human amnion-derived cells. Tyrosine hydroxylase transcription is enhanced by ascorbic acid treatment. Production of mRNA encoding various forms of cytochrome P450 in liver microsomes from guinea pigs is induced by high levels of L-AsA (Arrigoni and De Tullio, 2002). Ascorbic acid also induces transcription of the *fra-1* gene, which encodes a transcription factor of the FOS family that downregulates AP-1 target genes (Catani et al., 2001). Examples of this phenomenon, although studied infrequently, have also been found in plant systems. It has been shown that the maize *hrpg* gene is induced by L-AsA levels in cells (García-Muniz et al., 1998).

Although L-AsA is required by plants and animals, bacteria do not produce it. Some bacteria, such as *Penicillium,* have been shown to contain D-araboascorbic acid, while other organisms, such as *Saccharomyces cerevisiae* and *Candida albicans,* can synthesize the 5-carbon ascorbic acid analogue D-erythroascorbic acid which undergoes the same cleavage process associated with L-AsA to form oxalic acid and D-glyceric acid. (Lee et al., 1999).

Despite the fact that most of the important functions of L-AsA have been discovered in recent years, the importance of this molecule was recognized long before it was understood. Humans, like other primates, along with bats and guinea pigs, cannot produce their own ascorbic acid and must rely on dietary sources for this molecule. During the early days of global exploration, foods rich in L-AsA were often hard to come

by. Because of this, death due to scurvy was not uncommon, particularly while on long sea voyages. It was the Indians of Newfoundland who first taught settlers how to ward off this dreaded disease through the use of a tea made from Spruce needles, which are high in L-AsA.

James Lind performed his famous experiments on scurvy and published his *Essay on the most effectual means of preserving the health of seamen in the Royal Navy* in 1757 (Lind, 1953). While serving aboard the H.M.S. Salisbury, Dr. Lind selected crew members with similar symptoms. He proceeded to divide the men into six sets of pairs, gave each pair different 'treatments' and confirmed the superiority of citrus fruits to ward off the effects of scurvy. Because of this discovery, sailors of the 1700's came to be known as 'limey's.' Soon the importance of vitamin C in protection against scurvy was widely accepted and physicians urged all captains to bring aboard citrus fruits (such as limes) for the men during long trips.

Ascorbic acid was isolated in 1928 by a chemist named Sir Frederick Hopkins. Soon thereafter it was shown that the vital chemical properties of ascorbic acid are related to its structure (Figure 1). The molecule contains four hydroxyl groups at carbon positions 2, 3, 5 and 6. The -OH group in position 3 is acidic, while those in positions 5 and 6 behave as secondary and primary alcoholic residues, respectively (Cabral and Haake, 1988). The proton located at the carbon 3 is what gives ascorbic acid its acidity due to the conjugated ring system.

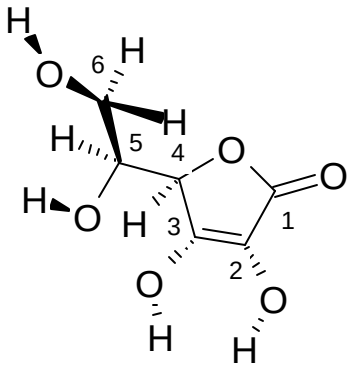


Figure 1. Structure of ascorbic acid. (a) The model of the chemical composition of ascorbic acid (made using ISIS™/Draw 2.4)

It is widely accepted that the ascorbate molecule has the ability to sequester electrons and donate them to molecules such as reactive oxygen species (ROS). By

doing so, ROS molecules are prevented from damaging lipids, nucleic acids and proteins. However, there is also research that suggests that the antioxidant activity of ascorbic acid involves a hydrogen transfer rather than an electron transfer (Cabral and Haake, 1988). The L configuration is the only biologically-active form of the molecule and the radical configuration of L-AsA can only be formed under physiological conditions.

Features of ascorbic acid such as its electroneutrality and its bicyclic configuration promote membrane permeability and may play an integral role in sodium and potassium ion transport by the radical form (Loewus, 1988a). The radical form of ascorbic acid is stable and relatively unreactive. After carrier-mediated transport into the plastids, the oxidation of ascorbic acid occurs when electrons are donated to hydrogen peroxide and monodehydroascorbic acid is formed. This molecule spontaneously dismutates into dehydroascorbic acid (DHA). When ascorbic acid reacts with molecular oxygen, ascorbate is oxidized, producing superoxide and dehydroascorbic acid, the latter being very unstable. The superoxide can then react with the ascorbate ion or its radical. L-AsA must be maintained in its reduced state to function effectively. During degradation, the lactone ring of DHA is broken down and diketo-D-gluconate (DKG) is formed (Koshiishi et al., 1998). This disassembled molecule serves as a precursor to molecules such as L-threonic acid, L-tartaric acid, oxalic acid and possibly glyceric acid (Loewus, 1988b). In animals additional compounds can be formed such as lyxonic acid, xylonic acid and xylose (Banhegyi et al., 1997).

It appears that ascorbic acid biosynthesis in biological systems is under tight control, suggesting that having too much could be as dangerous as not having enough. Transport of L-AsA is efficient because ascorbic acid is produced in the endoplasmic reticulum (ER) of liver cells in animals. Ascorbic acid and DHA are actively transported between animal cells. DHA is preferentially transported across the plasma membrane in plants and animals by the GLUT1 glucose transporter (Vera et al., 1995). All plant cells, in both photosynthetic tissues and non-photosynthetic tissues, have the ability to produce ascorbic acid *de novo*. Transport over large distances is not as vital as in animal cells but still relevant due to the observation that ascorbic acid production is highest in meristematic tissue (Sanchez-Fernandez et al., 1997). Research suggests L-AsA is probably transported from young to old tissues (Arrigoni et al., 1992).

Because humans cannot synthesize ascorbic acid, as do many other animals, significant time and money has been invested in the production of commercially-available forms of ascorbate. Ascorbic acid and its derivatives have been added to lotions, creams, drugs, and cosmetic products with the aim of improving the antioxidant activity. About half of the commercially produced ascorbic acid is used to make vitamin supplements and pharmaceutical products (Hancock and Viola, 2002). The other 50% is used in a variety of products such as make-up and lotions. About 40% is used in food and beverage processing as a preservative which not only increases the nutritional value but also prevents discoloration and protects flavor and aroma (Chauhan, 1998). The remaining 10% is used in animal feed to supplement growth of animals under stress.

The Reichstein process is the method by which most of the world's vitamin C is produced (Figure 2). The product is first isolated in a pre-purification stage using ion-exchange. The mild conditions during isolation result in a product of high purity and thermostability. The product is then further purified through continuous crystallization processes which produces a homogenous product. Since this process requires the use of hazardous chemicals along with high-energy steps, biotechnological approaches include the use of industrial microorganisms (Hancock and Viola, 2002).

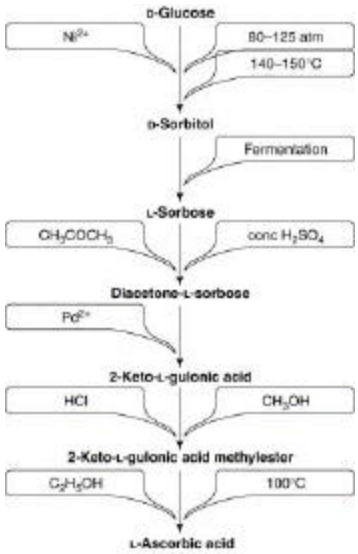


Figure 2. Reichstein process for the production of ascorbic acid (Hancock and Viola, 2002). The process of converting glucose to ascorbic acid on a commercial scale is done by this process. It includes seven steps, one of which is fermentation. Although this is the classical method of vitamin C production in industry, it requires the use of hazardous chemicals and high-energy steps. Consequently, scientists have been looking for new methods to produce this

In the present research, we utilized molecular tools to manipulate the L-AsA content of L-AsA deficient *A. thaliana* plant lines. Genetic engineering of plants has

become a promising tool in the improvement of human health. Genetic manipulation of commonly-consumed plants has already been used as an effective method of increasing certain vital molecules in the human diet. For example, rice endosperm (*Oryza sativa*) has been genetic-engineered to have an increased iron concentration in addition to improved nutrient absorption in the intestine (Lucca et al., 2000).

By first exploring biochemical pathways of model systems in the laboratory, data can be obtained to alter flux through the pathways to optimize levels of vital molecules. Ascorbic acid is present in plant tissues in millimolar concentrations. Depending on the level of stress the plant is under, ascorbic acid concentrations in wild type (WT) *A. thaliana* can range from 2 to 5 µmoles per gram of fresh weight in leaves. Levels in flowers can be twice that in the leaves. By increasing the levels of L-AsA in commonly-consumed plants such as lettuce, the shelf-life of these plants would be extended and their nutritional value increased. Increasing the level of L-AsA in crops would reduce, if not eliminate, the need for additives in animal feed and would also serve to make those crops more resistant to oxidative damage and stress in addition to increasing salt tolerance. To achieve this goal, more needs to be known about the vitamin C biosynthetic pathway in plants. It should be possible to uncover steps in ascorbic acid synthesis that would contribute to better health by using *A. thaliana* as a model system. This information could not only contributes to human health and the health of crops, but would also increases our understanding of the plant biosynthetic pathway(s) for this important molecule.

The L-AsA pathway

The complete L-AsA pathway for animals has been known since 1950. The earliest evidence of the progression of the pathway was acquired through feeding of chloreone-treated albino rats. Uniformly-^{14}C-labeled-D-glucose excreted in uniformly-labeled ascorbic acid in their urine (Jackel et al., 1950) indicated carbons from the original glucose molecule are retained. The animal biosynthetic pathways for L-AsA 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. The pathway proceeds through multiple intermediates, including D-glucuronate and L-gulono-γ-lactone (L-Gul), with an inversion occurring after the formation of glucuronate (Conklin, 1998).

A few years after the animal pathway was elucidated, a comparative study was done with normal rats and cress seedlings. Researchers found that exogenously-supplied D-glucuronolactone, D-galacturonic acid methyl ester, L-Gul and L-galactono-γ-lactone (L-Gal) were all effective in increasing ascorbic acid levels in both organisms. Of these four sugar acid lactones, D-galacturonic acid methyl ester and L-Gal were the best precursors in cress seedlings (Isherwood et al., 1954). This finding provided the first suggestion that two pathways could exist for the production of ascorbic acid in plants. Evidence provided by the Isherwood group also supports the theory that an oxidation pathway using D-glucose-6-phosphate is more likely to occur than one involving the use of free D-glucose to produce ascorbic acid (step 1, Figure 4). These feeding studies led researchers to propose a new, dominant pathway for ascorbic acid production in plants shown in Figure 3. Subsequently, it was found in pea seeds that D-galacturonic acid methyl ester was reduced, presumably to L-Gal, by an NADP-dependent oxidoreductase which had not be previously identified (Mapson and Isherwood, 1956). Only L-Gal was oxidized to ascorbic acid by crude enzyme preparations from plant material (Mapson et al., 1954; Mapson and Breslow, 1958).

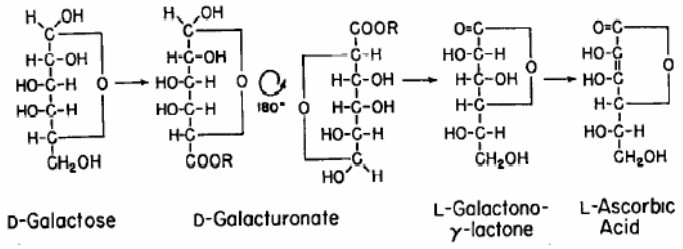


Figure 3. Conversion of D-galactose to L-ascorbic acid in plants (Isherwood et al., 1954). It was originally thought that this was the active pathway in plants for vitamin C production.

A series of studies on the conversion of 1-, 2-, and 6-^{14}C-D-glucose into ascorbic acid in strawberry led to the conclusion that the inversion shown to happen in animals did not occur in the plant pathway (Loewus and Seegmiller, 1956). In the radiolabeled-glucose experiment, the carbon in the 1 position of the glucose molecule became the carbon in the 1 position of ascorbic acid. Berries fed with 2- and 6-^{14}C-D-glucose

redistributed the label into carbon 6 or 1 in about 20% of the cases. Redistributions similar to the ones observed in these feeding experiments have been shown to routinely occur in studies of hexose products in plants given radiolabeled D-glucose (Shibko and Edelman, 1957). Retention of the C1 carbon was observed when this feeding was conducted in cress seedlings (Loewus and Jang, 1957). The carbon 1 in glucose remains the carbon 1 in ascorbic acid in plants. It was later discovered that the redistribution observed by Shibko and Edelman (1957) was due to pentose phosphate metabolism within the plant (Loewus, 1961), the normal process by which glucose is utilized to form precursors for ascorbic acid (Loewus, 1963). This study also provided that there was an oxidation of carbon 1 of D-glucose rather than 6 as is the case in animals.

At this time it was thought that D-galacturonosyl residues of pectin were derived from the same biosynthetic pathway as the D-galacturonic acid intermediate proposed by the Mapson group (Isherwood et al., 1954). Unpublished results obtained by Loewus and Kelly using ^{14}C tracer studies demonstrated that this was not the case. Radiolabeled 6-^{14}C-D-glucose was used to feed strawberries and the product retained the greatest portion of the radioactivity at the carbon 6 position. This portion was oxidized at the carbon 1 and the portion of the glucose that was used in the synthesis of pectin was oxidized at the carbon in position 6. Since the radioactivity was divided equally between D-fructose and D-glucose and the maximum redistribution of the radioactivity was at carbon 1 in all three products, it was assumed that both L-ascorbic acid and pectin are synthesized from a hexose precursor that was formed from the sucrose reserves in the strawberry.

The proposed precursor, D-galactose, was tested to confirm its presence in the biosynthetic pathway for ascorbic acid (Loewus and Jang, 1958). In these experiments, 1-^{14}C-D-galactose was used to feed strawberries. Most of the radioactivity appeared in the carbon 1 position of ascorbic acid, but significant redistribution occurred into carbon 6, similar to what had occurred with D-glucose. From this observation it was concluded that D-galactose is rapidly converted to hexose phosphate and then utilized in a similar fashion as D-glucose, proving the earlier Mapson pathway incorrect. It was proposed that plants possess two pathways for ascorbic acid production. Research then began to focus on the newly obtained evidence of D-glucose conversion into ascorbic acid. Radiolabeling studies were done on cress seedlings (Loewus and Jang, 1957), parsley

leaves and mung bean (Loewus, 1961; Belkhode and Nath, 1962). Similar results were found as in the strawberry experiments.

While some researchers were focusing on the production of L-AsA in plants, other groups were still trying to fully understand the production of ascorbic acid in animals. Rats were injected with 2-^{14}C-D-glucose and were either fed normally, or fasted with or without exercise (Loewus and Kelly, 1961). The resulting ascorbic acid was labeled primarily at carbon 5 and a greater redistribution of the label was noticed in positions other than 2 or 5 in the rats that fasted with and without exercise, indicating that stress affects the labeling pattern of the molecule as well as the amount of ascorbic acid produced.

A better understanding of how sugars are processed in higher plants was developed during the late 1950's. The conversion of D-glucose and D-galactose to D-galacturonosyl residues of pectin was described and the enzymatic steps leading to the oxidation of D-glucose to uridine diphosphate D-glucuronic acid (UDP D-glucuronic acid) were defined (Hassid et al., 1959). The conversion of UDP D-glucuronic acid to compounds such as UDP D-galaturonic acid, D-glucuronic acid and D-galacturonic acid was demonstrated (Hassid et al., 1959).

This new knowledge boosted a new interest in understanding the L-AsA biosynthetic pathway in plants. It was found that 1- and 6-^{14}C-D-glucuronolactone and were effective precursors to L-AsA (Loewus, 1961). The resulting labeling pattern revealed that the carbon 1 of the lactone must be reduced to form L-AsA and it was assumed that the carboxyl group of the uronic acid was conserved in the ascorbic acid product as it is in animal tissues. Despite these findings some researchers still were not convinced that D-glucuronolactone and L-gulonolactone were important precursors to ascorbic acid in higher plants (Loewus, 1963).

In response to the discovery of high levels of D-araboascorbic acid in *Penicillium* molds and the oxidation of D-gluconic acid in citrus fruit vesicle, a new pathway was suggested (Takahashi and Mitsumoto, 1961). This newest pathway started from D-glucose and proceeded through and split between D-glucono-γ-lactone and D-gluconic acid, the final intermediate being D-araboascorbic acid. This theoretical pathway was proven wrong by tracer studies performed in strawberry, in which it was demonstrated

that D-araboascorbic acid is not formed from D-glucose in strawberries (Loewus and Kelly, 1961).

The next non-inversion pathway proposed (Loewus, 1988a) suggested intermediates such as sorbosone and glucosone, although neither substrate was able to increase ascorbic acid levels when supplied exogenously to plants (Conklin et al., 1997). ^{14}C-tracer studies demonstrated that labeled glucosone appeared in a higher percentage of the ascorbic acid than did the labeled glucose. It was surmised that this observation could have been a result of the presence of the unlabeled glucosone which many have inhibited the conversion of glucose to ascorbic acid. An activity that catalyzed the conversion of sorbosone to ascorbic acid has been detected, however no enzyme has been isolated to date (Saito et al., 1990).

This new pathway did not garner much support from the scientific community More attention was given to a pathway including the oxidative step of L-Gal to L-AsA, which was proven to occur in plants through feeding of parsley leaves (Jackson et al., 1961; Baig et al., 1970). The gene encoding the enzyme that performs this conversion, L-galactono-γ-lactone dehydrogenase (GalLD), was later cloned and characterized from a variety of plant tissues (Østergaard et al., 1997b), and proved to significantly increase ascorbic acid levels when overexpressed in transgenic plants (Smirnoff and Pallanca, 1996). Shortly thereafter, the loss of the ^{3}H labeling at carbon 5 proved that there is an epimerization that accounts for the conversion to the L-configuration of the biologically significant form of the molecule (Loewus and Kelly, 1961).

Additional support for the importance of the GalLD enzyme in the biosynthetic pathway for L-AsA was published more recently. Transgenic lines of tobacco (*Nicotiana tabacum* cv. Bright Yellow 2) were generated expressing antisense RNA for *GalLD.* A significant decline in GalLD was noted and the lines were shown to retain only 70% of the WT L-AsA level. The rate of cell growth and division also decreased significantly in comparison to the WT (Tabata et al., 2001).

Barber (1971) solved an additional piece of the puzzle by demonstrating that GDP-D-mannose-3,5-epimerase can produce L-galactose in plants. This activity was later detected in peas (*Pisum sativum*) and *A. thaliana,* as well as in free L-galactose (Wheeler et al., 1998). It was soon discovered that plant extracts were also able to

produce L-Gal and, inturn, L-AsA from GDP-mannose when NAD^+ and cytochrome *c* are supplied (Wheeler et al., 1998).

Conklin *et al.* (1999) have demonstrated the importance of mannose as an intermediate in ascorbic acid biosynthesis in plants. After identifying a line of ascorbic acid-deficient *A. thaliana* mutants (*vtc1*), 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. When the *vtc1* mutant plants were transformed with the WT gene complementary to this region, the ascorbic acid levels increased from a low value back to WT. Additional support for the importance of this enzyme came from analysis of antisense lines *A. thaliana* deficient in the production of GDP-mannose pyrophosphorylase (step 4, Figure 4). A 50-70% decrease in L-AsA was noted as compared to the WT (Keller et al., 1999).

A different, more generally accepted non-inversion pathway has been proposed for L-AsA production in plants (Wheeler et al., 1998). 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 discovered prior to the proposal of the new pathway (Loewus, 1999). Although not all 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. Feeding studies have shown that both L-galactose and D-mannose serve as sufficient precursors to L-AsA synthesis.

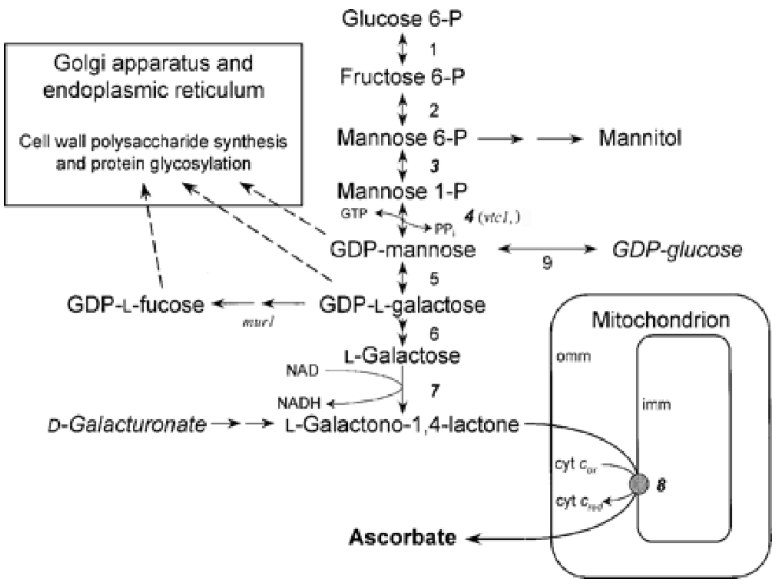


Figure 4. Proposed ascorbic acid biosynthetic pathway for plants (Wheeler et al., 1998; Smirnoff et al., 2001). The diagram shows the currently accepted model of ascorbic acid production in higher plants. A possible alternative pathway to the production of L-galactono-1,4-lactone is shown in italics. The *vtc* 1 mutant (Conklin et al., 1997) and the *mur* 1 mutant (Bonin et al., 1997) are also shown. 1, Hexose-phosphate isomerase (EC 5.3.1.9); 2, Mannose-6-phosphate (Phosphomannose) isomerase (EC 5.3.1.8); 3, Phosphomannomutase (EC 5.4.2.8); 4, Mannose-1-phosphate guanylytransferase (GDP-D-Mannose pyrophophorylase; EC 2.7.7.13); 5, GDP-mannose 3,5-epimerase (EC 5.1.3.18); 6, Sugar-phosphatase (EC 3.1.3.23); 7, L-Galactose 1-dehydrogenase; 8, Galactonolactone dehydrogenase

An important factor in the development of this new pathway was the identification of L-galactose-γ-dehydrogenase (L-GalDH; step 7, Figure 4). It has been identified in both pea and *A. thaliana* and appears to be specific for L-galactose (Wheeler et al., 1998). It has been shown that the enzyme will catalyze the formation of L-AsA from sorbosone at a very slow rate, making it possible that this could be the activity that Loewus purified in an attempt to investigate the presence of sorbosone in the pathway. The importance of L-GalDH was further confirmed in *A. thaliana* plants expressing the antisense form of the gene, in which there was a 70% decrease in L-AsA content relative to WT. The antisense plants also had a higher accumulation of L-galactose when grown under high light conditions, suggesting that the synthesis of L-galactose from GDP-mannose had increased. The L-GalDH enzyme appears to have little effect on the control of the pathway because tobacco overexpressing the gene did not have an increased L-AsA pool. In low light conditions there is not an observable increase in the concentration of L-AsA. It was only in high light that an dramatic increase in the L-AsA content of the tissue could be noted (Gatzek et al., 2002).

Support for alternative pathways including experiments with GLO

Alternatives have been proposed that differ slightly from the Wheeler L-AsA pathway. It was demonstrated that molecules such as L-Gul, methyl-D-galacturonic acid and D-glucurono-γ-lactone, which are not part of the Smirnoff-Wheeler pathway can also serve as precursors for L-AsA production in *A. thaliana*. In addition, D-galacturonic acid methyl ester was found to increase L-AsA production 12-fold over controls in *A. thaliana* plant cell cultures (Davey et al., 1999). A 7-fold increase in L-AsA production has been achieved in lettuce and tobacco by transformation with the gene that encodes the terminal enzyme in the animal biosynthetic pathway for ascorbic acid, L-gulono-γ-lactone oxidase (GLO, E.C. 1.1.3.8; Jain and Nessler, 2000). This is only possible if the precursor L-Gul is present to be converted into L-AsA or if GLO can utilize a different substrate *in vivo* for L-AsA production.

GLO was shown to be the enzyme that oxidizes L-Gul in the final step of the animal L-AsA pathway and was first solubilized from rat liver microsomes (Nakagawa and Asano, 1970) and later isolated from chicken kidney microsomes (Kiuchi et al., 1982) and goat liver (Nishikimi et al., 1976). Vertebrates phylogenetically more complex than fish possess this enzyme. Mammals and some birds have this enzyme in their liver whereas less complex organisms such as primitive birds, reptiles and amphibians have GLO present in the kidney (Kiuchi et al., 1982). It is now known that GLO is localized to the ER in all of these cells (Nandi et al., 1997).

The GLO enzyme is well characterized. The molecular weight of GLO is approximately 50 kD as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Nishikimi et al., 1976; Kiuchi et al., 1982). A flavin adenine dinucleotide is attatched through the methyl group at the C(8) position of the isoalloxazine ring to the N(1) position of a histidyl residue (Kenney et al., 1976; Kiuchi et al., 1982). GLO converts its substrate L-Gul into 2-oxo-L-gulono-γ-lactone. It does this when the flavin accepts two electrons from the substrate and is then oxidized by molecular oxygen (Nishikimi and Yagi, 1991). Hydrogen peroxide is produced in the process and the 2-oxo-L-gulono-γ-lactone molecule then spontaneously converts to L-AsA.

As stated earlier, expression of the rat cDNA has been shown to increase the level of ascorbic acid in lettuce and tobacco plants up to 7-fold (Jain and Nessler, 2000). It was also determined that supplying L-Gul as a precursor to non-transformed plants raised the ascorbic acid levels. Feeding these plants L-Gal (step 8, Figure 4), the terminal precursor in the proposed plant pathway, raised the levels over 40 times, whereas L-Gul, the animal precursor, only increased the content in leaves 25-30% (Jain and Nessler, 2000). Thus, the precursors from the proposed plant pathway prove to be more valuable to the terminal enzyme in a plant system than the animal precursor.

To fully understand the implications of these results, the terminal enzyme in the plant pathway, GalLD, must be revisited. It is known that this enzyme is present in the mitochondrial membranes of higher plants (Mapson et al., 1954; Mutsuda et al., 1995). Some forms can also oxidize very small amounts of L-Gul. It was shown that the enzyme isolated from sweet potato cannot utilized L-Gul. Purified enzymes from cauliflower and spinach are completely specific for the plant substrate because no reduction of cytochrome c, the enzyme's electon acceptor, was observed. It has also been noted that GalLD cannot utilized other electon acceptors such as FAD, NAD, NADP or molecular oxygen (Østergaard et al., 1997b). GalLD falls into the flavin class of enzymes in which the active sites involve electron transport between different prosthetic groups on the enzyme surface (Mapson and Breslow, 1958). It has also been suggested that two distinct isoforms of this enzyme exist in plants (Østergaard et al., 1997a).

Research conducted recently with D-galacturonic acid reductase (GalUR; step 2, Figure 11), a gene isolated from strawberries (*Fragaria*), also brings light to another alternative route to making L-AsA. Researchers cloned and characterized GalUR and discovered its overexpression in *A. thaliana* greatly increases the ascorbic acid level (Agius et al., 2003). GalUR catalyzes the enzymatic reduction of D-galacturonic acid to galactonic acid, which can then be converted into L-Gal by an aldono-lactonase to form L-AsA. Strawberry transformed with the antisense version of the gene contained significantly less L-AsA than the WT, indicating that this pathway makes a significant contribution to the general L-AsA pool.

These facts suggest that either the pathway proposed by Wheeler contains a branch point or that the synthesis of L-Gul is the final intermediate in a second pathway

to L-AsA in plants. Due to the large increase in L-AsA in plants expressing GLO, it is also evident that negative feedback is not playing a role in the newly-proposed secondary pathway. If it were, these levels would not have increased so dramatically.

Data regarding a feedback system in the Smirnoff-Wheeler pathway have been contradictory. In one study, fresh potato tubers were fed glucose. The cells contained high levels of endogenous L-AsA and showed no increase in content during the first 10 days. As the level of L-AsA dropped during storage, the cells regained the ability to produce the compound from the supplied glucose. Since administration of L-Gal increased the levels of L-AsA synthesized dramatically, it was surmised that a negative feedback system was present in the Wheeler pathway upstream of GalLD (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 L-AsA production over a 6-h period (Tabata et al., 2002).

A. thaliana vtc mutants

The *A. thaliana* EMS mutants isolated on the basis of ozone sensitivity and identified to be deficient in L-AsA production (Conklin et al., 1999) are key to understanding the vitamin C pathway in plants. The first and best characterized of these lines, mentioned earlier, is the *vtc1* (formerly *soz1*; *vtc*, *V*itamin *C*) plant line which retains only 30% of the WT level of L-AsA. A radio-labeled D-mannose tracer study and locus mapping were done, along with a GDP-mannose pyrophosphorylase assay and complementation analysis to confirm the activity of the gene product. When the *VTC1* cDNA sequence was compared to that of the *vtc1* mutant line, a single conversion of cytosine to thymine was observed. The resulting point mutation would convert a highly conserved proline to a serine. The remaining high levels of GDP-mannose pyrophosphorylase mRNA suggests that the mutation affects enzyme activity or stability. To confirm that the *VTC1* locus encodes GDP-mannose pyrophosphorylase, a *vtc1* plant line was transformed with the wild type *VTC1*. The L-AsA level in the transformed homozygous line was comparable to that of the wild type. The *VTC1* locus in the

genome encodes for GDP-mannose pyrophosphorylase (step 4; Figure 4), which catalyzes the conversion of D-mannose to GDP-mannose (Conklin et al., 1999).

Other ozone-sensitive mutants were also isolated and shown to be deficient in the production of L-AsA. They were designated as *vtc 1-2, vtc 2-1, vtc 3-1,* and *vtc 4-1*. A nitroblue tetrazolium (NBT) assay was used to estimate the levels of L-AsA in leaf tissue of the mutant plant lines. NBT is an electron transfer dye which is reduced by L-AsA, resulting in an insoluble blue-purple colored precipitate. Plants were assayed for the equivalent (NBT+) or reduced (NBT-) of WT levels of L-AsA. Crosses were done between each of the mutant lines and wild type of both Col-0 and Ler ecotypes (Conklin et al., 2000). The crosses between *vtc 1-2, vtc 2-2,* and *vtc 3-1* with the Col-0 WT segregated in a 3:1 ratio of NBT+:NBT- of the F_2 progeny. In contrast, when Col-0 WT was used in crosses with *vtc 2-1,* a higher number of NBT- progeny was produced in the F_2 generation. Regarding *vtc 4-1,* more NBT- progeny resulted. Crosses done between the mutant lines and Ler ecotype resulted in the expected 3:1 ratio of F_2 progeny with all mutant lines (Conklin et al., 2000).

Although reasons for these observed segregation ratios from crosses done using Col-0 ecotype are unclear, the results from the Ler ecotype suggests that the deficiency in these plants is conferred by single monogenic recessive traits (Conklin et al., 2000). The mutant loci were mapped using polymorphic F_2 mapping populations generated from the crosses between the mutants and WT Col-0 *A. thaliana* plants (Conklin et al., 2000). Locations for these loci are shown in Figure 5.

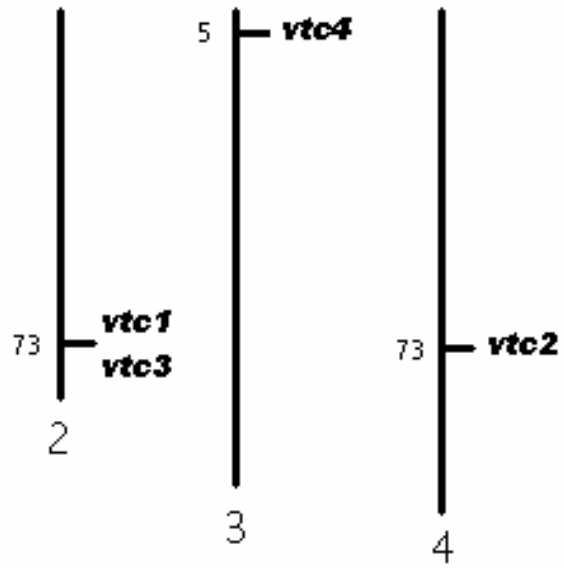


Figure 5. Relative mapped positions of *vtc* mutations in the *A. thaliana* genome (Conklin et al., 2000)**.** Numbers along the left side represent the loci of each mutation in centimorgans on latest recombinant inbred genetic map for *Arabidopsis*. Larger numbers along the bottom represent each chromosome number. This map was created using microsatellite markers on *vtc/vtc* individuals from the F_2 polymorphic mapping populations segregating for *vtc1-1, vtc2-1, vtc3-1,* and *vtc4-1*. The mutations are located at 71.7 for *VTC2, VTC3* is located at 73.01, and *VTC*4 is shown at position 6.83. *VTC1* was previously mapped at 0.9cM centromere distal from CAPS marker m429.

It has been hypothesized that *vtc2* mutation negatively affects a biosynthetic activity in the ascorbic acid pathway which becomes rate-limiting. It is also possible that the *VTC2* locus has a regulatory function that affects the production of L-AsA and has a developmental component (Conklin et al., 2000). Activity levels of phosphomannose mutase (step 3, Figure 4), GDP-D-mannose-3,5-epimerase (step 5, Figure 4), L-galactose dehydrogenase (step 7; Figure 4) and GalLD (step 8; Figure 4) are identical in *vtc2-1, 3-1,* and *4-1* and to the WT (Smirnoff et al., 2001), leaving only two remaining steps in the Smirnoff-Wheeler pathway. Although it has not been proven to date, it is hypothesized that the *VTC2* and *4* loci could be involved in the conversion of GDP-L-galactose to L-galactose (step 6, Figure 4) and/or cofactors that regulate the sugar phosphatase. *VTC2* has been cloned but the sequence reveals a gene of unknown function (Smirnoff et al., 2001). Research is currently being conducted to help us understand the altered L-AsA pathway in these mutants.

In this study WT and ascorbic acid-deficient *Arabidopsis thaliana* lines supplied by Dr. Patricia Conklin were transformed with the *glo* cDNA. Homozygous lines of these transformants were generated and the ascorbic acid levels were compared to the non-transformed WT and mutant plants. Although the WT plants containing *glo* did not show a significant increase in ascorbic acid production, all five of the *vtc* mutant lines constitutively expressing the *glo* cDNA had an increased ascorbic acid content relative to WT level. These data suggest that an alternative pathway is present in plants that does not require the steps in the Smirnoff-Wheeler (1998) pathway to produce L-AsA.

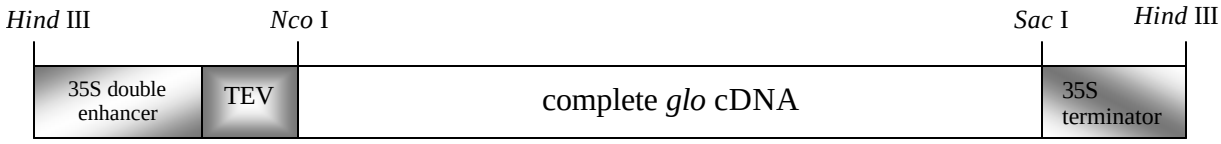
Materials and Methods

Transformation of *A. thaliana* plants

A. thaliana seeds were planted in Sunshine Mix #2 soil (Wetsel Inc., Harrisonburg, VA) and grown for transformation (Clough and Bent, 1998) in a room with a 16-h photoperiod at 30% humidity. Plants were grown for approximately 4 weeks before the unopened inflorescences were removed. This induced the plant to produce more buds and increased the overall number of unopened flower buds for transformation two days later.

Agrobacterium tumefaciens strain GV3101 was transformed via heat shock with the vector pBIN19 containing the *glo* gene construct (Jain and Nessler, 2000). A 3 mL cell culture was grown to an OD_{600} of approximately 0.6 AU in YEP media containing 100 mg/L rifampicin (Sigma, St. Louis, MO) and 50 mg/L gentamicin (Fluka BioChemika, Basal, Switzerland) to select for the *Agrobacterium tumefaciens* strain GV3101. Cells were pelleted at 3000 x g for 5 minutes and resuspended in 100 µl of ice-cold 20 mM $CaCl_2$ (Fisher Scientific, Fairlawn, NJ). Approximately 100 ng of plasmid DNA was added to the suspension and immediately frozen in liquid nitrogen. The cells were thawed in a 37°C dry bath for 5 minutes. Following this heat shock, 400 µl of YEP medium was added to the cell and the culture recovered for 4.5 hours in a 28°C shaking incubator. The YEP media contained 2% (w/v) BACTO™peptone (Becton Dickinson, Sparks, MD), 1% (w/v) yeast extract (USB, Cleveland, OH), and 85.5 mM NaCl (Fisher Scientific), pH 7.0. Cells were plated on YEP medium containing 50 mg/L kanamycin (Agri-Bio, N. Miami, FL), 100 mg/L rifampicin and 50 mg/L gentamicin to select for transformed cells.

Figure 6. The *glo* construct (Jain and Nessler, 2000). The gene construct was made and used to transform both WT and mutant *A. thaliana* Col-0 plants. The *glo* cDNA gene fragment was kindly provided by Dr. Nishikimi (Koshizaka et al., 1988) in a truncated form and was extended to the full length form using very long PCR primers. The gene was placed into pRTL2 where it was placed between the 35S double enhancer with the TEV leader and the 35S terminator (Jain and Nessler, 2000). The entire construct was then put into the binary vector pBIN19 and transformed into *Agrobacterium* strain GV3101 so it could be introduced into *A. thaliana*.



A 500 mL culture of the transformed cells was grown for 48 h in YEP media under the same selection as stated above. When the OD_{600} of the cell culture reached 2.2 AU, the cultures were centrifuged at 3,200 x g for 30 minutes and resuspended to an OD_{600} of 0.8 AU in a solution containing 5% sucrose (Fisher Scientific) and 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX). The volume of the cell suspension was approximately 1 L. The above-ground parts of each pot were dipped into the *Agrobacterium* suspension at room temperature for approximately 15 minutes. The pots were set on their sides and covered for 24 hours, then grown under the conditions stated previously to maturity.

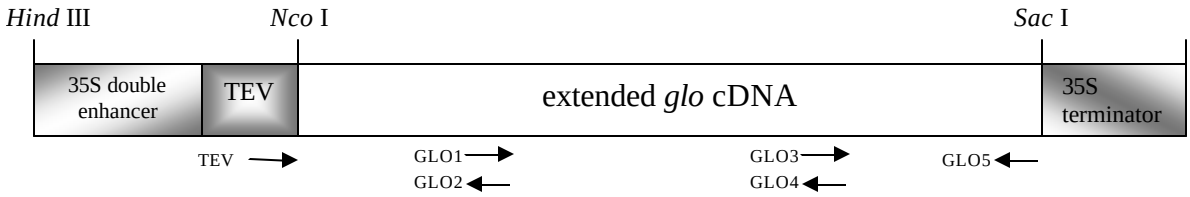
The seeds from these transformed plants were harvested and planted on MS medium containing 100 mg/L kanamycin and 500 mg/L carbenicillin (Agri-Bio; Figure 7). Kanamycin was used to select for plants containing the plasmid of interest and carbenicillin was used to prevent the growth of residual *Agrobacterium*. MS medium contains 1X Murashige and Skoog salt and vitamin mixture (Murashige and Skoog, 1962), 30% (w/v) sucrose (Fisher Scientific), and 1.6 mM $MgSO_4$ (Fisher Scientific), pH 5.8. This same process was followed to transform and generate transgenic lines of each of the *vtc* mutants supplied by Dr. Patricia Conklin.

Preliminary large-scale screening of transformants by PCR

Numerous PCR reactions were performed using various combinations of six primers (Figure 8), along with various PCR conditions to obtain optimal conditions for the various primers. GLO1 (5'CGGCATCCTGGCCACTCAGGTG3') and GLO2

(5'CACCTGAGTGGCCAGGATGCCG3') were the first primers on the 5' ends of the cDNA homologous to the internal sequence of the cDNA. They were reverse complements of each other and were situated approximately 500 bp into the cDNA sequence. GLO5 (3'TTAGTAGAAGACTTTCTCCAGG3') was a 3' primer homologous to the very end of the gene. Primers GLO3 (5'GCCATGCCTCGTGGGCTGG3') and GLO4 (5'CCAGCCCACGAGGCATGGC3') were a combination of internal primers that were reverse complements of each other situated approximately 500 bp from the end of the gene. A primer homologous to the end of the TEV leader (5'CAAACGAATCTCAAGCAATCAAG3') had been developed prior to this research (Lorence, A., personal communication).

Figure 7. PCR primers. Five primers were developed to amplify regions of the *glo* gene to confirm transformation of the plant lines. The 3' primers were GLO2, GLO4 and GLO5. The 5' primers used were GLO1 and GLO3. They were arranged to be used for seauencing the cDNA and for large screens in PCR reactions.



The PCRs included an initial denaturation step for 3 minutes at 94°C. The cycling parameters were as follows: denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. This cycle was repeated 35 times. The last cycle included a final extension step at 72°C for 10 minutes. Various annealing temperatures were tested between 50°C and 65°C in an attempt to optimize the PCR conditions. Primers were also designed to amplify the kanamycin resistance gene in the plasmid (*nptII* gene). The sequence used to develop primers was kindly supplied by Dr. Fabricio Medina-Bolivar (Virginia Tech, Blacksburg, VA). Amplification of the *nptII* gene would indirectly confirm transformation because the antibiotic resistance gene would have been integrated into the plant genome along with the *glo* transgene. Cycling parameters as stated above were used with an annealing temperature of 45°C.

DNA for this screening process was obtained by using the Extract-N-Amp™ Plant PCR kit (Sigma) according to the supplied protocol. A paper hole-punch was used to cut a disc of leaf tissue from a young *A. thaliana* plant. The disc was carefully collected using forceps and placed into a 2.0 mL centrifuge tube. The hole punch and forceps were rinsed with 70% ethanol between samples, as suggested by the published protocol. One hundred microliters of extraction solution was added to the leaf disc and the tube was placed at 95°C for 10 minutes. One hundred microliters of dilution solution was added and the sample was used directly in a PCR reaction.

Generation of GLO transformed lines and genomic DNA isolation

Several independent generations of lines were grown as stated above. Plants grown to propagate lines were germinated on selective MS medium described above containing 200 mg/L kanamycin to select for transformed plants. The plants were transplanted to soil after 2-4 weeks. Plants were grown to maturity under 24-h photoperiods in a Percival growth chamber at 25°C and seed was collected. Each line was propagated until the third generation to identify homozygous lines.

Approximately 25 seeds of each line were planted on selective MS medium. Germination of all seeds indicated the line was homozygous. Once the third generation of plants was reached, DNA from each plant line was isolated using the DNeasy Plant DNA isolation kit using the protocol supplied (Qiagen, Valencia, CA). Tissue for the DNA isolation protocol was taken when the plants had grown for 2 weeks to 1 month. When samples were taken of lines that had been growing for less than three weeks, roots, in addition to photosynthetic tissue, were generally included.

Isolated DNA was further purified by ethanol precipitation. The total volume of DNA samples was dried in a Speed-Vac to an approximate volume of 200 µl. Forty microliters of 10 M ammonium acetate (Fisher Scientific) and 400 µl of ice-cold ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY) were added to the sample. The samples were mixed and centrifuged at 10,000 x g for 7 minutes and the supernatant was discarded. The pellet was washed with 70% and 100% ethanol, then dried in a Speed-

Vac for approximately 5 minutes to remove all residual ethanol. Samples were resuspeneded in 100 µl of dH_2O and DNA concentrations were obtained using a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). These DNA samples were used for DNA gel blotting.

DNA gel blotting

Between 2 and 3 µg of DNA from each plant line, extracted as described above, was digested with the restriction enzyme *Xho*I (Promega, Madison, WI), which does not cut within the *glo* construct. The digested DNA was separated on a 1% agarose gel. The positive control used for the first blots was a WT genomic DNA sample combined with approxiately 25 ng of plasmid DNA purified from the bacteria used to transform the plants. Later blots used a positive control that was a genomic sample from a plant line shown to contain the gene of interest.

DNA was blotted onto a Hybond-N+ charged nylon membrane (Amersham Biosciences, Piscataway, NJ) using a protocol adapted from a previously published procedure (Sambrook and Russell, 2001). Briefly, the gel was soaked in several volumes of alkaline transfer buffer [0.4 M NaOH (Sigma) and 1 M NaCl] with gentle agitation from a rotary platform for 15 minutes at room temperature. The solution was changed and the gels were soaked for an additional 20 minutes. The Hybond-N+ charge nylon membrane was cut to the appropriate size (equal to the gel) and wet with deionized water. It was then soaked in the alkaline transfer buffer for 10 minutes at room temperature.

DNA was transferred from the gel to the nylon membrane by capillary action. The tray from the gel box was used as a stand in a Pyrex dish. Two pieces of 3 MM Whatman 3030-392 chromatography paper (Whatman International Ltd., Maidstone, England) were draped over the sides of the tray to act as a wick. In this manner, buffer poured into the pyrex dish traveled up to the bottom of the gel by capillary action. The gel was inverted and place on top of the Whatman paper. The nylon membrane was lined up and placed directly over the gel, followed by two more pieces of Whatman paper cut to the size of the gel and membrane. A stack of paper towels was placed on top of the

Whatman papers to assist in the capillary action. Using this set up, the buffer absorbed by the lower layer of Whatman paper was pulled through the gel, membrane, Whatman paper and paper towel, causing the DNA to be transferred from the gel to the membrane. A glass plate was placed on top of the paper towel stack to allow the addition of a 400-g weight to add pressure to the stack. The apparatus was left overnight at room temperature. When the transfer was complete, the set up was disassembled and the membrane was soaked in neutralization buffer II [0.5 M Tris-HCl [pH 7.2] (Sigma) and 1 M NaCl] for 15 minutes at room temperature. To immobilize the DNA, the membrane was irradiated at 254 nm in a Spectrolinker XL-1000 UV crosslinker (Spectronics Corp., Westbury, New York) at 1200 x 100 $\mu J/cm^2$. Membranes were wrapped in aluminum foil and stored at –20°C for no longer than one month.

Membrane hybridization

The probe used to hybridize to the DNA gel blot was made using the truncated *glo* cDNA (Koshizaka et al., 1988). The fragment was cut from the pUC19 vector using the restriction enzyme *Eco*RI (Promega). The radioactive probe was made using the Prime-It® Random Primer Labeling Kit (Stratagene, La Jolla, California) and ^{32}P-labeled dCTP (PerkinElmer Life Sciences, Boston, MA) with 40 ng of the DNA insert. After labeling according to the manufacturer's protocol, the radioactive DNA was separated from unincorporated nucleotides using a Sephadex G-25 TE spin column (Millipore, Bedford, Massachusetts). Approximately 0.75 mL of TE buffer [pH 8.0] was added to the column. The column was allowed to hydrate for 30 minutes, then centrifuged for 1 minute at 700 x g to remove excess buffer. The total volume of the random labeling reaction was added to the column and spun for 4 minutes at 700 x g. The column was washed with 200 µl of additional TE buffer to fully elute the probe. Half of the total volume of eluate (approximately 100 µl) was used to probe each membrane.

Membranes were pre-hybridized at 65°C with 15 mL of hybridization buffer and 300 µl of salmon testes DNA (Sigma). The hybridization buffer contained 5X Denhardt's reagent (Denhardt, 1966; Sambrook and Russell, 2001) [0.01%(w/v) Ficoll (Sigma), 0.01%(w/v) Polyvinylpyrrolidone (Sigma) and 0.01%(w/v) acetylated BSA

(Fisher Scientific)] with 5X SSC buffer (Sigma) and 0.5% sodium dodecyl sulfate (SDS; Sigma). The membranes were pre-hybridized for at least two hours prior to addition of the probe. After blocking, pre-hybridization buffer was removed and fresh buffer was added. The probe was added directly to the buffer in the tube (35 x 150 mm, Fisher Scientific) and remained rotating in the hybridization oven (National Lab Net, Edison, NJ) at 65°C overnight. The next day, membranes were washed twice with 1X SSC and 0.1% SDS in the tubes for 15 minutes. The membranes were then washed once in the bottle and twice in a pyrex dish for 15 minutes using 0.1X SSC and 0.1% SDS. Kodak X-Omat AR-5 Scientific Imaging Film (Eastman Kodak Co., Rochester, NY) was used to visualize radioactive areas on the membranes. The membranes were exposed from 1 to 8 days depending on the level of signal to ensure all radioactive signal was detected.

Assay of L-AsA content in plant tissue

NBT assay.

Prior to developing homozygous lines of the plants containing the *glo* gene, a nitro blue tetrazolium (NBT; Sigma) assay was used to determine whether the plant lines had high levels of L-AsA compared to non-tranformed lines. This assay was developed by Dr. Patricia Conklin while studying the *vtc* mutants (Conklin et al., 1999). *A. thaliana* leaves <10 mm in length were removed from each plant. Individual leaves were pressed onto Whatman chromatography paper with a curved metal spatula. Approximately 10 µl of a 1 mg/ml aqueous NBT solution (about 1.2 nmoles) was pipetted onto the imprint of the leaf on the chromatography paper. After approximately 7 minutes, a formazan precipitate formed a purple halo around each extract according to the amount of L-AsA diffused from the pressed leaf.

L-AsA spectrophotometric assay.

The ascorbic acid assay was adapted by Dr. Boris I. Chevone (Luwe et al., 1993). Leaf tissue was harvested from 3-week-old *A. thaliana* plants. Plants used in this assay

were grown under greenhouse conditions with approximately 14-h photoperiods at 25°C and 30% RH. Between 100-500 µg of rosette leaf tissue were frozen in liquid nitrogen and ground using a mortar and pestle. The ground tissue was transferred to a 2-mL microcentrifuge tube. Without allowing the tissue to thaw, approximately 100 µg of tissue was transferred to a 2.0-mL tube containing 0.75 mL of a freshly prepared 5% glacial *m*-phosphoric acid (Fisher Scientific) solution to prevent oxidation of the ascorbic acid in the tissue. The samples were further ground using a PowerGen 125 homogenizer equipped with an Omni Tip Generator Probe (Fisher Scientific) for 30 seconds. The samples were centrifuged at 12,000 x g for 5 minutes. The supernatant was transferred to a clean centrifuge tube and centrifuged at 12,000 x g for 2 minutes. The supernatant was transferred to a clean centrifuge tube and used to assay ascorbic acid.

The SmartSpec 3000 was zeroed with a solution containing 0.95 mL phosphate buffer and 50 µL 5% *m*-phosphoric acid. A control reaction was performed by adding 1 µL of a 35 mM solution of L-ascorbic acid (Sigma) made in 5% *m*-phosphoric acid to 0.95 mL of phosphate buffer [45 mM KH_2PO_4 (Fisher Scientific) and 55 mM K_2HPO_4 (Fisher Scientific), pH 6.8]. This mixture was read and then oxidized using 1U of ascorbate oxidase (Sigma) to confirm that the enzyme was working efficiently. The spectrophotometric readings decreased from 0.5-0.0 AU within 30 seconds.

Fifty microliters of the prepared sample were mixed with 0.95 mL of phosphate buffer in a 1 mL quartz cuvette (Bio-Rad Labs, Hercules, CA). One unit of ascorbate oxidase was added to the prepared sample. The cuvette was covered with parafilm and inverted 2-3 times. Samples were measured on the SmartSpec at 265 nm. Repeat readings were taken after about 2 minutes until the values stabilized. The cuvette was rinsed twice with dH_20 between samples.

Fifty microliters of prepared sample were mixed with 0.95 mL of phosphate buffer in a 1 mL quartz cuvette. One microliter of a 26 mM solution of DL-Dithiothreitol (Sigma) was added to the sample to convert oxidized L-AsA to the reduced form. The cuvette was covered with parafilm and inverted 2-3 times. Repeat readings were taken after about 10 minutes until the values stabilized. The cuvette was rinsed twice with dH_20 between samples. The following equation was used to estimate the total L-AsA in the plant tissue.

[(Δ in absorbance) x (20) x (0.75 mL of *m*-phosphoric acid)] / (14.3 extinction coefficient)

This equation gives the micromoles of ascorbic acid per gram of fresh weight (g FWT). These calculated values are shows in Table 1 (a-f).

Statistical analysis was done using a student *t*-test. The mean of four values from each transformed line was compared to the mean of four non-transformed control lines and the line transformed with pBI121. The standard deviation of each plant line was generated using Microsoft® Excel.

Results

Transformation of *A. thaliana* with the *glo* transgene and media selection

A. thaliana transformation by the floral dip method was successful, with an efficiency rate of approximately 1%. This percentage was slightly lower for the transformation of mutant line *vtc3-1*. In some cases, offspring from one line grown on medium containing kanamycin displayed different levels of resistance. A plant was considered resistant if it was able to fully germinate and grow roots into the selective medium. If it was not obvious that an individual plant was transformed (i.e. if it was severely stunted or had a poorly developed root system), the seedling was taken from selective medium, the roots were removed and the shoot was placed back on fresh selective medium. If the plant grew and developed new roots, it was considered to carry the kanamycin resistance gene.

Two types of medium were used to determine the optimal growing conditions for the plant lines. Plant lines were originally grown on MSSV medium which consists of minimal nutrient Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with a mixture of vitamins and microelements optimized for *A. thaliana* growth. Selection on this media was not distinct and leaf color was light yellow-green. To better distinguish transformed plants from non-transformed plants, a wide range of kanamycin was tested. The same line was grown on 50 mg/L increments of kanamycin from 50 mg/L to 300 mg/L as well as 400 mg/L. The optimal concentration chosen was 100 mg/L kanamycin. At this concentration, non-transformed plants, both *vtc* mutants and WT *A. thaliana,* did not survive, but lines later shown to be carrying the T-DNA insert did.

After first screening some of the T_0 lines of the WT transformants on MSSV medium containing 100 mg/L kanamycin, the selective media was switched to MS. On the MS media, the plants grew at a faster rate and the leaf color was a deep green. Each time transformed lines were grown on selective media, non-transformed controls were planted to confirm selection and avoid escapes. The negative controls are either the WT or the non-transformed mutant lines. The positive control was a homozygous *A. thaliana* line transformed with *GUS* gene in the pBI121 vector kindly supplied by Dr. Brenda S. J.

Winkel (Virginia Tech, Blacksburg, VA). The pBI121 vector was derived from the pBIN19 vector used in the *glo* gene construct, and thus represents an appropriate control.

Verification of transgene integration

Preliminary Large-scale screening of transformants by PCR

The entire rat *glo* cDNA in the binary vector was sequenced from the middle of the TEV leader to the beginning of the terminator sequence. A set of 5 primers were designed for this purpose. Each primer was homologous to different parts of the published *glo* cDNA sequence (GenBank Accession number NM_022220). The primers were also used for PCR reactions. PCR was initially used to screen the transgenic *A. thaliana* plant lines to confirm the presence of the transgene. By first using a quick and inexpensive method to screen transformed plant lines the mostly likely positives could be selected for analysis by DNA gel blotting. Unfortunately, the results from these reactions were often inconsistent and considered unreliable. It was decided that it would be more efficient and direct to forego this PCR step.

DNA gel blots

To confirm incorporation of the *glo* construct into the genomes of WT and mutant *A. thaliana* plants, a DNA gel blotting protocol was used. Approximately 2 µg of genomic DNA from each putative transformant was digested with the *Xho*I restriction endonuclease. The digested DNA was separated on an agarose gel and blotted onto a positively charged nylon membrane. The radioactive probe made for the hybridization was produced using the truncated EcoRI-EcoRI *glo* fragment obtained from Drs. Nishikimi and Yagi as the template. Membranes were left on film between 1-8 days, depending on the level of signal, in a cassette at -20°C. If the hybridization was inconclusive, membranes were stripped and reprobed. Figure 9 shows a DNA gel blot that was performed on 4 independently transformed lines. Lane 1 is the 1 kb λ DNA ladder (Promega). Lane 2 is the positive control, a WT DNA sample plus 50 ng of

plasmid DNA isolated from the bacterial culture used to transform the plants. By combining the WT genomic DNA sample with the plasmid DNA prior to digestion, one can determine if the WT DNA background affects the hybridization of the homologous DNA. Lane 3 contains the negative control, WT DNA alone. All transformed plant lines considered positive displayed either a distinct band on the autoradiograph (such as lane 4, Figure 9) or a higher molecular weight smear (such as lanes 6 and 7, Figure 9). If most lanes on a blot appeared to have a smear near the high molecular weight area, the membrane was exposed to a new piece of film for a shorter period of time. Plant lines that displayed very faint or indistinct bands on the film were not selected for further analysis.

Selection of homozygous lines containing the *glo* trangene

In order to prevent gene dosage effects, only lines determine to be homozygous were grown in soil for the L-AsA assay. These plant lines demonstrated clear resistance to kanamycin at 100 mg/L (Figure 8) and were determined to be homozygous based on Mendelian ratios of offspring of the T_3 generation grown on selective media. As stated earlier, some lines did not show clear resistance and were screened again after the roots were removed. Homozygous lines of transgenic *vtc3-1* were difficult to develop due to a reduced production of seeds assumed to be a side effect of mutagenesis.

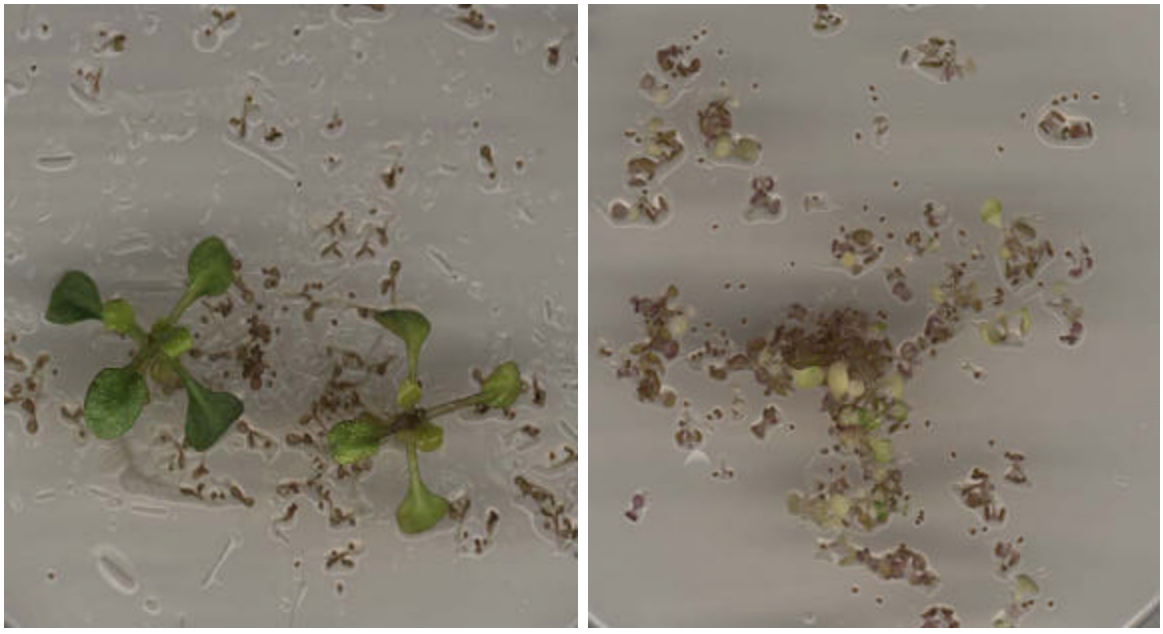
Ascorbic acid levels of *glo* transformed plants

In order to compare physiologically relevant L-AsA values, plants grown under greenhouse conditions were assayed for L-AsA levels. These plants were either planted directly in soil or transplanted from media containing kanamycin or media without antibiotics in the case of the WT and non-transformed *vtc3-1*. The *vtc3-1* mutant and control lines transferred from selective media to soil appeared healthier (e.i. leaves were green on the top surface) then plants germinated directly on soil but had smaller rosette leaves. Approximately 20 seeds were germinated in each pot. Two tissue samples were harvested from each pot and ground. Two pots were sampled for each line.

Approximately 100 µg of rosette leaf tissue was used in the L-AsA assay. The L-AsA values obtained are shown in Table 1 (a-f).

Overall, the largest average increase of L-AsA levels among the plant lines was approximately 25% over WT (Table 1, a). The plants transformed with *glo* did not have statistically significant increases in L-AsA levels, as determined by a *t*-test. However, the trangenic plants appeared to grow taller and matured at a faster rate than the control plant lines. The WT and pBI121 controls contained very similar levels of L-AsA. All lines of *vtc* mutant lines transformed with *glo* showed an increasing trend of L-AsA concentrations when plotted. Only one line of the *vtc3-1* with *glo* had statistically higher levels of L-AsA than the WT and pBI121 controls determined using a *t*-test.

Figure 8. Selection of *A. thaliana* transformants. Seeds of the transformed plants were grown on selective MS media containing 100 mg/L kanamycin to select for the lines containing the *glo* T-DNA construct and 500 mg/L carbenicillin to prevent residual *Agrobacterium* growth after dipping. The image on the left shows two transformed offspring in the T1 generation. The image on the right shows wild type plants grown on selection. Offspring were continuously planted on selection until 100% of seeds of a line grew on the selective media, indicating that the line was homozygous.



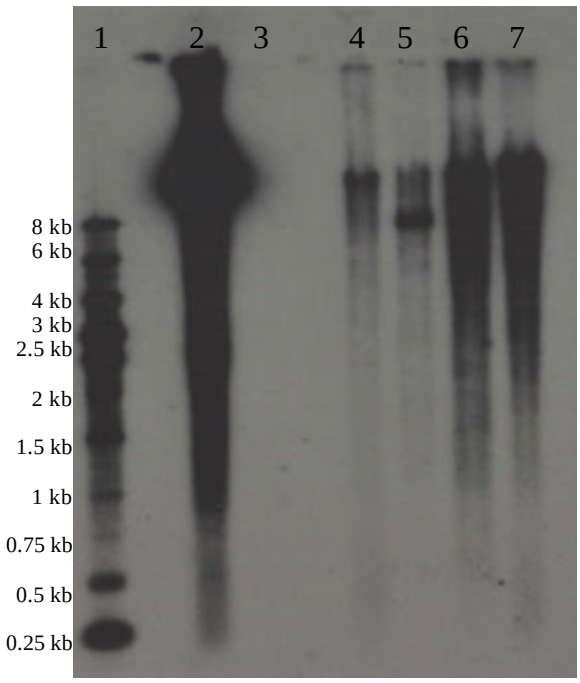


Figure 9. Confirmation of *A. thaliana* transformation. To prove that the kanamycin resistant lines contained the gene construct, DNA gel blots were performed using high stringency procedures (Sambrook and Russell 2001). Genomic DNA was digested with the restriction enzyme *Xho*I and run on a 1% agarose gel. The DNA was blotted from the gel onto a charged nylon membrane and probed with a radioactively labeled *glo* cDNA fragment. Lane one contains the molecular weight marker. Lane 2 holds the positive control which is digested genomic DNA from a wild type line with about 50 ng of plasmid DNA used to transform the plant lines added prior to digestion. Lane 3 is the negative control; digested genomic DNA from the WT line. Lanes 4-7 contain digested genomic DNA from various transformed lines.

Figure 10. NBT (nitroblue tetrazolium) assay (Conklin et al., 2000). Preliminary indication of ascorbic acid levels in the plants was given by this 'squash' test. By pressing a leaf of the test plant on to chromatography paper and pipetting NBT directly onto the leaf, NBT is reduced by the L-AsA diffusing into the paper from the leaf tissue and turns blue-purple. This assay can give a general idea of the amount of L-AsA in the different lines. In the figure below, a faint halo of color can be seen around the WT plant. No halo is seen for the *vtc 2-1* mutant line. The two transformed lines to the right display darker regions of color than both of the control leaves indicating higher levels of L-AsA.

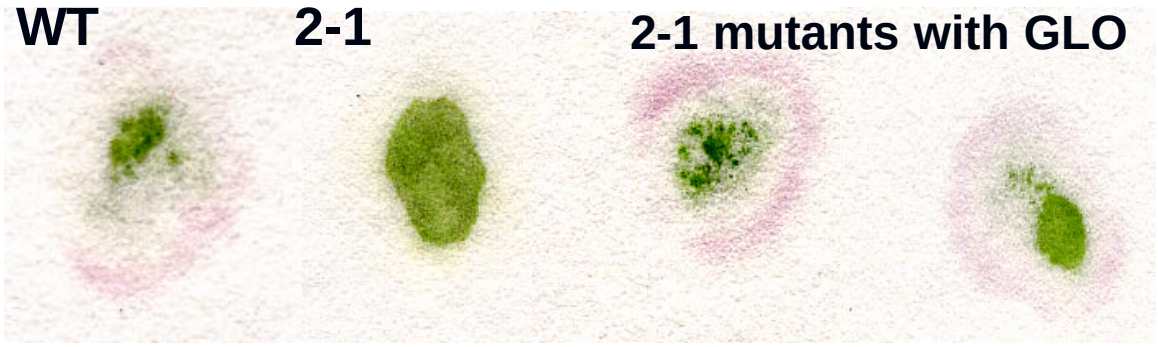
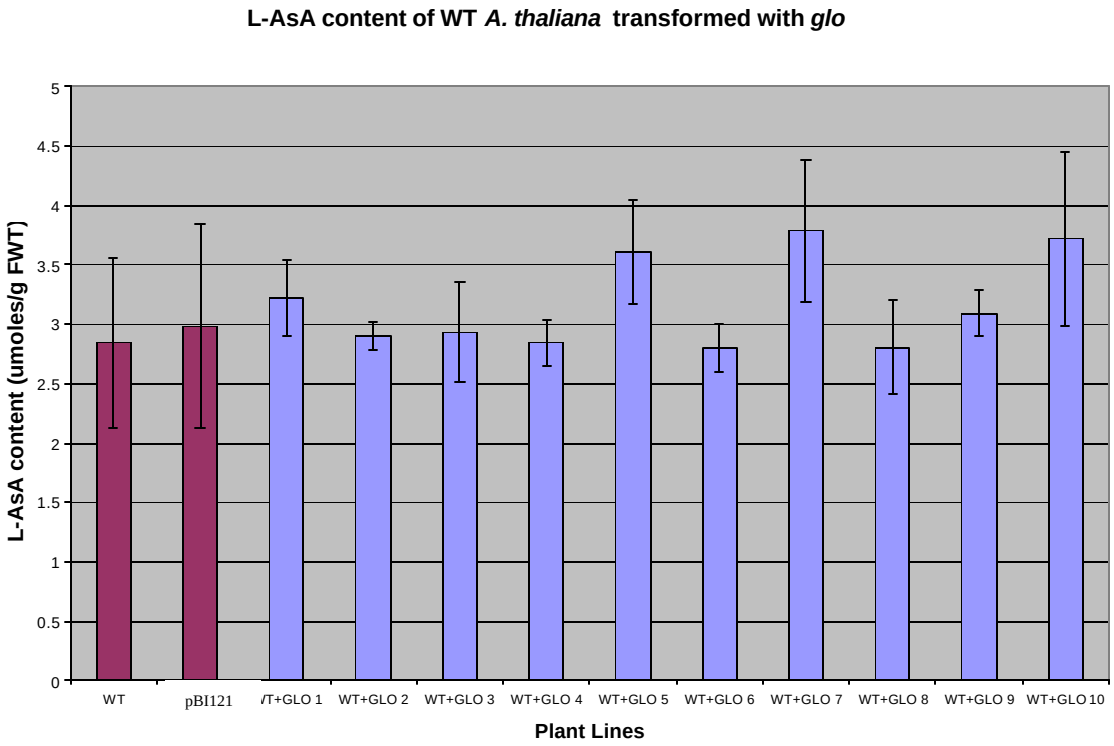
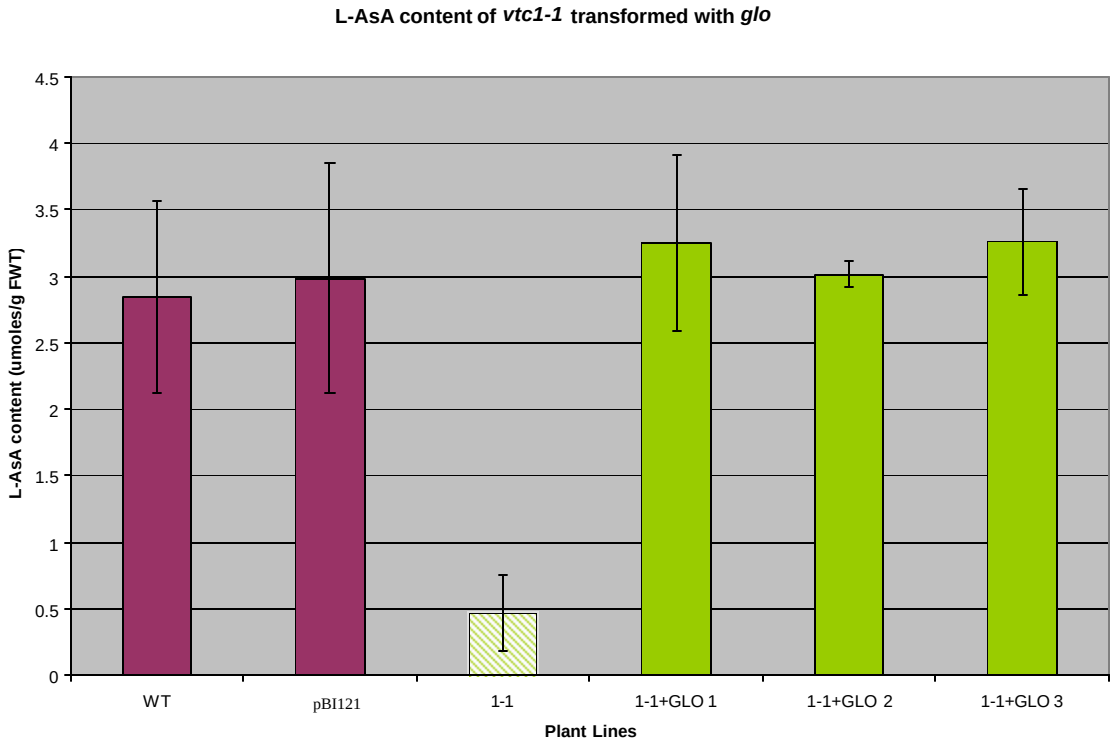


Table 1. L-AsA values of plant lines transformed with GLO. The table below shows the ascorbic acid content of the plant lines assayed. All tissue was collected after being grown for 21 days.
(a) WT plants transformed with *glo*. (b) *vtc1-1* mutant plant lines transformed with *glo*. (c) *vtc2-1* mutant plant lines transformed with *glo*. (d) *vtc2-2* mutant plant lines transformed with *glo*. (e) *vtc3-1* mutant plant lines transformed with *glo*. (f) *vtc4-1* mutant plant lines transformed with *glo*.

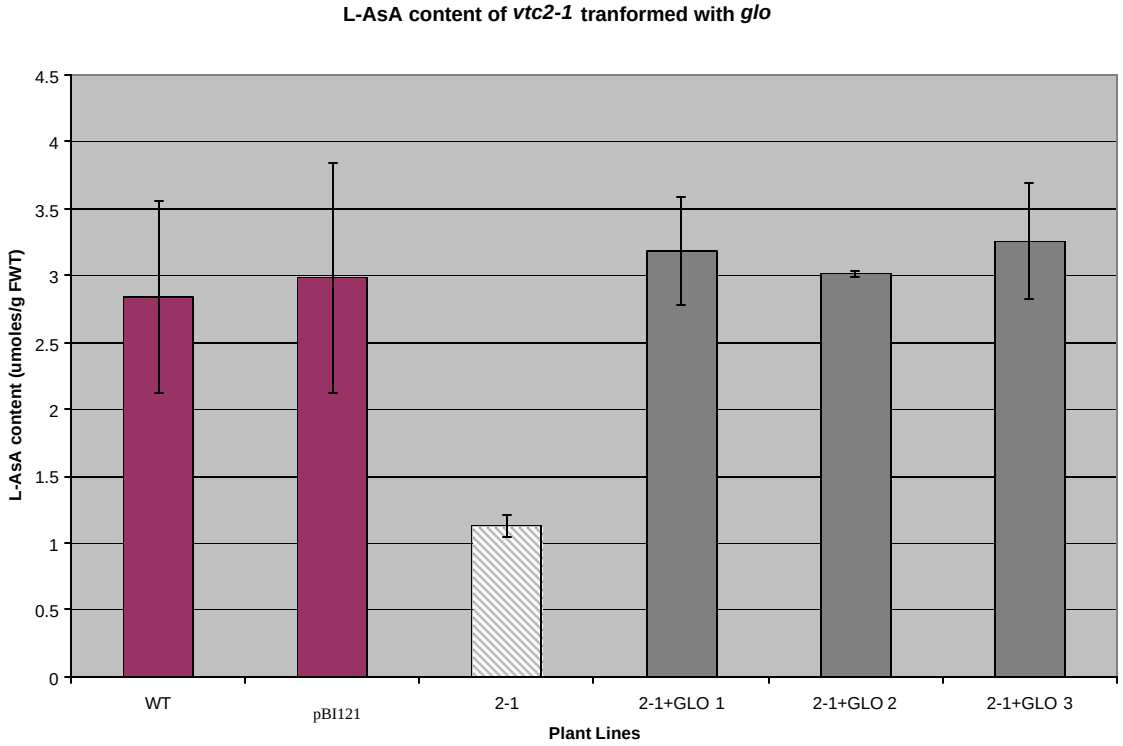
(a)



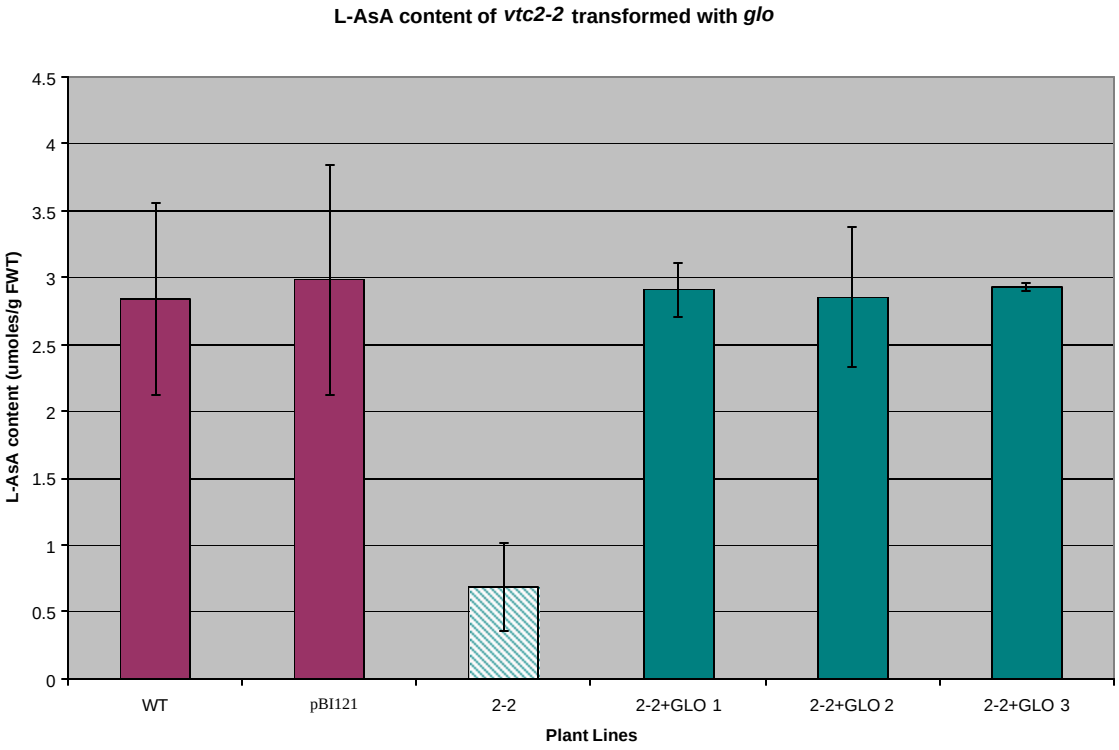
(b)


L-AsA content of vtc1-1 transformed with glo
L-AsA content (umoles/g FWT)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
pBI121
1-1
1-1+GLO 1
1-1+GLO 2
1-1+GLO 3
Plant Lines

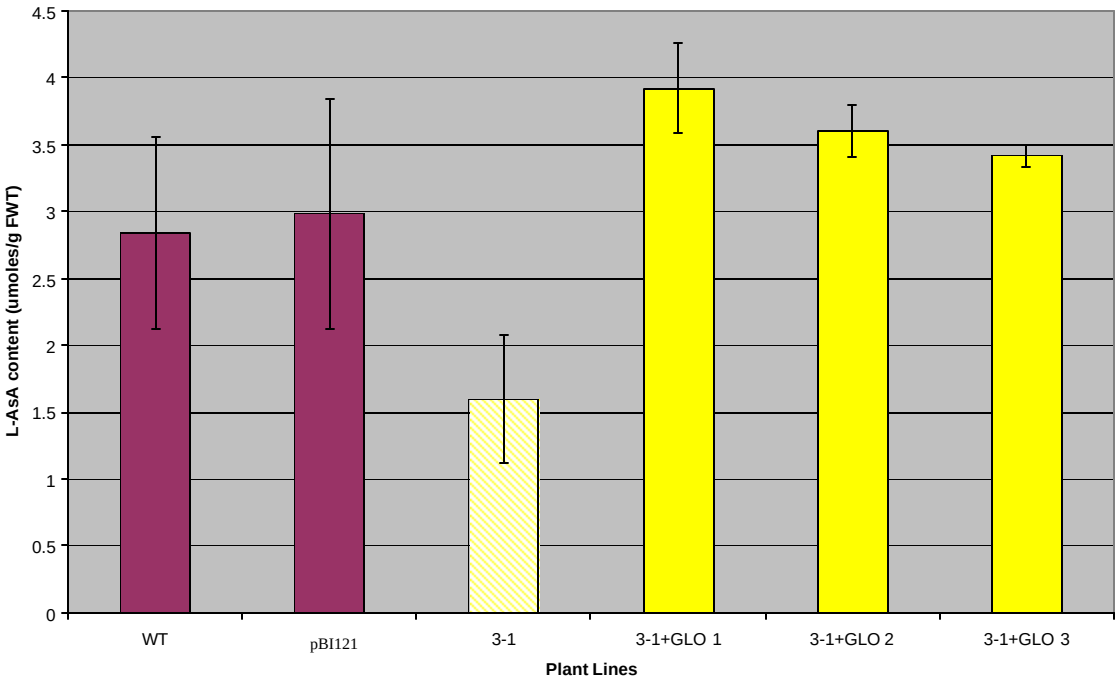
(c)


L-AsA content of vtc2-1 tranformed with glo
L-AsA content (umoles/g FWT)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
pBI121
2-1
2-1+GLO 1
2-1+GLO 2
2-1+GLO 3
Plant Lines

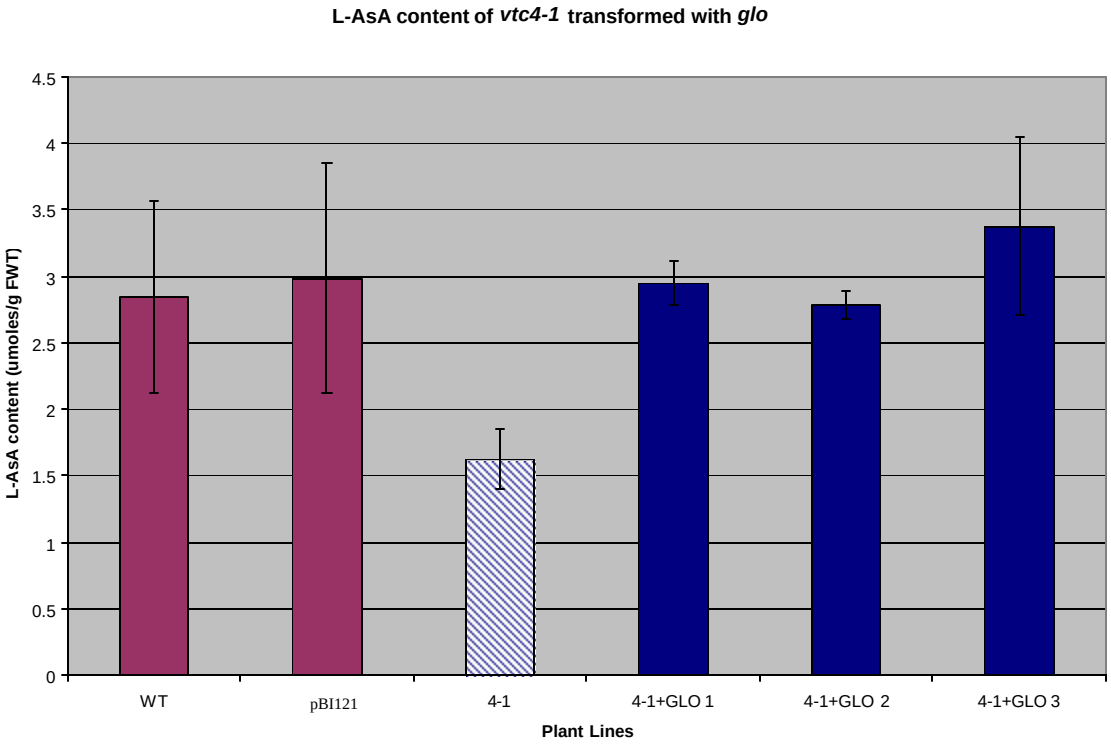
(d)


L-AsA content of vtc2-2 transformed with glo
L-AsA content (umoles/g FWT)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
pBI121
2-2
2-2+GLO 1
2-2+GLO 2
2-2+GLO 3
Plant Lines

(e)


L-AsA content of vtc3-1 tranformed with glo
L-AsA content (umoles/g FWT)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
pBI121
3-1
3-1+GLO 1
3-1+GLO 2
3-1+GLO 3
Plant Lines

(f)


L-AsA content of vtc4-1 transformed with glo
L-AsA content (umoles/g FWT)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
pBI121
4-1
4-1+GLO 1
4-1+GLO 2
4-1+GLO 3
Plant Lines

Discussion

Transformation of *A. thaliana* with the *glo* transgene and media selection

A. thaliana plants were grown on both MS media (as described in Materials and Methods) and MSSV media containing MS medium (Murashige and Skoog, 1962) supplemented with a vitamin mixture for *A. thaliana* growth. The vitamin mixture included 55.5 µM *myo*-inositol, 26.42 µM glycine, 4.06 µM nicotinic acid, 2.43 µM pyridoxine and 0.3 µM thiamine HCl. Plant lines seemed much healthier on the MS media. The leaf color was a deeper green and the plants grew at a faster rate. This could be a result of additional vitamins and nutrients present in the complete MS media. The complete MS mixture used contains more than three times the sucrose in MSSV and nearly twice the amount of cobalt chloride ($CoCl_2$) and cupric sulfate ($CuCO_4$). Plants require copper for production of proteins and reproduction. Cobalt is used in nitrogen fixation, but probably not essential. The complete media also contains over 60 times more thiamine-HCl (vitamin B1), which is important for growth (Murashige and Skoog, 1962). In addition to 100 times more nicotinic acid, necessary for NAD and NADP, the complete media also has 200 times more pyridoxine·HCl (vitamin B6), which helps to breakdown and synthesize amino acids. The most probable explanation for the increased growth rate and healthier tissue on the complete MS media is due to the increased sucrose content.

Some lines grown on MS media containing 100 mg/L kanamycin were not clearly resistant to the antibiotic, yet they did not quickly die and display the phenotype of a non-resistant plant. Most non-transformed plants developed 2 cotyledons then turn white a few days after germination. Some transformed plants were screened for a second time by root removal, as described in the results section, and developed short roots. These plants were considered to be transformed but assumed to have a low level of expression of the kanamycin resistance gene. The *vtc2-1* mutants segregated on kanamycin prior to transformation with the *glo* gene, indicating the presence of a resistance gene or a mutation that rendered them relatively kanamycin insensitive. Mutant lines *vtc1-1* and *2-2* containing the *glo* construct grew considerably faster under controlled conditions than did the non-transformed mutant lines.

To accurately assess the increase of L-AsA in stable transformants, it was necessary to isolate homozygous lines. Plant lines were propagated until the T_2 generation because it is at this stage that lines have segregated. Homozygous lines were chosen based on the germination ratio of the offspring. If all offspring of a particular line successfully germinated on the MS media containing 100 mg/L of kanamycin, the parent lines (and subsequent lines) were considered to be homozygous. Development of homozygous lines allowed us to investigate the outcomes of maximum gene expression, normalize gene dosage, and propagate the lines without continuous antibiotic selection.

Confirmation of transformation

Large scale screening by PCR

Six primers were designed to sequence the construct and for PCR reactions (Figure 7). The sequence of the gene was confirmed and the primers were proven effective for sequencing purposes. In an attempt to limit the number of DNA blots needed to validate transformed lines, PCR conditions were developed to quickly screen putative transformants. Unfortunately, the results of both the *glo* and *nptII* PCR reactions were very inconsistent and unreliable. It is not clear as to whether the inconsistency was due to the method of DNA isolation or due to the primers themselves. Therefore, plants were screened by DNA blot analysis in lieu of PCR screening.

DNA gel blots

The DNA gel blotting experiment proved to be the most reliable method for identifying transgenic *A. thaliana* plants. The blots were performed to confirm the presence of the *glo* gene as it was integrated into the genome of the *A. thaliana* plant lines. Because the probe used was completely homologous to most of the gene inserted into the plant line, background hybridization was extremely low. High stringency washes were used to further ensure low background hybridization. As is evident, no hybridization took place with the WT DNA sample. This indicates that nothing in the *A.*

thaliana WT genome is homologous to the probe and eliminates the occurrence of a false positive. Because equal amounts of DNA (2 µg) were loaded into all lanes, the possibility of mass effect was eliminated.

Lanes 4 and 5, corresponding to independent WT-*glo* transformant lines 14 and 11, show distinct, individual bands indicating the presence of a *glo* gene integrated into the plant genome. A single band indicates a single transformation event and integration of only one copy of the gene. In most cases, the results were inconclusive regarding integration of one or more copies of the *glo* gene because the hybridization signal appeared in the high molecular weight DNA.

L-AsA concentrations

L-AsA levels of the plants transplanted from selective media to soil were assayed after they were allowed to acclimate to the controlled light conditions for at least 5 days because it has been shown in the literature that it takes approximately 2-3 days for plants to adapt to new light conditions (Gatzek et al., 2002). All plant lines were grown under the same greenhouse conditions. They were exposed to approximately 14 h of ambient light for 21 days. All tissue samples were taken from *A. thaliana* plants under investigation at the same age and at the same time of day. All L-AsA values were assayed for rosette leaf tissue. These parameters are vital because it has been reported that L-AsA values vary widely with factors such as light conditions (Grace and Logan, 1996; Tabata et al., 2002), time of day, tissue type and age (Bartoli et al., 2000). Small changes in stress level can change the L-AsA content greatly.

Transformed WT A. thaliana

Expression of the *glo* cDNA did not significantly increased the steady-state level of L-AsA in WT *A. thaliana*. L-AsA values of all 10 lines assayed were within the range of the WT and pBI121 control plants (Table 1). L-AsA content in the lines having the largest increase was 3.786 µmoles/g FWT, which is an approximately 25% increase over the average WT value. All lines were in the rosette stage and had not begun to bolt at the

time of tissue collection. This eliminates the possibility of obtaining a lower rosette leaf L-AsA content due to transport to developing tissues.

Although under normal circumstances L-AsA is an antioxidant, it has been shown that at high concentrations, L-AsA can also act as a powerful oxidant. Because the lowest unoccupied molecular orbital of L-AsA has low energy, it easily reacts with free radicals. The free radical, if it is donating an electron as in most cases, must put an electron in the lowest unoccupied orbital of L-AsA. When the electron is accepted, the overall energy of the L-AsA molecule stays about the same. If there is a high concentration of L-AsA rather than free radicals, L-AsA will be in the position to give away its free electron, rather than accepting one. In this case, L-AsA becomes an oxidizing agent rather than an anti-oxidant (Cabral and Haake, 1988). Because of this, high levels could be toxic to the plant cell.

There are several possible explanations for the observed limited increase in L-AsA content. Because L-AsA could become harmful to cells rather than protective when the oxidizing power of the molecule cannot be used, intermediates, along with L-AsA production could be highly regulated causing silencing of the transgene. There could be suppression of the L-AsA levels if there is too much flux through the pathway. There could also be suppression of intermediate steps if too much of one product in the pathway is accumulating. It is known that high concentrations of some intermediates, such as D-mannose, are toxic to plant cells (Harris et al., 1989). Cells respond rapidly to high levels of L-Gal and L-galactose suggesting that the conversion to D-mannose is tightly controlled (Davey et al., 1999). It is known that under stressful conditions levels of L-AsA dramatically increased, because higher levels of this compound are required to regulate free radicals in the plant. Perhaps a significant increase would be seen in *A. thaliana* plants transformed with *glo* as compared to controls under stressful conditions.

Another explanation for the modest increase of ascorbic acid in the *A. thaliana* plant lines is that the GLO enzyme is non-specific. It has been shown that the GLO enzyme will catalyze the reaction of L-Gal to L-AsA at a reasonably high rate. As stated earlier, it is known that increased levels of the L-Gal substrate lead to elevated levels of L-AsA (Smirnoff and Pallanca, 1996; Arrigoni and De Tullio, 2002) and that there does not seem to be regulation at this step in the pathway (Arrigoni and De Tullio, 2002),

although evidence exists to the contrary (Tabata et al., 2002). In addition, recent evidence shows that when the GalLD cDNA from cauliflower is expressed in tobacco plants, despite a 2- to 3-fold increase in enzymatic activity, the L-AsA level is only increased about 30% (Bauw et al., 1998). This observation suggests that the substrate is more limiting than the enzyme in this plant system.

If GLO was catalyzing this same reaction, ascorbic acid levels would not be expected to significantly increase when expressed in tobacco plants. However, it has been shown that tobacco lines expressing GLO achieve L-AsA levels more than 8-fold over the control plants (Jain and Nessler, 2000). This high level of product could be the result of enzyme efficiency. The terminal enzyme in the plant pathway (GalLD) could be more efficient in *A. thaliana* than in tobacco, in which case, expressing an enzyme that catalyzes the same reaction (step 4, Figure 11) would increase the L-AsA product in tobacco, but not in a species that has an efficient enzyme, such as *A. thaliana*. In the tobacco plant, there would still be substrate present to be converted into L-AsA whereas in the *A. thaliana* the L-AsA would not increase because the substrate (L-Gal) would have been converted by the intrinsic, efficient GalLD. This is probably not the case due to the overexpression of GalLD in tobacco (Bauw et al., 1998). If increasing the amount of terminal enzyme in tobacco was unable to increase the L-AsA content greater than 30%, it is unlikely that there is a surplus of L-Gal present.

All evidence provided to this point seems to indicate that GLO is acting in a different fashion to increase L-AsA levels. The L-AsA increase that was observed in tobacco was not observed in the *A. thaliana* plant lines expressing *glo* which suggests that an alternative pathway is enhanced in the tobacco plants and not in the *A. thaliana* plant lines by the expression of *glo*.

Transformed vtc mutants

In contrast to transformed WT plants, the *vtc* mutant lines did have a significant increase in L-AsA values, comparable to those of the WT and pBI121 controls. It has been hypothesized that the *VTC2* and *VTC4* loci mutations involve either, the enzyme or a regulatory function of the conversion of GDP-L-galactose to L-galactose (step 10-12,

Figure 11) and/or cofactors which regulate the protein that performs this conversion. The observation that *vtc2-1* and *2-2* have L-AsA levels comparable to the *vtc1-1* mutant in the seedling stage but after 6 weeks, retain only about 10% of the WT level in the rosette leaves suggest that the *VTC2* locus maybe be involved in a biological function which causes carbon to be available in young leaves but limiting in older tissues (Conklin et al., 2000). It has been suggested that the *vtc3-1* line might have a reduced ability to convert mannose to L-AsA (Conklin et al., 2000). It is known that mutant lines *vtc2-1*, *vtc3-1*, and *vtc4-1* have WT levels of phosphomannose mutase (step 7, Figure 11), GDP-D-mannose-3,5-epimerase (step 9, Figure 11), L-galactose dehydrogenase (step 12, Figure 11) and GalLD (Smirnoff et al., 2001).

An important consideration is the availability of the substrate and compartmentalization of the enzymes. In this study, GLO was expressed using the 35S promoter. This promoter causes the *glo* gene to be expressed constitutively at high levels. In animals, GLO is targeted to the ER lumen (Nandi et al., 1997), so the substrate, L-Gul, must enter the ER and the final product, L-AsA, is then exported from this membrane system. A different compartmentalization is found for the terminal enzyme in plants. It has been shown that GalLD (step 8, Figure 11), is localized to the mitochondria (Mutsuda et al., 1995), and the enzyme that catalyzes the production of the substrate for GalLD, L-GalDH (step 12, Figure 11), is located in the cytosol (Gatzek et al., 2002). This indicates that the majority of the L-AsA pathway in plants is located in the cytosol, while only the terminal step is catalyzed in the mitochondria.

When transformed into a plant system by *Agrobacterium*-mediated transformation, GLO is not sorted to the mitochondria, due to lack of mitochondrial targeting signal. Based on animal studies, it is likely that the GLO enzyme is targeted to the ER, where it is either retained, sorted to an unknown membrane compartment or sent to the cell wall by the default pathway. Since L-Gal is made in the cytosol, it is possible that it diffuses into the ER where GLO acts on it, if GLO is retained in the ER, and produces L-AsA. However, the concentration of L-Gal in the cytosol would need to be rather high for an increase in L-AsA. Thus, the L-Gal would be diverted from mitochondria to the ER. Evidence does exist for multiple isoforms of the GalLD enzyme. Rather than only being present in mitochondria, it seems that GalLD may also be present

in the cytosol (Oba et al., 1994; Østergaard et al., 1997b). In this case, there may be moderate levels of L-Gal in the cytosol and GLO could be using part of this pool as a substrate. It is more likely that the L-Gal being produced in the cytosol is still being imported into the mitochondria and converted into L-AsA there, while a small percentage of the L-Gal is being converted to L-AsA by GLO.

An explanation of elevated levels of L-AsA in the *vtc* mutant plants expressing the GLO enzyme could be that GLO increases the flux through another biosynthetic pathway to L-AsA production. Because many alternative pathways could exist, as shown in Figure 11, there are multiple possibilities for branch points. There could also be an alternative pathway similar to the animal pathway (steps 13-20, Figure 11) which is completely independent of the plant pathway.

All of these mutations were overcome by the presence of the GLO enzyme. If GLO is catalyzing the reaction with L-Gal as the substrate, having surplus enzyme could be shifting the flux in the pathway to WT levels of L-AsA. This level could be maintained through the amount of L-Gal available for conversion. This is an unlikely explanation since these mutant plants have WT levels of GalLD. It would be expected that the presence of WT levels of this terminal enzyme would be sufficient to shift the pathway into producing more L-Gal substrate without the requirement of GLO.

The increase observed with the presence of GLO suggests that an alternative pathway may be present which can by pass the conversion of GDP-L-galactose to L-galactose (step 10, Figure 11) and thus raise levels of L-AsA without the requirement of L-galactose and, in turn, without the requirement of subsequent steps. If present, this alternative route to L-AsA production seems to be heavily regulated in *A. thaliana* and not in tobacco due to the great increase in L-AsA seen in tobacco but not in *A. thaliana*.

This data could point to an alternative pathway to L-AsA production in tobacco that is not present in *A. thaliana*. Compounds produced in tobacco plants but not in *A. thaliana* could serve as intermediates in an L-AsA pathway and would explain why such high levels were observed in the tobacco plants with GLO. An alternative pathway in tobacco may be completely independent from the published pathway, or the two may share common intermediates.

It is interesting to note that in feeding studies in WT tobacco and WT transformed with the *glo* construct, L-Gal significantly increased the L-AsA levels in the plant tissue as expected from the Wheeler pathway, but the feeding of L-Gul to the control plants also increased the L-AsA levels in the WT plants by approximately 10-fold (Jain and Nessler, 2000). *A. thaliana* cell lines also have been shown to be able to utilize L-Gul as a substrate to enhance L-AsA production (Davey et al., 1999). Although it has not been determined, it is expected that the *A. thaliana* GalLD is specific for the L-Gal substrate. To date, only a handful of GalLD enzymes have been characterized. The Mapson group did not observe any conversion of L-Gul to L-AsA by GalLD from pea seedlings (Mapson et al., 1954). The cauliflower (Østergaard et al., 1997b) and spinach (Mutsuda et al., 1995) homologs were shown to be absolutely specific for L-Gal, and the sweet potato GalLD was able to convert L-Gul at a rate of 1% (Oba et al., 1995). The partially purified white potato GalLD is the most non-specific, catalyzing L-Gul at a rate of 20% (Oba et al., 1994). For both the recombinant and native forms of the tobacco GalLD, L-Gul was only 7% as effective as a precursor for L-AsA as L-Gal (Yabuta et al., 2000).

Although the specificity of the *A. thaliana* GalLD gene homolog has not been experimentally tested, this gene seems to be remarkably conserved in other plant species. If we assume that the *A. thaliana* GalLD is as specific as the other enzymes, published data showing an increase in L-AsA levels when plant systems are fed L-Gul would most likely be due to an alternative pathway, rather than enhancement of the proposed Smirnoff-Wheeler pathway. In the Jain and Nessler L-Gul feeding experiments (2000), the increased levels of L-AsA can not be attributed to the tobacco GalLD using L-Gul at a rate of 7%. Although the enzyme may have used a very small fraction, this cannot account for the observed increase in L-AsA. Increased L-AsA levels in L-Gul-fed tobacco supports an alternative pathway in this plant system. An alternative pathway different from the tobacco is also probable due to the increase in L-AsA production through L-Gul feeding (Davey et al., 1999). It is also possible that cells in suspension, as in the Davey, et al. (1999) studies are able to utilize an alternative pathway when provided substrates such as L-Gul, whereas plant systems are unable to do this as a result of regulation.

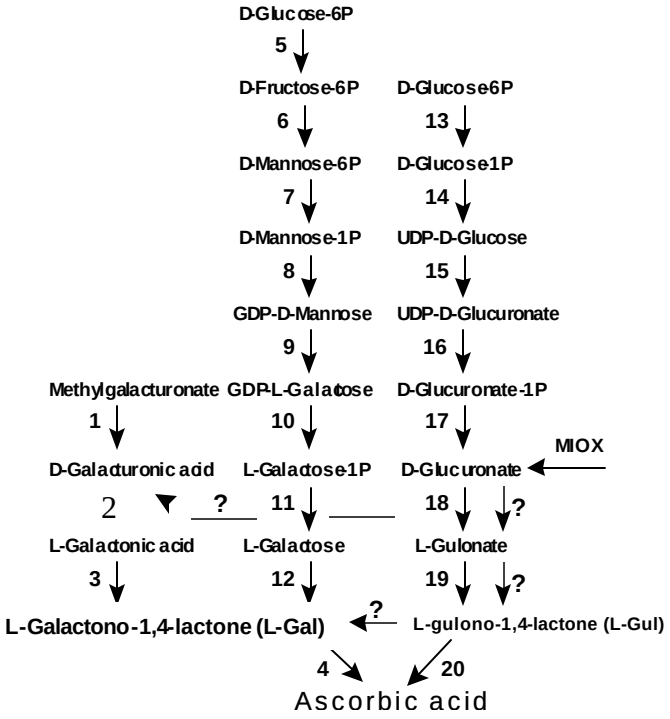


Figure 11 Alternative ascorbic acid pathways (Nessler, C., Lorence, A., pers. com.). Proposed biosynthetic pathways of L-ascorbic acid in plants (reactions 1-12), and animals (reactions 13-20). Enzymes catalyzing the numbered reactions are: 1, methyleste-rase; 2, D-galacturonate reductase (GalUR); 3, aldono-lactonase; 4, L-galactono-1,4-lactone dehydrogenase (GalLD); 5, glucose-6-phosphate isomerase; 6, mannose-6-phosphate isomerase; 7, phosphomannomutase; 8, GDP-mannose pyrophosphorylase; 9, GDP-mannose-3,5-epimerase; 10, phosphodiesterase; 11, sugar phosphatase; 12, L-galactose-1-dehydrogenase (GalDH); 13, phosphoglucomutase; 14, UDP-glucose pyrophosphorylase; 15, UDP-glucose 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 *myo*-Inositol (MI) oxygenase (MIOX), and the possible pathway from MI to AsA (broken

It is also possible that substrate specificity or affinity could be affected by glycosylation. Glycosylation can affect the three dimensional structure of a protein, and thus possibly altering the activity (Dove, 2001). The GLO sequence has two predicted N-glycosylation sties at positions 226 and 401 of the amino acid sequence (obtained from NetNGlyc 1.0). Most non-cytoplasmic proteins are glycosylated (Doyon et al., 2002) and proteins with signal peptides are more likely to be exposed to the N-glycosylation machinery than proteins lacking a signal peptide and thus, are more likely to be glycosylated *in vivo.* It is also known that glycosylation in plant systems is different from animals. For example, plant glycoproteins lack the characteristic galactose and sialic acid-containing complex *N*-glycans found in mammals (Bakker et al., 2001). Because it has been proven that glycosylation can affect the activity of an enzyme in some cases (Delagebeaudeuf et al., 1996), and not in others (Warrena et al., 2002), there is no way to predict the effect of altered glycosylation patterns of the GLO enzyme expressed in a plant system.

The data presented here suggest that, in addition to current knowledge derived from the *vtc* mutant lines, an alternative pathway for L-AsA biosynthesis is present in plants that may or may not share common intermediates with the Smirnoff-Wheeler pathway. It is apparent that if such a pathway is present, it only supplies a fraction of the total L-AsA pool in plant tissue based on published feeding studies with L-Gul and the observation that the *vtc* mutants are only able to recover to WT levels of L-AsA production.

Future research

To confirm the results, the controlled growth of the transformed lines should be repeated for example, at 25°C with a 16-h photoperiod in a controlled environment. By doing so, physiologically-relevant values can be compared to the levels obtained in a greenhouse. Since it has been suggested that regulation of enzymes in the biosynthetic pathway for L-AsA may be light modulated, the *glo*-expressing plant lines could be grown under high light conditions (Gatzek et al., 2002). This stress would also affect the regulation of L-AsA production.

RNA gel blots and RT-PCR experiments will be performed on the tissue samples from the GLO expressors to develop a clearer picture of the correlation between the gene expression and levels of L-AsA. These lines will be studied further and a metabolic profile created to determine what other intermediates or compounds are present in high concentrations in plant lines expressing GLO. A metabolic profile should also be developed from plant tissue grown under physiological conditions and compared with the profile developed from plants grown in a more stressful environment. It would be important to look at the levels of L-Gal in the *A. thaliana* plant lines and compare those values to concentrations in tobacco and lettuce plants.

To conclusively determine if GLO can increase ascorbic acid levels by enhancing an alternative pathway in plants without the requirement of the terminal step, GalDH knockouts could be used. Antisense lines with significantly decreased activity are available (Gatzek et al., 2002) and could be transformed with the *glo* gene construct containing a different antibiotic selection from the antisense plasmid. The lines can then

be selected and homozygous lines of *A. thaliana* plants expressing both the *glo* and antisense version of the *GalDH* gene can be developed. L-AsA levels can be assayed under controlled growth conditions and compared to those of untransformed lines with and without the antisense expression.

Because it may be difficult to interpreted results obtained from a double transformation, especially one that is antisense expression, knockout lines may prove easier to work with. A Salk T-DNA insertion mutant line has been identified with a mutation in the *GalDH* gene. Salk line 056664 has a T-DNA insertion at position 15135630 of the *A. thaliana* genome which falls in an exon of the *GalDH* gene. After confirming that this knockout line has significantly decreased levels of L-AsA, it could be transformed with the *glo* gene construct to see if those levels increase. If elevated levels of L-AsA are detected, GLO must be utilizing a substrate from another biosynthetic pathway for L-AsA. When the mutated genes at loci *vtc2, 3* and *4* are cloned and characterized this research can be revisited.

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Vita

Jessica Ann Radzio was born and raised in Martinsville, NJ and is the oldest of two children. She attended Bridgewater-Raritan Regional High School in central NJ and graduated in 1997. After high school, Jessica was accepted into the Biology program at Virginia Polytechnic Institute and State University. She spent her sophomore year attending the University of Alaska at Anchorage through an exchange program and worked at the Matanuska Maid Dairy during the course of her stay. Jessica returned to Virginia and completed her Bachelor of Science degree in the summer of 2001 with a concentration in Microbiology/Immunology. She also acquired a double major in Interdisciplinary Studies, with minors in Chemistry, Women's Studies and Religion. In the fall of 2001, Jessica entered the Master's program at Virginia Tech under the direction of Dr. Craig Nessler in the Dept. of Plant Pathology, Physiology and Weed Science. She completed her Master's degree in the fall of 2004 with a focus in Plant Physiology. Jessica is currently working at the University of Pittsburgh conducting research related to structure-function relationships in HIV-1 reverse transcriptase.