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Improving the Nutritional Value of Crops through Enhancement of L-Ascorbic Acid (Vitamin C) Content: Rationale and Biotechnological Opportunities

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L-Ascorbic acid (AsA, vitamin C) is an essential human nutrient that must be obtained in the diet, with the vast majority being obtained from plant foods. A vitamin C-deficient diet results in the onset of scurvy, which can have lethal consequences. However, vitamin C has also been implicated in the prevention of chronic diseases such as heart disease, stroke, cancer, and several neurodegenerative diseases. Although the supporting evidence for these claims is disputed, the dietary allowances for vitamin C have been recently increased in several countries, including the United States. This scenario, together with the general perception by consumers of vitamin C as being of benefit in the prevention of several lifestyle diseases and associated with general "well-being", contributes to a market rationale for enhancing the vitamin C content of crops. In recent years, there has been substantial progress in the understanding of vitamin C biochemistry in plants with a number of structural genes cloned. Here these findings are reviewed, and a description of how such knowledge could be applied to the nutritional enhancement of crops is given.

KEYWORDS: L-Ascorbic acid; human nutrition; crop improvement; biosynthesis

INTRODUCTION

L-Ascorbic acid (AsA) is the most abundant soluble antioxidant found in plants and is also an essential nutrient for humans and a few other animals. Together with flavonoids, polyphenolics, and lipophilic compounds such as α-tocopherol (vitamin E), AsA contributes to the overall intake of "free radical scavengers" or "antioxidative metabolites" in the human diet. Diets rich in fruits and vegetables are associated with decreased risk of certain cancers and cardiovascular diseases, and this has been attributed to the high concentrations of antioxidants in such foods (*1*). Increasing awareness, particularly in the developed world, of the impact of the diet on "lifestyle" diseases is driving a consumer-led demand for increased food choice in relation to its nutritional value, of which food antioxidant capacity is a major component. This has resulted in the rapid expansion of functional food and nutraceutical markets, estimated to have a world value of between 10 and 30 billion U.S. dollars in 2001 (*2*). The development of more "nutritionally dense" foodstuffs has now become a primary target for research organizations and funding agencies in the agrifood sector. This trend is likely to be further fueled by the emergence of high-throughput technologies such as nutritional genomics, which enable for the first time systemic approaches to the study of human nutrition. By exploiting metabolomic and genomic advances in food science, nutrition, and human physiology, nutrigenomics is beginning to provide a molecular understanding of how common dietary chemicals affect human and animal health by altering the expression and/or structure of the genome (*3*). What is expected is that nutritional genomics will eventually open the way for "personalized nutrition", just as pharmacogenomics has led to the concept of "personalized medicine" and "designer drugs".

These developments are driving global efforts aimed at the development of new crop varieties with improved nutritional value, now perceived as a rewarding strategy in the agriculture−biotechnology sector as performance and productivity levels have stabilized. Vitamin C is a clear target for improvement as although synthetic vitamin C can be readily added to food, there is an increasing awareness and resistance among consumers to the use of additives in food materials and a tightening of the legislation on food and drink fortification. Plants are by far the most important sources of dietary vitamin C intake, which is clearly recognized as being beneficial by consumers. Marketing strategies emphasizing the vitamin C intake from the consumption of specific crop products (e.g., potato and fruit juices) are commonplace. Recent scientific advances in understanding AsA biosynthesis in plants provide unparalleled opportunities for the development of AsA-enhanced crops. These advances are summarized here.

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CHEMICAL AND BIOLOGICAL PROPERTIES OF L-ASCORBIC ACID

Vitamin C was first isolated in 1928 (*4*), its structure established in 1933 (*5*), and the compound named L-ascorbic acid in recognition of its protective role against scurvy. The main biological attribute of AsA is its reducing ability (redox potential = 0.05 V at pH 7). AsA is an extremely effective antioxidant due to its capacity to donate electrons, and furthermore due to the relatively low reactivity of its oxidation products, it is able to act as an efficient free radical scavenger. The ene−diol structure involving the second and third carbons is sensitive to oxidation in the presence of molecular oxygen and metal ions or thorough interaction with superoxide anions, hydroxyl, tocopheryl, alkoxyl, peroxyl, and phenoxyl radicals, the glutathione thiol radical, and reactive nitrogen radicals such as nitrogen dioxide, nitroxide, and peroxynitrite (*6*). AsA oxidation yields the free radical monodehydroascorbate (MDHA), which is poorly reactive and decays spontaneously by disproportionation to AsA and dehydroascorbic acid (DHA). Although MDHA is not particularly stable, its relative nonreactivity is a necessary quality for any antioxidant, which acts by quenching radical propagation reactions. This enables AsA to effectively scavenge reactive oxygen species (ROS), which can participate in radical-transfer propagation reactions and are responsible for the formation of aggressive organic peroxides. The accumulation of ROS caused by oxidative stress results in damage to DNA, proteins, carbohydrates, and lipids, excessive rise of intracellular "free" Ca^{2+}, and disruption of energy metabolism. Many degenerative conditions in humans including atherosclerosis, myocardial infarction, and cancer as well as aging are thought to be linked with ROS-induced damage (*7*). In plant chloroplasts, high generation of superoxide anions occurs as a result of oxygenic photosynthesis. Here oxidative damage is prevented by the combined action of Cu,Zn-superoxide dismutase (SOD; EC 1.15.1.1) and AsA peroxidase (APX; EC 1.11.1.11), as chloroplasts are devoid of catalase (*8*). Oxidized products formed (MDHA and DHA) are then enzymically recycled to AsA at the expenses of NADPH or (MDHA) directly by photosystem I (PSI) via reduced ferredoxin (*9*). AsA also quenches singlet oxygen formed at the photosynthetic site and immediate surroundings by transfer of energy from chlorophyll. This antioxidant potential is coupled with a reducing ability, which is important in the regeneration of α-tocopherol from the α-chromanoxyl radical (*10*).

AsA has a high propensity to reduce transition metals in solution. This has implications for the dietary absorption of metals; for example, AsA increases non-heme iron absorption by increasing its solubility (*11*). However, this property also makes AsA a potential pro-oxidant as it can propagate Fenton reactions and trigger radical-induced peroxidation cascades in vitro (*12*). Whether this function is relevant under physiological conditions is dubious as in functional biological systems, metal ions are not free in solution but largely sequestered by metal-binding proteins (*13*). Indeed, one of the best known functions of AsA in biological systems is the reduction of metal ions at the active site of certain metalloenzymes (oxygenases and dioxygenases), thus promoting their activity. In animals, important reactions catalyzed by oxygenases and dioxygenases include collagen hydroxylation, synthesis of carnitine and catecholamine, tyrosine catabolism, α-amidation of peptide hormones and hormone-releasing proteins, and synthesis of homogentisic acid during tyrosine metabolism (*14*). In plants, such enzymes are involved in extensin processing, glucosinolate catabolism, and biosynthesis of ethylene and gibberellin (*9*). AsA is also involved in the regeneration of zeaxanthin from violaxanthin in the xanthophyll cycle (*10*).

L-Ascorbic Acid and Human Nutrition. Humans, nonhuman primates, guinea pigs, bats, teleost fish, and some birds are unable to synthesize vitamin C and depend on dietary intake (*15*). In vitamin C-dependent mammals and fish, deprivation of this nutrient eventually results in a deficiency disease (scurvy), which is life threatening and the symptomatology (tissue and capillary fragility) of which is directly attributable to a defect of the hydroxylation steps during collagen biosynthesis and processing. It is assumed that at some point during their evolution, these animals lost the competence for vitamin C synthesis, which is otherwise widespread in vertebrates. In humans, primates, and guinea pigs there is clear evidence that this deficiency arose as a result of mutations in the gene encoding L-gulono-γ-lactone oxidase (L-GulLO; EC 1.1.3.8), the terminal enzyme of the biosynthetic pathway in animals (*16*; **Figure 1**). The evidence is that loss of vitamin C biosynthesis has occurred several times independently during vertebrate evolution and has become established in particular environments where this metabolic function was superfluous. It is conceivable that loss of AsA biosynthetic capacity in primate ancestors might have been compensated for by an elevated vitamin C intake via a highly foliarphagic or frugivorous diet. Estimates of vitamin C intake from such raw material suggest that several grams may have been ingested per day (*17*). Dietary intake is expected to have progressively declined during the successive steps in human evolution, which involved a shift to a more energy-rich diet comprising roots, nuts, dry seeds, and meat (all poor sources of vitamin C) and which also coincided with a reduction in gut size and a matching expansion of the brain (*18*). The introduction of food processing, for example, cooking, is also likely to further reduce the dietary AsA intake due to its heat lability and water solubility (*9*). Even so, estimates of dietary vitamin C intake from the Paleolithic diet (400−600 mg/day) are still 5−10-fold higher than modern dietary intake (*19*).

Current national vitamin C recommended daily allowances (RDAs) range between 30 and 110 mg/day for healthy adults (*20*, *21*). The discrepancy between these values and the presumed AsA intake in the primate ancestors for which endogenous biosynthesis was superfluous has fueled a raging debate. A main point of contention is the method used for estimating vitamin C RDA. Vitamin C is transported into cells in the oxidized form (DHA) via facilitative glucose transporters (GLUTs) (*22*, *23*) and as the reduced form by sodium-dependent transporters (SVCTs) (*24*), and plasma vitamin C concentrations are maintained between 10 and 100 μM with any excess excreted by the kidney (*25*). The average half-life of vitamin C in the adult human is ∼10−20 days, with a turnover of 1 mg/kg of body weight and a body pool of ∼20 mg/kg at a plasma concentration of 50 μmol/L (*26*). Thus, in the absence of continuous intake, scurvy ensues and, originally, RDA levels were established by determining the amount of vitamin C required to prevent the onset of the disease after ∼1 month of a vitamin C-free diet (*25*). However, because scurvy can be classified as a clinical deficiency, some argue that these levels are not sufficient to counteract "subclinical" deficiency, which has no clear symptomatology but may have implications for the development of many common degenerative diseases such as atherosclerosis, myocardial infarction, cancer, and aging, although evidence from intervention trials does not yet support such arguments (*27*). Additionally, it has recently been established that specific SVCT isoforms (SVCT2) are implicated in

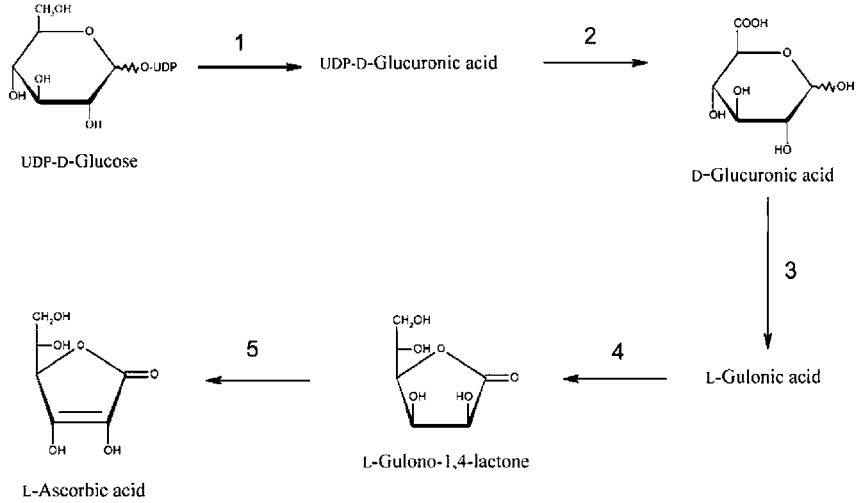

Figure 1. L-Ascorbic acid biosynthetic pathway in animals. Animals synthesize AsA in an "inversion" pathway in which C1 of glucose becomes C6 of AsA. In animals unable to synthesize AsA, multiple mutations occur in the gene encoding L-gulono-1,4-lactone oxidase, the ultimate biosynthetic step. 1, UDP-D-glucose dehydrogenase (EC 1.1.1.22); 2, UDP-D-glucuronic acid hydrolase; 3, D-glucuronic acid reductase (EC 1.1.1.19); 4, aldonolactonase (EC 3.1.1.18); 5, L-gulono-1,4-lactone oxidase (EC 1.1.3.8). Where EC numbers are omitted, they have not been assigned.

the transport of vitamin C into body organs such as adrenal glands, lung, pancreas, neurons, spleen, testis, ovary, eye (corpus luteum), bones, seminal fluid, and neutrophils, where vitamin C concentrations can reach between 100- and 1000-fold that of plasma levels (*28*). Although vitamin C accumulation in these organs seems to serve a specific purpose (i.e., it does not have a storage function), the optimum functional concentration has not been established. Recently, the vitamin requirements in the United States have been reassessed (*21*) on the basis of the kinetics of vitamin C uptake and secretion, that is, the intake required to maintain neutrophil concentration at 80% saturation with minimal urinary excretion (*25*). As a result, the RDAs have been raised from 60 mg/day to 90 mg/day for men and to 75 mg/day for women (*29*). The departure from scurvy prevention as a key parameter for determining the vitamin C RDA has not escaped criticism, particularly because there is no clear consensus on the validity of the other parameters adopted (*30*). Many of the protective effects exerted by vitamin C on physiological functions have been extrapolated from in vitro experiments, whereas direct evidence from dietary supplementation is far from convincing (*20*). As the health benefits of high dietary vitamin C intake cannot easily be separated from those of other antioxidants and bioactive compounds contained in fruits and vegetables (*1*, *31*), high vitamin C intake or plasma levels could be simply markers of good diet rather than a true protective factor (*20*). Pro-oxidant effects of vitamin C supplementation have also been reported (*32*), although the physiological relevance of these studies has been questioned (*6*). Since the original claims by Pauling on the need for vitamin C megadoses (*33*) it seems that no other dietary constituent can inspire such a fierce debate with claims and counterclaims. Despite the obvious confusion and controversy generated by this debate, consumer preference for foods high in natural vitamin C seems to be well established.

L-Ascorbic Acid in Plants. AsA is the most abundant soluble antioxidant in plant cells, and plants are the most important sources of AsA in the human diet. The reason for the high rate of synthesis in plants and its accumulation especially in chloroplasts relate to its protective role during oxygenic photosynthesis and photoprotection via its role in the xanthophyll cycle. In chloroplasts the AsA concentration can reach 50 mM (*34*), that is, 1000 times higher than in human plasma. AsA is a key component of the oxidative defense system evolved in plants to scavenge ROS generated during oxygen generation and break oxidation chains. The "constitutive" role of AsA in photosynthetic biology is illustrated by the widespread high AsA content in leaves. Foliar analyses of 211 "nonwoody" angiosperm species showed an average AsA content of 161 mg/100 g of fresh weight (FW) (*35*), whereas the foliar content of 41 species of "woody" shrubs and trees was even higher at 292 mg/100 g of FW (*36*). In general, the AsA content of non-photosynthetic plant tissues is much more variable. Dry seeds are generally devoid of AsA (*37*), whereas the contents of hydrated storage organs such as fruits and vegetables show a much greater interspecific variation than leaves. For example, exotic fruits such as the kakapo plum or the camu-camu contain over 5000 and 3000 mg/100 g of FW, respectively (*38*, *39*), whereas other fruits such as the common plum apricots or apples contain 1000-fold lower levels (*40*). With regard to vegetables, brassicaceae are good providers (50−100 mg/100 g of FW) as well as peas and peppers. Onions and root vegetables are generally low in AsA. Potatoes are not a particularly rich source of AsA (10−15 mg/100 g of FW) but are the major source of dietary AsA intake in much of Europe by virtue of the quantity consumed (*41*). More complete listings of the AsA content of common foodstuffs are provided elsewhere (*9*, *40*).

We know too little with regard to AsA metabolism in non-photosynthetic tissues to hypothesize the evolutionary reasons for the large variability in AsA content in different fruits and vegetables. By virtue of the AsA pro-oxidant properties in the presence of free transition metals, it is tempting to speculate that high AsA content may help protect against the attack of certain pathogens. Alternatively, the high AsA content of some fruits may be rewarding for seed-dispersing animals, some of which depend on dietary AsA for survival. In any case, the widely ranging AsA levels found in storage organs indicate, on the one hand, that normal metabolism in a large number of heterotrophic cells (including plant cells) can occur with minimal amounts of AsA. On the other hand, the very high AsA content of some fruits indicates that this bioactive compound may accumulate to very high concentrations without exerting toxic effects. These considerations support the feasibility for achieving substantial enhancement of AsA content in crop products via genetic approaches.

ADVANCES IN PLANT L-ASCORBIC ACID BIOTECHNOLOGY

The large natural diversity in AsA content in crop plants has been exploited in numerous attempts to enhance its levels by selective breeding. In particular, high levels of AsA are seen as an important trait in certain fruits such as *Ribes nigrum* (black currant) and *Malus* (apple) (*42*, *43*). Similarly, due to the relatively low AsA content and large quantities consumed in Western diets, *Solanum tuberosum* (potato) and *Lycopersicon esculentum* (tomato) are seen as suitable targets for improvement by breeding (*44*, *45*), although in practice this has resulted in negative traits such as a reduction in yield in tomato (*46*). One important aspect that must be considered in all breeding programs is the effect of environment. Numerous authors have reported strong effects of environmental conditions on crop AsA content (reviewed in ref *47*); however, no consistent patterns emerge, and it is therefore necessary to determine the response of any new varieties to specific growing conditions. Newly available genetic and genomic resources and technologies should provide a more effective means for the identification of genetic tools [e.g., molecular markers, quantitative trait loci (QTL)] for accelerated breeding programs. Map-based cloning has been used to identify loci and clone genes associated with AsA biosynthesis in *Arabidopsis thaliana* mutant lines (*48*), and quantitative genetic approaches are being used to identify QTL regulating AsA content in the same species (*49*). There is evidence for the coordinated regulation of AsA biosynthesis and turnover (*50*−*52*), implying the involvement of transcription factors, although this remains to be demonstrated. More rapid advances have been made in plant AsA biochemistry and have led to the identification of genes directly involved in AsA biosynthesis and degradation (reviewed below). These breakthroughs will provide the future basis for targeted genetic strategies for the development of new varieties with enhanced AsA content.

L-ASCORBIC ACID BIOSYNTHESIS

Despite the long understood importance of AsA in both human nutrition and plant stress tolerance, it is only relatively recently that progress has been made in understanding the mechanism(s) of biosynthesis of this compound in plants. Early work demonstrated that L-galactonolactone (L-GalL) was the most efficient AsA precursor in cress seedlings supplied with an array of sugar acid lactones (*53*); additionally, supply of L-gulonolactone (L-GulL) and D-glucuronolactone resulted in enhanced AsA content. D-Galacturonic acid (D-GalUA) methyl ester was also an efficient precursor of AsA in cress seedlings. Further work with isolated pea mitochondria demonstrated conversion of L-GalL to AsA. The enzyme preparation was specific for AsA formation from L-GalL, and L-GulL was not converted (*54*). The authors proposed a pathway similar to the animal biosynthetic pathway (**Figure 1**) in which the carbon skeleton of the primary substrate is inverted in the final product (*55*). In the case of plants the proposed reaction sequence was D-galactose → D-galacturonic acid → L-galactonic acid → L-ascorbic acid (*53*). However, the proposed pathway received no substantiation from studies with labeled monosaccharides as only very limited molecular inversion of the original substrate was observed during AsA synthesis (*56*, *57*).

L-Galactose Pathway. A major breakthrough in the identification of the primary AsA biosynthetic pathway in plants was the finding that supply of the rare sugar L-galactose (L-Gal) to plant tissues resulted in the accumulation of large amounts of AsA (*58*). This observation has subsequently been reproduced in many plant systems (*59*−*64*). On the basis of their observation, Wheeler et al. (*58*) proposed that the endogenous formation of L-Gal proceeded according to the Smirnoff−Wheeler pathway outlined in **Figure 2**. In support of their hypothesis the authors found that incorporation of radioactivity from D-[U-^{14}C]mannose (D-Man) into L-[^{14}C]AsA was 10-fold higher than that from D-[U-^{14}C]glucose (D-Glc) in *A. thaliana* leaves. They suggested that GDP-D-mannose-3,5-epimerase (GDPM-3,5-epimerase; EC 2.7.7.22), an enzyme first identified in the 1970s (*65*, *66*), was involved in the synthesis of GDP-L-Gal. Additionally, the authors demonstrated hydrolysis of GDP-L-Gal to L-Gal in the presence of pea and *A. thaliana* extracts and identified a novel enzyme capable of oxidizing L-Gal to L-GalL in the presence of NAD in plant extracts. Notably, the proposed pathway does not involve the inversion of the carbon skeleton of hexose precursors and can fully explain previous data obtained with radiolabeled precursors.

Noncommitted Steps. The early steps of the pathway, leading to the formation of GDP-L-Gal, are shared between a number of pathways including cell wall polysaccharide formation and protein glycosylation (*67*). No evidence is available directly correlating enzymes involved in the metabolism of phosphorylated D-Man intermediates, phosphomannose isomerase (PMI; EC 5.3.1.8) or phosphomannose mutase (PMM; EC 5.4.2.8), with AsA biosynthesis. Activities of these enzymes are generally very low in plant tissues to the extent that metabolic selection protocols based on the lack of growth of plant cells in D-Man coupled with ectopic overexpression of phosphomannose isomerase to permit growth on this substrate (*68*) have been developed. During screening of mutagenized *A. thaliana* populations for increased ozone sensitivity, a mutant containing ∼30% of wild-type levels of AsA (*vtc1*; 69) was found to be defective in AsA biosynthesis, and the genetic lesion was mapped to the gene encoding GDP-D-Man pyrophosphorylase (GDPM PPase; EC 2.7.7.22) (*70*). Subsequent analyses showed that a point mutation resulted in a proline to serine amino acid change in the enzyme, resulting in the mutagenized line having only ∼65% of the activity of the wild type (*71*). Similarly, down-regulation of GDPM PPase activity in potato via antisense technology resulted in reduction of enzyme activity up to 60% in leaves and 35% in tubers, with corresponding reductions of AsA concentration of 55 and 30% in the respective tissues (*72*). The strong negative impact on AsA levels ensuing from reduced GDPM PPase activity implies that the enzyme exerts a strong control over AsA biosynthesis, although the possibility that other physiological processes such as protein glycosylation are affected and contribute to the phenotype cannot be excluded. On the other hand, in *Prototheca moriformis*, a colorless microalga which synthesizes AsA in a pathway identical to that of higher plants, the AsA biosynthetic flux appears to be controlled by the activity of GDPM-3,5-epimerase. Analyses of mutagenized strains containing up to ∼4-fold higher AsA levels than the wild-type strain showed a strong correlation (R^2 = 0.9482) between GDPM-3,5-epimerase activity and rates of AsA synthesis in 10 strains (*64*). The recent cloning of a GDPM-3,5-epimerase gene from *A. thaliana* (*73*) should enable the testing of the relevance of this enzyme activity on the AsA biosynthetic flux in higher plants.

Committed Steps. Characterization of two heterotrophically grown AsA hyperaccumulating mutant strains of the green microalga *Chlorella pyrenoidosa* (*74*) showed that up-regulation of conversion of D-Man to L-Gal was correlated with enhancement of GDP-L-Gal pyrophosphatase (GDP-L-Gal PPPase)

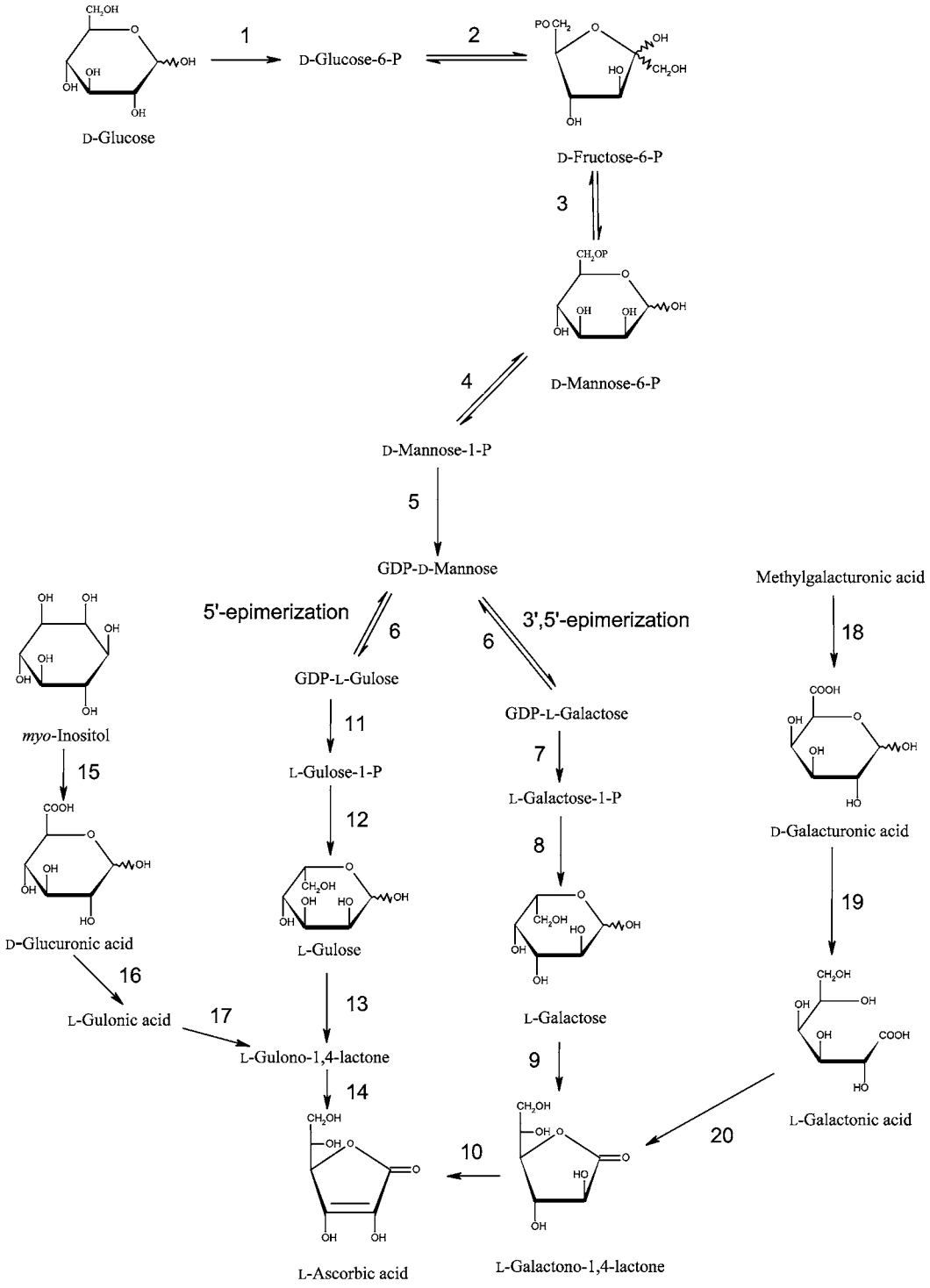

Figure 2. Pathways of L-ascorbic acid biosynthesis in plants. Three potential pathways of AsA biosynthesis have been in identified in plants. The Smirnoff–Wheeler pathway (reactions 1–10) utilizes GDP-D-mannose and L-galactose as key intermediates. It was recently demonstrated that in addition to formation of GDP-L-galactose, GDP-D-mannose epimerase (reaction 6) can produce GDP-L-gulose in vitro. This observation led to proposals for an alternative route (L-gulose shunt) in which L-gulose and L-gulono-1,4-lactone are pathway intermediates (reactions 11–14). An alternative biogenesis for L-gulono-1,4-lactone (*myo*-inositol pathway) was recently proposed following the isolation of a gene encoding *myo*-inositol oxygenase from *A. thaliana* (reactions 15–17). A third pathway proposed in the 1950s (D-galacturonic acid pathway) in which D-galacturonic acid is reduced to L-galactonic acid with inversion of the carbon chain (reactions 18–20) was initially dismissed as labeling experiments failed to demonstrate significant inversion. However, a gene encoding D-galacturonic acid reductase was recently cloned from strawberry and demonstrated to be involved in AsA synthesis. 1, hexokinase (EC 2.7.1.1); 2, phosphoglucose isomerase (EC 5.3.1.9); 3, phosphomannose isomerase (EC 5.3.1.8); 4, phosphomannose mutase (EC 5.4.2.8); 5, GDP-D-mannose pyrophosphorylase (EC 2.7.7.22); 6, GDP-D-mannose 3′,5′-epimerase (EC 5.1.3.18); 7, GDP-L-galactose pyrophosphatase; 8, L-galactose-1-phosphate phosphatase; 9, L-galactose dehydrogenase; 10, L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3); 11, GDP-L-gulose pyrophosphatase; 12, L-gulose-1-phosphate phosphatase; 13, L-gulose dehydrogenase; 14, L-gulono-1,4-lactone dehydrogenase; 15, *myo*-inositol oxygenase (EC 1.13.99.1); 16, D-glucuronic acid reductase (EC 1.1.1.19); 17, aldonolactonase; 18, (pectin) methylesterase (EC 3.1.1.11); 19, D-galacturonic acid reductase; 20, aldonolactonase (EC 3.1.1.18). Where EC numbers are omitted, they have not been assigned.

activity in both strains (*75*). No other enzyme activity of the Smirnoff−Wheeler pathway was significantly altered in both mutant strains. An enzyme displaying GDP-L-Gal PPPase activity was subsequently purified from *A. thaliana* roots, and the amino acid sequence was used to clone the corresponding gene. Functional characterization of the gene in transgenic plants is in progress in our laboratory. L-Gal-1-phosphate phosphatase (L-Gal-1-Pase), the subsequent enzyme in the pathway, was recently purified from kiwifruit and *Arabidopsis* (*76*). The enzyme from both sources was shown to be highly specific and displayed minimal activity against a wide range of alternative phosphorylated substrates. Functional analysis of the subsequent pathway enzyme was achieved following purification of L-Gal dehydrogenase (L-GalDH) from *A. thaliana* (*63*). Expression of the corresponding gene sequence in the antisense orientation resulted in up to 70% reduction of enzyme activity. Under low light conditions, the AsA concentration of transgenic plants was unchanged; however, under high light conditions, AsA content was reduced by up to 55% compared with the wild type. These data suggest that under many environmental conditions L-Gal availability, not L-GalDH activity, limits AsA biosynthesis, a hypothesis supported by the findings that supply of L-Gal to plant tissues markedly enhances the AsA content (*60*, *62*). Further support for this contention comes from the observation that ectopic overexpression of *Arabidopsis* L-GalDH in tobacco results in up to 3.5-fold increases in enzyme activity but no change in AsA content (*63*). Unlike L-GalDH expression, which appears to be unregulated by environmental signals or product concentration (*63*), the expression of L-GalL dehydrogenase (L-GalLDH; EC 1.3.2.3) appears to be regulated by light in *A. thaliana* (*77*) and tobacco (*78*) and by product concentration in tobacco BY-2 cells (*52*). A purported relationship between L-GalLDH activity and AsA synthesis (*52*, *59*, *77*−*79*) has led to suggestions that this step may be a suitable target for manipulation of AsA biosynthesis in plants. However, the finding that supply of L-GalL to many plant tissues results in increased levels of AsA (*53*, *60*−*62*, *80*) suggests that substrate formation rather than its oxidation rate is the limiting factor in AsA biosynthesis. On the other hand, it has recently been reported that overexpression of tobacco L-GalLDH in BY-2 cells under the constitutive CAMV35S promoter resulted in up to 4-fold increased enzyme activity and a 60% increase in the AsA pool size (*81*). One complication that may be encountered in trying to raise crop AsA content by manipulation of L-GalLDH activity is its intimate association with the mitochondrial electron transport chain with which it competes for availability of oxidized cytochrome *c* (*82*).

Thus, despite the relative novelty of the pathway, almost all of the enzymes have been purified and the corresponding genes cloned. The involvement of some of these genes in the AsA biosynthetic pathway in vivo has been demonstrated by analysis of AsA-deficient mutants or transgenic plants in which individual enzyme activities were reduced. It is notable that no lines with AsA reduction below ∼30% of wild-type levels have been observed. This may suggest that residual enzyme activities were sufficient to allow a substantial biosynthetic flux or that lower levels of AsA are generally lethal.

Evidence for Additional Pathways. *L-Gulose Shunt.* Kinetic characterization of purified and recombinant GDPM-3,5-epimerase unexpectedly showed that the enzyme was able to convert GDP-D-Man not only into GDP-L-Gal but also into GDP-L-gulose (GDP-L-Gul) in vitro (*83*). *A. thaliana* suspension cells supplied with either L-Gul or L-GulL exhibited enhanced AsA content. It has been known since the 1950s that the animal pathway precursor, L-GulL, can result in enhanced AsA content when supplied to plants, and recent metabolic profiling of *Arabidopsis*, potato, and tobacco demonstrated the presence of L-gulonic acid (*84*). Wolucka and van Montagu (*83*) proposed that L-Gul and L-GulL act as the physiological precursors of AsA in plants (**Figure 2**), and the channeling of substrates into this end-product or into cell wall biosynthesis (as L-fucose or L-Gal residues) is controlled by binding of chaperones. Support for this proposal was obtained in transgenic tobacco and lettuce plants transformed to express the rat L-GulLO gene encoding the last enzyme in the animal AsA biosynthetic pathway. Leaf AsA contents were enhanced by up to 7-fold in both species expressing the gene (*85*). However, the complementation of *A. thaliana vtc1-1* mutant lines (in which GDPM PPase activity is inhibited) by expression of the rat L-GulLO gene has been taken as an indication that a route of L-GulL biogenesis alternative to the Smirnoff−Wheeler pathway must exist (*86*). Considering the absolute specificity for L-GalL of L-GalLDH (*87*) and the fact that no GulLO orthologue has yet been identified in plants, the race is open to identify and characterize the plant gene responsible for the conversion of L-GulL to AsA in plants.

myo-Inositol Pathway. An alternative derivation of L-GulL may be via the oxidation of *myo*-inositol (MI) to D-glucuronic acid followed by reduction to L-gulonic acid (**Figure 2**). Recently, a bioinformatics approach was used to search for homologues of the pig *myo*-inositol oxygenase (MIOX; EC 1.13.99) encoding gene in the *Arabidopsis* genome. Four homologues were found on chromosomes 1, 2, 4, and 5 (*miox1*, *-2*, *-4*, and *-5*). One of theses sequences (*miox4*) was cloned, expressed in *Escherichia coli*, and demonstrated to have high specific activity (*88*). Constitutive expression of *miox4* in homozygous transgenic *Arabidopsis* lines resulted in up to 3-fold enhancement of AsA levels in leaves, demonstrating the potential utility of the gene for producing enhanced AsA in crops.

D-Galacturonic Acid Pathway. Early work demonstrated that supply of methyl D-galUA to cress seedlings increased plant AsA content (*53*), and this result was subsequently reproduced in *Arabidopsis* cell suspension cultures (*60*). The cloning of a gene encoding D-galUA reductase (GalUAR) from strawberry (*89*) prompted a re-examination of this biosynthetic route. GalUAR expression was found to correlate with the AsA content during fruit development, prompting the suggestion that in strawberry fruit breakdown of pectin and release of D-GalUA during ripening may be an important source of substrate for AsA synthesis. The finding that ectopic overexpression of GalUAR in *A. thaliana* induced a 3-fold increase in AsA content (*89*) indicates that this biosynthetic route may be more widespread. However, as in the previous case, there are doubts on the physiological relevance of this route, which requires molecular inversion of D-galUA during its conversion into AsA (**Figure 2**).

The data outlined above suggest a number of possible pathways of AsA biosynthesis in plants (**Figure 2**). There is good evidence supporting the widespread distribution of the Smirnoff−Wheeler pathway, although attempts at up-regulating the AsA biosynthetic flux via manipulation of genes within this pathway have so far met with limited success. The possibility of a tight regulation of the flux, perhaps via fine-control mechanisms, should not be dismissed (*83*). The physiological relevance of the other biosynthetic routes proposed is less clear. These may be vestigial pathways or secondary routes that are activated under specific conditions via coarse control (explaining

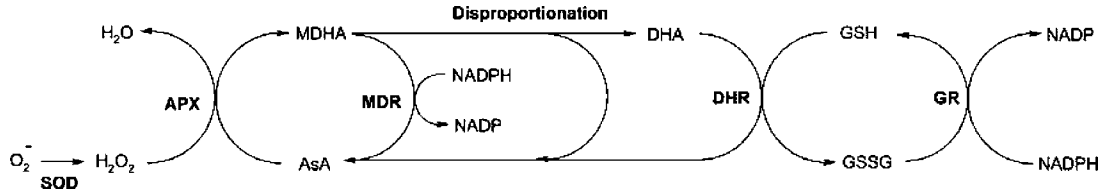

Figure 3. Ascorbate–glutathione cycle. Plants use the ascorbate–glutathione cycle for detoxification of reactive oxygen species, particularly in the chloroplast, which lacks catalase. In this pathway, superoxide is dismutated to hydrogen peroxide via the activity of superoxide dismutase (SOD, EC 1.15.1.1). AsA acts as a cofactor for the reduction of hydrogen peroxide to water via the activity of ascorbate peroxidase (APX, EC 1.11.1.11), resulting in the oxidation of AsA to monodehydroascorbate (MDHA). MDHA either spontaneously disproportionates to AsA and dehydroascorbate (DHA) or is reduced to AsA via the action of monodehydroascorbate reductase (MDHAR, EC 1.6.5.4). DHA is reduced to AsA via the action of dehydroascorbate reductase (DHAR), which uses the sulfur-containing tripeptide glutathione (GSH) as a cofactor. Oxidized glutathione (GSSG) is reduced by NADPH (produced by PS1) in a reaction catalyzed by glutathione reductase (GR, EC 1.6.4.2).

their increased sensitivity to genetic manipulation). Additional work is needed to establish whether multiple biosynthetic routes coexist within the cell or whether different biosynthetic pathways are linked to specific tissues, developmental stages, or external influences. To achieve optimal methods for enhancing the AsA content of crops, it will be necessary to expand our knowledge regarding such questions.

L-ASCORBIC ACID RECYCLING

One of the primary functions of AsA in plants is to protect against ROS and in particular in the removal of hydrogen peroxide (H_2O_2) via APX. In C_3 plants it has been estimated that typical rates of H_2O_2 production are $\sim$14.2 μmol m^{-2} s^{-1} in mesophyll cells during photosynthesis (*90*), and given that two molecules of AsA are required to reduce H_2O_2 to water, this represents a significant potential drain on the AsA pool. Oxidized products formed (MDHA and DHA) are enzymically recycled to AsA at the expense of NADPH or MDHA directly by PSI via reduced ferredoxin (MDHA). Enzymic reduction of DHA to AsA occurs via a NADPH-dependent pathway involving the reduced form of the sulfur-containing tripeptide glutathione (GSH) and known as the AsA−GSH cycle (**Figure 3**). In general, it appears that plants have several isoforms of enzymes involved in the AsA−GSH cycle; for example, *A. thaliana* has nine loci encoding putative AsA peroxidase, five loci encoding DHA reductase (DHAR; EC 1.8.5.1), five loci encoding MDHA reductase (MDHAR; EC 1.6.5.4), and two loci encoding GSH reductase (GR; EC 1.8.1.7) (*91*). Added complexity is brought about by the observation that different isoforms of these enzymes are expressed in different cell compartments and that single genes target specific enzymes to multiple cell compartments (mitochondria and chloroplasts) using mechanisms such as alternative splicing (*92*) or dual targeting of enzymes to both cell compartments (*91*). Despite these potential difficulties, a number of researchers have expressed transgenes of the AsA−GSH cycle in plants in an attempt to improve photosynthetic efficiency and stress resistance (*93*). Intriguingly, up-regulation of the plant's AsA recycling capacity can also induce steady-state increases in AsA content. Chen et al. (*94*) observed up to 3.9-fold increase in leaf AsA content in tobacco and maize as a result of ectopic overexpression of wheat DHAR, and this was accompanied by an increase in the AsA redox ratio (AsA:DHA) from 1.5 to 4.0. Interestingly, the AsA level also significantly increased in maize kernels (a sink tissue), although in lines where DHAR activity was >100-fold higher than that in wild type. In both tobacco and maize, levels of GSH were doubled, suggesting coordination between these two antioxidants.

LONG-DISTANCE L-ASCORBIC ACID TRANSPORT

Several authors have reported the presence of AsA in plant phloem (*62*, *95*, *96*), and active uptake of AsA (*96*) and AsA precursors (*62*) into the phloem of source leaves has also been demonstrated in a number of species. Radiotracer experiments in conjunction with autoradiography have demonstrated translocation of AsA from source to sink tissues in *M. sativa* and *A. thaliana*. Furthermore, artificial enhancement of AsA content in source leaves of potato results in increased AsA levels in developing tubers (*97*). These observations suggest that phloem AsA transporters may represent a key target for the improvement of AsA content in sink organs such as fruits and tubers. Although the kinetics of AsA transport have been studied across a number of plant membranes such as the plasmalemma, the chloroplast membrane, the thylakoid membrane, and the tonoplast (*98*), to date no AsA transporter genes have been cloned from plants. On the basis of sequence homology with mammalian vitamin C (nucleobase) transporters, a family of 12 genes has been identified in the *A. thaliana* genome, and work is currently being undertaken to characterize the protein products (U. I. Flügge, Universitat zu Koln, personal communication), although no data have yet been published in the scientific literature. Work in our laboratory has demonstrated AsA biosynthesis in plant phloem and active uptake of AsA biosynthetic intermediates by source leaf phloem (*62*); an alternative mechanism may therefore be to engineer the enhanced uptake of AsA precursors by the phloem.

CONCLUSIONS

The mounting evidence of the protective role exerted by antioxidants on major life-threatening diseases, combined with an increased awareness among consumers of the impact of dietary constituents on health and "lifestyle" ailments, is generating fertile ground for research and commercial opportunities for the development of more nutritional foodstuffs. The development of nutritionally enhanced food crops by virtue of increased natural vitamin C content represents a promising target, as the health-giving attributes of this bioactive compound are well-known to the consumer. To achieve this goal, we need to identify the genetic bases of AsA accumulation in crop plants and in particular in their edible parts. Although little progress has been made on the development of tools for accelerated breeding of new AsA-enhanced varieties, our understanding of plant AsA biochemistry in plants has advanced considerably in recent years. Several genes involved in AsA biosynthesis and recycling have been cloned, and transgenic plants have been generated that contain enhanced levels of AsA. The demonstration of long-distance AsA transport and phloem AsA biosynthesis in plants also provides additional targets for genetic

improvement. It remains to be seen whether the improvements observed in model systems such as *A. thaliana* or tobacco can be translated into viable strategies for enhancing the AsA content of nutritionally important crop plants. In addition to enhanced nutritional value, it is expected that AsA-enhanced crop products may have added benefits such as extended shelf life and increased stress tolerance by virtue of the multifaceted properties and functions of AsA.

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