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Increasing ascorbate levels in crops to enhance human nutrition and plant abiotic stress tolerance

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Ascorbate (or vitamin C) is an essential human micronutrient predominantly obtained from plants. In addition to preventing scurvy, it is now known to have broader roles in human health, for example as a cofactor for enzymes involved in epigenetic programming and as regulator of cellular iron uptake. Furthermore, ascorbate is the major antioxidant in plants and underpins many environmentally induced abiotic stress responses. Biotechnological approaches to enhance the ascorbate content of crops therefore have potential to improve both human health and abiotic stress tolerance of crops. Identifying the genetic basis of ascorbate variation between plant varieties and discovering how some 'super fruits' accumulate extremely high levels of ascorbate should reveal new ways to more effectively manipulate the production of ascorbate in crops.

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Introduction

Ascorbate (or vitamin C) is a major antioxidant produced by plants and its primary role is to protect the cell from the damaging effects of reactive oxygen species (ROS) that are produced as a result of normal cellular metabolism, especially during photosynthesis [1,2]. Ascorbate also plays an important protective role under stress conditions where damage to cellular machinery causes increased production of ROS [1]. Changing global climate patterns are predicted to increase extreme environmental conditions, such as high temperatures and drought. Developing abiotic stress tolerant crops will therefore be critical to ensuring food security for an increasing world population. As plants with increased ascorbate levels are more tolerant to stress, this is a promising way to generate more resilient crops [3]. Furthermore, ascorbate is an essential human micronutrient, and while severe deficiencies are rare, optimum levels of ascorbate are not commonly consumed even in Western countries [4]. Therefore increasing the ascorbate levels of crop plants should also provide a way to improving human nutrition. The various pathways by which ascorbate is synthesized have been elucidated, and the relative importance of the different pathways is becoming more clear [5]. However, there is still much to learn about how plants regulate these pathways to control ascorbate levels. The recent discovery that plants are able to control ascorbate levels through a translational feedback mechanism has revealed a new level of regulation that potentially offers another way of increasing ascorbate levels [6••]. Here, we review recent research efforts to increase ascorbate in leaves (primarily aimed at stress tolerance) and edible plant parts (aimed at improved nutrition).

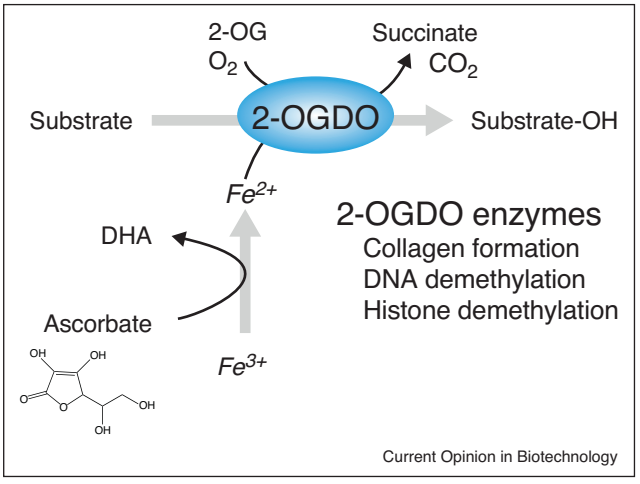
Why do humans need dietary ascorbate?

While most mammals are able to make ascorbate, it is classified as a vitamin (or essential micronutrient) because humans have lost the ability to synthesize it. This is due to mutations within the *L-gulono-γ-lactone oxidase* (*GLO*) gene, which in other mammals encodes the enzyme catalysing the last step in the ascorbate biosynthetic pathway [7]. The mutations rendering the GLO enzyme nonfunctional occurred ~61 million years ago in our primate ancestor we share with tarsiers, monkeys and apes [7]. Two other mammalian lineages; guinea pigs and bats have also lost the ability to synthesize ascorbate as a result of mutations within the *GLO* gene [7]. At the time these mutations occurred, the three ancestral groups would have had ascorbate-rich diets of fruit and/or vegetation. Under these conditions there would have been little selective pressure to maintain the ability to synthesize ascorbate (there is also no evidence that there was a

selective advantage to losing it) [7]. However, the neutrality of this genetic mutation is entirely dependent on the environment. When humans do not consume sufficient ascorbate (*i.e.*, less than a total of ~10 mg per day), the consequences of lacking a functional *GLO* gene are devastating. Over human history dietary ascorbate deficiency has resulted in millions of people suffering a miserable death due to scurvy [8]. Indeed the name ascorbate is a derivative of the term 'antiscorbutic': having the effect of preventing scurvy. Our primate ancestor likely consumed excess ascorbate, similar to modern gorillas and spider monkeys who consume ~25 and ~100 mg ascorbate/Kg body mass/day, respectively. Today, eating sufficient fruit and vegetables to consistently obtain more than the minimum level of ascorbate can be a challenge for many people in the developed and developing world [4,9]. While scurvy is uncommon today, many people throughout the world obtain less than the Institute of Medicine recommended daily allowance (RDA) for ascorbate of about 1 mg/Kg/day. A USA health and nutrition survey revealed that 7% of adults had serum ascorbate concentrations regarded as deficient (<11.4 μmol/L) and 16% had serum concentrations regarded as low (11.4–16.5 μmol/L) [9]. The survey also revealed indications of latent scurvy, which includes low energy and weakness.

Research is revealing the importance of ascorbate in human health well beyond simply preventing scurvy. Ascorbate plays two key roles in the cell; it is a general antioxidant and it serves as a cofactor for dioxygenase (the Fe^{2+}/2-oxoglutarate-dependent dioxygenases; 2-OGDO) and monooxygenase enzymes (Figure 1). The symptoms of scurvy are classically associated with the role ascorbate plays in the activity of the 2-OGDO enzyme, collagen prolyl 4-hydroxylase (CP4H) [10]. Ascorbate reduces the reactive core iron atom from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state, which is required for CP4H to hydroxylate the proline residues of collagen that form stable collagen fibers. Ascorbate is also needed as a cofactor to reduce Fe^{3+} to Fe^{2+} for other 2-OGDO enzymes that play important roles in the cell (Figure 1). For example, two classes of 2-OGDO enzymes involved in epigenetic programming; the methylcytosine dioxygenases (known as ten-eleven translocation (TET) dioxygenases) that are responsible for DNA demethylation [11,12] and the Jumonji C (JmjC) domain containing histone demethylases [13]. The link between ascorbate and epigenetics has profound implications on how dietary ascorbate might impact on human health. Epigenetic programming plays a key role in embryonic development, the progression of cancer and age-related diseases and there is evidence ascorbate influences all of these processes [10•,14•]. Therefore continually obtaining sufficient dietary ascorbate is likely important for long-term human health.

Figure 1



The importance of ascorbate for the activity of Fe^{2+}/2-oxoglutarate-dependent dioxygenase (2-OGDO) enzymes.
The catalytic activity of 2-OGDO enzymes requires ferrous (Fe^{2+}) iron, which is generated from ferric (Fe^{3+}) by the conversion of ascorbate (vitamin C) to dehydroascorbate (DHA). Hydroxylation of substrate molecules by 2-OGDO enzymes involves the conversion of 2-oxoglutarate (2-OG) and O_2 to produce succinate and CO_2. The roles of some 2-OGDO enzymes important for human health are shown.

Ascorbate has also been shown to play a key role in regulating iron uptake. Iron deficiency anemia is the most common and widespread micronutrient deficiency, affecting around two billion people worldwide, especially women and children [15]. It has been long known that ascorbate enhances uptake of non-haem iron from the diet [16]. Vegetable derived non-haem iron is typically less easily absorbed than haem iron from meat, with uptake of haem iron also being less prone to being influenced by gastric pH. However, rather than simply acting in the gut to convert iron into a form that is more readily absorbed, ascorbate has now been shown to regulate cellular iron uptake [17]. While the exact mechanisms by which ascorbate enhances iron uptake are yet to be fully understood, there is good evidence that increasing the consumption of foods rich in ascorbate reduces iron deficiency anemia [18,19].

Reasons to develop crops with elevated ascorbate levels

As the nutritional benefits of ascorbate in food are identified and validated the case to biofortify common food sources with ascorbate increases. The major commodity crops in both developed and developing countries are notable for the low amount of ascorbate they contain (Table 1). With the exception of potatoes and sweet potatoes, all of the major food staples have less than 10 mg ascorbate per 100 g fresh weight and seed-based staples have very low levels of ascorbate. Postharvest storage and cooking further reduces ascorbate levels in

Table 1

Ascorbate levels[a] and production volume[b] of major global commodity crops[c]

Commodity	Ascorbate (mg/100 g FW)	Production (tonnes × 10^{12})
Rice, paddy	0.2	738
Wheat	0.2	671
Barley	0.6	134
Apples	4.6	76
Maize	4.8	873
Onions, dry	6.4	83
Watermelons	8.1	105
Bananas	8.7	102
Tomatoes	10	162
Potatoes	11.4	365
Sweet potatoes	17.1	108
Cassava	20	269
Soybeans	29	241

[a] According to USDA food composition database [51].
[b] According to FAO [52].
[c] Excluded from the table; sugarcane and sugar beet, as they are used for processed sugar; and the FAO food category 'vegetable, fresh' as this includes a range of crops with differing ascorbate levels (but generally >50 mg ascorbate/100 g fresh weight).

the food we eat [20,21]. One way to increase dietary ascorbate intake is to develop crops that contain higher ascorbate levels using biotechnological approaches (summarized in Table 2 and discussed in the next section).

As well as being important for human nutrition, ascorbate plays a vital role in plants as the major antioxidant that protects cells against the damaging effects of ROS produced as consequence of aerobic metabolism. The overproduction of ROS, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO^-) is a common consequence of many abiotic stresses [22]. Ascorbate, the most abundant water-soluble antioxidant in plants, can directly mitigate the damaging effects of ROS as a ROS scavenger or indirectly as a substrate for the ascorbate peroxidase enzyme (APX) [23]. Notably, ascorbate plays a core role in the ascorbate-glutathione cycle, a major antioxidant system of plant cells [1]. Through crop domestication ascorbate levels may have been inadvertently diluted in favor of increased growth and yield. This could have important ramifications for crop performance, particularly in terms of stress tolerance and plasticity in response to environmental changes [2]. The biotechnological manipulation of ascorbate levels has revealed that even minor increases improves tolerance to a range of stresses (Table 2).

Approaches to elevate ascorbate levels in crops

A large number of biotechnological interventions have been made in order to elevate ascorbate levels with varying degrees of success (see Table 2 for recent examples). These approaches have focused on overexpressing genes encoding enzymes involved in ascorbate biosynthesis or ascorbate recycling and overexpressing regulatory genes.

Increasing ascorbate via overexpression of biosynthetic genes

The major route for ascorbate synthesis in plants is through the L-galactose pathway (also known as the Wheeler–Smirnoff pathway) [24] (Figure 2). A number of studies have demonstrated that the rate-limiting step in this pathway is the conversion of GDP-L-galactose to L-galactose-1-P, which is catalyzed by the enzyme GDP-L-galactose phosphorylase (GGP) [5]. As the cell also uses GDP-D-mannose for the synthesis of other compounds (and to a much lesser extent GDP-L-galactose) (Figure 2), GGP catalyses the first ascorbate-specific step in the pathway. Overexpressing the *GGP* gene consistently results in two–sixfold increases in ascorbate in wide range of plant species (Table 2). For example, Bulley *et al.* [25] overexpressed *GGP* in tomato and strawberry using a strong constitutive promoter (35S promoter) and obtained fruit with up to sixfold (111 mg/100 g FW) and twofold (131 mg/100 g FW) increase in fruit ascorbate levels, respectively. The tomato fruit with the highest levels of ascorbate lacked seeds for unknown reasons but the strawberry fruit developed normally [25]. *GGP* has also been overexpressed in potato [25] and this resulted in tubers with an up to threefold increase in ascorbate levels (up to 36 mg/100 g FW).

Alternative pathways

While there is now good evidence that the L-galactose pathway is responsible for the majority of ascorbate synthesized in plants, other pathways might also be important in some situations. An alternative pathway of ascorbate biosynthesis is through conversion of cell wall polysaccharide derived galacturonate to L-galactono lactone which feeds into the terminal step of the L-galactose pathway (Figure 2). Overexpression of a strawberry *L-galacturonic acid reductase* gene (*GalUR*) in potato tubers and tomato fruit elevated ascorbate 2.5- and 1.4-fold above control levels, respectively [26,27] (Table 2). The relative importance of the galacturonate pathway appears to vary according to tissue type and developmental stage [28,29]. Another potential pathway to ascorbate, through myo-inositol via glucoronate and finally L-gulono-1,4-lactone (Figure 2), has been investigated by over expressing and knocking out *myo-inositol oxygenase* (*MIOX*) genes. This work indicates that myo-inositol does not significantly contribute to ascorbate production [30,31]. Another approach has been to overexpress the *D-Arabinono-1,4-lactone oxidase* (*ALO*) gene from yeast and rats in plants. As well as utilizing D-arabinono-1,4-lactone to produce D-erythroascorbate, the ALO enzyme can use L-galactono-1,4-lactone to produce ascorbate [3,32–35] (Table 2).

Table 2

Recent examples of biotechnological approaches to increase ascorbate in plants for biofortification or stress tolerance

Gene transformed	Species transformed	Tissue examined	Max fold increase	Stress tolerance	Reference
Galactose pathway					
GGP	Tomato	Fruit	6.2		[25]
GGP + *GPP*	*Arabidopsis*	Leaves	4.0		[53]
GGP + *GLDH*	*Arabidopsis*	Leaves	3.5		[53]
GGP	Potato	Tubers	3.0		[25]
GGP	*Arabidopsis*	Leaves	2.9		[53]
GGP	Rice	Leaves	2.5	Salt	[54••]
GGP	Strawberry	Fruit	2.1		[25]
GLDH	Tobacco	Leaves	2.1	Salt & herbicide	[55]
GLDH	*Arabidopsis*	Leaves	1.8		[53]
GPP	*Arabidopsis*	Leaves	1.6		[53]
GME	*Arabidopsis*	Leaves	1.6	Drought, salt, & acid	[56]
GME	*Arabidopsis*	Leaves	1.4		[53]
GME	Rice	Leaves	1.4	Salt	[54••]
GMP	*Arabidopsis*	Leaves	1.4		[53]
GDH	*Arabidopsis*	Leaves	1.3		[53]
GMP	Tomato	Fruit red	1.3		[35]
GLDH	Lettuce	Leaves	1.3		[57]
GMP	*Arabidopsis*	Leaves	1.2		[58••]
GME	*Arabidopsis*	Leaves	1.1		[39]
GMP + *GME*	Tobacco	Young leaves	1.0		[59]
GMP + *GME*	Tobacco	Old leaves	1.0		[59]
Alternative pathways					
ALO (yeast)	Stylo	Leaves	3.1	Chilling	[32]
GalUR	Tomato	Fruit	2.5	Herbicide, salt, & drought	[60]
GalUR	Tomato	Fruit & leaves	2.0	Herbicide, salt, & cold	[61]
ALO (rat)	*Arabidopsis*	Leaves	1.7	Salt, cold, heat, & herbicide	[3]
ALO (yeast)	Tobacco	Leaves	1.6	Herbicide, high light, & Al toxicity	[33]
MIOX	*Arabidopsis*	Leaves	1.5	Salt, cold, heat, & herbicide	[62]
ALO (rat)	Tomato	Fruit	1.5	Herbicide, salt, & drought	[34]
GalUR	Tomato	Fruit	1.4		[27]
MIOX	Tomato	Fruit red	1.3		[35]
ALO (yeast)	Tomato	Fruit red	1.1		[35]
Recycling pathway					
DHAR	Tomato	Leaves & fruit	1.9	Salt & herbicide	[36]
MDAR	Tobacco	Leaves	1.8	Salt	[37]
DHAR	Tomato	Leaves	1.5	Salt & chilling	[38]
DHAR	*Arabidopsis*	Leaves	1.4		[39]
DHAR	*Arabidopsis*	Leaves	1.3		[40]
Regulatory factors					
AtERF98	*Arabidopsis*	Leaves	1.7	Salt	[63]
SlHZ24	Tomato	Fruit & leaves	1.6	Herbicide	[64••]
KONJAC	*Arabidopsis*	leaves	1.4		[58••]
CSN5B[a]	*Arabidopsis*	Seedling	1.4		[65]
SlDof22	Tomato	Fruit	1.3	Salt	[66]
CML10[a]	*Arabidopsis*	Seedlings	−1.4		[67•]
VTC3[a]	*Arabidopsis*	Leaves	−3.8	Heat & light stress	[68]

[a] Phenotype of mutant rather than overexpressing line.

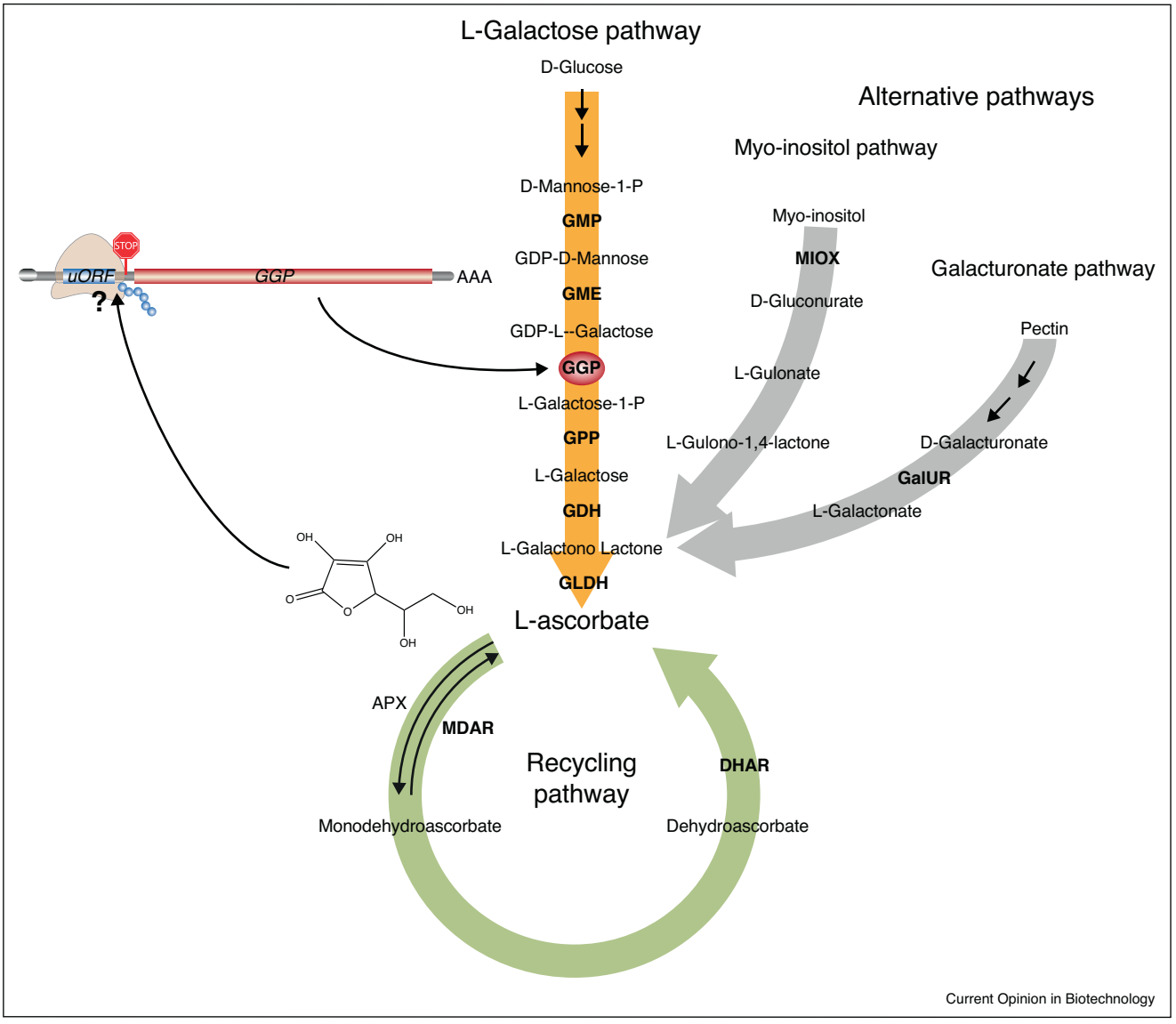
Increasing ascorbate recycling

Non-biosynthetic interventions have also been successful at elevating ascorbate levels in plants. Oxidized ascorbate in the form of dehydroascorbate is less stable than reduced ascorbate and quickly lost. Overexpressing the ascorbate recycling genes *monodehydroascorbate reductase* (*MDAR*) and *dehydroascorbate reductase* (*DHAR*) typically increased ascorbate around 1.2- to 2-fold and has been shown to increase abiotic stress tolerance [36–40] (Table 2).

Altering the regulation of ascorbate biosynthesis

Altering the regulation of the ascorbate production might be a more effective way of increasing ascorbate levels. However, despite progress in elucidating the biosynthetic pathways, only a few regulatory genes have

Figure 2



Summary of the pathways involved in ascorbate biosynthesis and recycling.
The L-galactose pathway is the major route for ascorbate biosynthesis in plants. The enzyme GGP is the rate-limiting step of this pathway; and the gene encoding GGP is regulated at the level of translation via an upstream open reading frame (uORF) which inhibits translation of the downstream GGP coding region in the presence of high ascorbate, via an unknown mechanism [6••]. The ascorbate recycling pathway and potential alternative pathways for ascorbate production are shown. The enzymes that are described in the text are shown in bold.

been identified [5] (Table 2). Further work is needed to understand how these various signaling pathways function to control ascorbate levels.

It has been discovered recently that the translation of *GGP* is feedback controlled by ascorbate [6••]. Under high ascorbate, translation of the *GGP* mRNA is reduced and under low ascorbate translation increases. This regulation is through a highly conserved upstream open reading frame (uORF) in the *GGP* 5′ upstream untranslated region (5′UTR) mRNA [6••]. Unusually, this uORF initiates from an ACG (Thr) rather than an AUG (Met) codon. Disabling this uORF in a model system not only increased translation of reporter genes controlled by the uORF in the 5′UTR, but also results in increased ascorbate in transiently transformed leaves in a *GGP promoter-uORF-GGP* construct when comparing the functional uORF to a disabled uORF [6••]. Disabling this uORF would be an obvious target of gene editing as just changing a single nucleotide is sufficient to inactivate the uORF (*e.g.*, mutating the ACG start codon).

Conclusions and prospects for the future

Biotechological approaches to increase ascorbate levels in plants have predominantly focused on using a strong constitutive promoter to overexpress single genes encoding enzymes within the ascorbate biosynthesis or recycling pathways (Figure 2). This has resulted in somewhat modest increases in ascorbate levels (at most ~150 mg/100 g FW). (Table 2). Other examples of plant metabolic engineering have revealed that increasing the level of a single potentially rate-limiting enzyme does not usually dramatically alter pathway flux [41]. Therefore future efforts to engineer crops with increased ascorbate should focus on co-expressing multiple steps in the pathway. Consistent with this idea, transient expression of two of the L-galactose pathway genes, *GGP* and *GDP-D-mannose epimerase* (*GME*) genes in *Nicotiana benthamiana* leaves has resulted in the greatest increase in ascorbate levels to date, ~12-fold (>300 mg/100 g FW) [6,42]. Overexpressing *GME*, which catalyzes the production of GDP-L-galactose – the substrate of GGP – by itself had little effect on ascorbate. This suggests that the epimerase reaction only becomes limiting when the production of ascorbate is significantly raised above normal levels. Stably transformed lines co-expressing *GGP* and *GME* have not been reported. Similarly, co-expression of ascorbate biosynthetic and ascorbate recycling genes might result in large pools of reduced ascorbate and should be investigated.

Ascorbate production is regulated and maintained within a normal physiological range through various transcriptional, translational, and post-translational regulatory mechanisms (Table 2). A better understanding of these regulatory mechanisms is needed. In addition, attempts to increase ascorbate levels have only examined steady state levels (Table 2) and it is not known if the rate of ascorbate degradation also increased in these plants. Fruit from a few plant species contain extremely high ascorbate levels (~500 to 3000 mg/100 g FW) (*e.g.*, *Myrciaria dubia* [camu–camu] [43], *Malpighia glabra* [acerola] [44] and *Actinidia eriantha*, a wild kiwifruit variety [42]). The existence of these 'superfruits' indicates that it is possible to significantly increase fruit ascorbate levels. Discovering how the regulation of ascorbate production is altered in these plants to allow such high levels will provide new insights into how to increase ascorbate levels in commercial fruit species.

While this review has focused on biotechnological approaches, traditional breeding also has potential to increase ascorbate levels. Genetic analysis of ascorbate levels in a number of food crops, including tomatoes [45], and apple [46], indicate that it is a quantitative trait with a few loci potentially explaining a significant proportion of the variation in ascorbate levels [46–48]. The genes encoding the ascorbate biosynthetic and recycling enzymes are often present in multiple copies and can have particular tissue-specific or developmental roles [47,49,50]. Identifying these loci and understanding how specific genetic variants lead to increased ascorbate should shed light on how ascorbate levels are controlled [47]. The development of genetic markers linked to these loci would also allow for more efficient breeding of varieties with elevated fruit ascorbate levels, since selection could be done at the seedling stage rather than having to wait for several years for mature fruiting plants.

Since the first proposed biosynthetic framework for plant ascorbate synthesis in 1998 [24], significant progress has been made in understanding and manipulating the production of ascorbate in plants. Gaining a better understanding of ascorbate regulation and the mechanisms that underlie the variation in its levels in different plants should lead to new ways to manipulate ascorbate levels in crops. This knowledge, together with new methodologies such as gene editing, will hopefully provide simple and effective strategies for increased ascorbate levels; for example, by disrupting negative regulators of ascorbate biosynthesis, such as the putative uORF that regulates *GGP* translation [6••].

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