# Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase

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L-Ascorbic acid (vitamin C) in fruits and vegetables is an essential component of human nutrition. Surprisingly, only limited information is available about the pathway(s) leading to its biosynthesis in plants. Here, we report the isolation and characterization of *GalUR*, a gene from strawberry that encodes an NADPH-dependent D-galacturonate reductase. We provide evidence that the biosynthesis of L-ascorbic acid in strawberry fruit occurs through D-galacturonic acid, a principal component of cell wall pectins. Expression of *GalUR* correlated with changing ascorbic acid content in strawberry fruit during ripening and with variations in ascorbic acid content in fruit of different species of the genus *Fragaria*. Reduced pectin solubilization in cell walls of transgenic strawberry fruit with decreased expression of an endogenous pectate lyase gene resulted in lower ascorbic acid content. Overexpression of *GalUR* in *Arabidopsis thaliana* enhanced vitamin C content two- to threefold, demonstrating the feasibility of engineering increased vitamin C levels in plants using this gene.



Since vitamin C (defined as ascorbic acid and its oxidation product dehydroascorbate) was first isolated, there have been numerous reports on the physiological and metabolic processes in which it is involved[1]. Ascorbic acid is crucial to the maintenance of a healthy immune system and is required for the synthesis of collagen, carnitine, and neurotransmitters. In general, it acts as an enzyme cofactor, free radical scavenger, and donor and acceptor in electron transfer reactions[2]. As a consequence, its most vital role in the human body is as a water-soluble antioxidant.

The biosynthetic pathway of L-ascorbic acid in animals involves intermediates of the D-glucuronic acid pathway[3] (Fig. 1). In plants, ascorbic acid biosynthesis occurs by a different pathway[4–6], with L-galactose being the immediate precursor of the key intermediate L-galactono-1,4-lactone[2,7] (Fig. 1). This pathway proceeds via GDP–D-mannose and GDP–L-galactose, as indicated by radiolabeling data[8]. Another proposed biosynthetic pathway in plants includes D-galacturonic acid as a metabolic precursor of L-galactono-1,4-lactone[9] (Fig. 1). Although radiotracer[10] and biochemical evidence, including data demonstrating the enzymatic reduction of derivatives of D-galacturonic acid to L-galactonic acid by plant extracts[11], supported this alternative pathway, there had previously been no confirming molecular data. Here we demonstrate that the biosynthesis of L-ascorbic acid in ripe strawberry fruit can occur through D-galacturonic acid. This supports previous suggestions that this pathway could constitute a carbon salvage mechanism in certain organs after the breakdown of cell walls, such as occurs during fruit ripening[2,12]. Pectins are a major cell wall component whose main building blocks, homogalacturonan and rhamnogalacturonan I, release D-galacturonic acid upon hydrolysis. We show that vitamin C levels can be increased in *A. thaliana* plants by overexpressing the strawberry gene *GalUR*, encoding a D-galacturonic acid reductase. This enzyme converts D-galacturonic acid into L-galactonic acid, which is readily converted to L-galactono-1,4-lactone, the immediate precursor of ascorbic acid.

## Results

**Cloning of a gene (*AKR*) encoding aldo-keto reductase from strawberry.** The cultivated strawberry (*Fragaria* × *ananassa*) is an important small fruit crop in temperate regions. Ripening of the fruit occurs over a short time period and is accompanied by a change in the expression of many genes[13]. Ripe strawberry fruit are rich in ascorbic acid, containing an average of 60 mg per 100 g fresh weight, although this varies among cultivars[14]. It has been reported that ascorbic acid content increases as fruit ripens[2,14]. Early studies reported the conversion of D-galacturonic acid to L-ascorbic acid in ripening strawberry fruit[10]. A key enzymatic activity of this pathway was an NAD(P)H-dependent reductase that was present in the soluble fraction of some plant extracts[11]. We confirmed the presence of D-galacturonic acid reductase activity in the crude extracts of ripe strawberry fruit with a mean value of 16.2 nmol $min^{-1}$ per milligram of protein.

Using differential display, we identified numerous genes expressed in ripe strawberry (*Fragaria* × *ananassa*) fruit[13]. Of all the cDNAs analyzed, several shared significant homology to cytosolic NAD(P)H-dependent aldo-keto reductases (AKRs)[15]. After assembling the cDNA fragments, we obtained two contigs, indicating that they encoded two different AKRs (AKR1 and AKR2), both with the consensus motifs for this family of proteins clearly identifiable[15].

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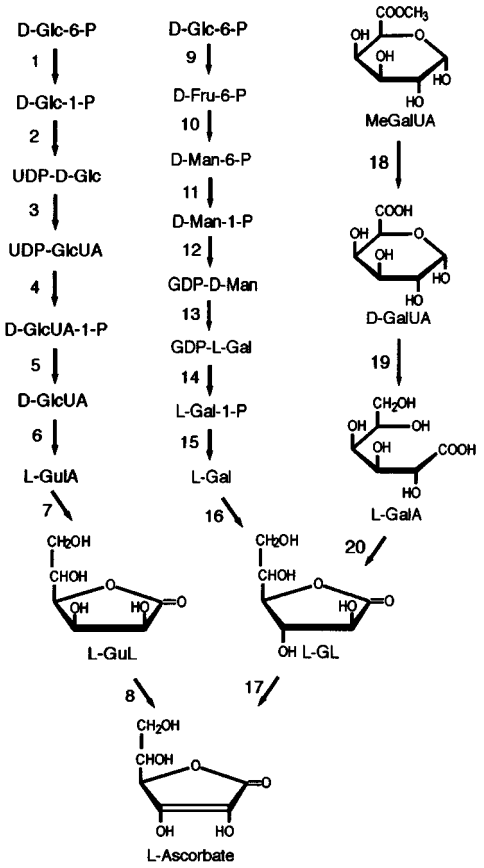


**Figure 1.** Proposed biosynthetic pathways of L-ascorbic acid in animals (reactions 1–8) and plants (reactions 9–20). Glc, glucose; GlcUA, glucuronic acid; GulA, gulonic acid; GuL, gulono-1,4-lactone; Fru, fructose; Man, mannose; Gal, galactose; GL, galactono-1,4-lactone; GalUA, galacturonic acid; GalA, galactonic acid. Enzymes catalyzing the numbered reactions are: 1, phosphoglucomutase; 2, UDP-glucose pyrophosphorylase; 3, UDP-glucose dehydrogenase; 4, glucuronate-1-phosphate uridylyltransferase; 5, glucurono kinase; 6, glucuronate reductase; 7, aldono-lactonase; 8, gulono-1,4-lactone dehydrogenase; 9, glucose-6-phosphate isomerase; 10, mannose-6-phosphate isomerase; 11, phosphomannomutase; 12, GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase); 13, GDP-mannose 3,5-epimerase; 14, phosphodiesterase; 15, sugar phosphatase; 16, L-galactose-1-dehydrogenase; 17, L-galactono-1,4-lactone dehydrogenase; 18, methylesterase; 19, D-galacturonate reductase; 20, aldono-lactonase.

AKR1 has high identity to quinone oxido-reductases (data not shown). The proteins most similar to AKR2 (GenBank accession no. AF039182) are a codeinone reductase from the opium poppy (*Papaver somniferum*)[16] and chalcone reductases from soybean (*Glycine max*), alfalfa (*Medicago sativa*), and a tropical legume (*Sesbania rostrata*); however, this similarity was mainly restricted to the consensus motif characteristic of AKRs (data not shown).

***AKR2* encodes a D-galacturonic acid reductase.** In the ascorbic acid biosynthetic pathways proposed in higher plants, three reactions can be identified as putatively catalyzed by an AKR enzyme: the oxidation of L-galactose using $NAD^+$ as the cofactor and the reduction of either D-glucuronic or D-galacturonic acid, both using NADPH as the cofactor (Fig. 1). We assayed the enzyme activity of a recombinant AKR2, obtained as a glutathione S-transferase (GST)-fused protein in *Escherichia coli*, with the three substrates but did not detect any activity. However, this could be due to a requirement for post-translational modification of the gene product. To assure the availability of active enzyme, we immunopurified the protein from strawberry fruit using antibodies generated against the recombinant protein, but were unable to obtain a significant amount of pure protein. This was most likely due to the high polysaccharide content of ripe strawberry fruit extracts.

As an alternative strategy, we expressed *AKR2* ectopically in *A. thaliana* plants. Western analysis of 17 independent transgenic lines revealed several lines with high levels of AKR2 (lines 3, 4, 8, 10, 11, and 17; Fig. 2A). We assayed the enzymatic activity in the crude extracts of three transgenic lines (lines 3, 10, and 17), using as controls both the wild type and a line transformed with the empty vector pSOV2 (ref. 17; Table 1). We found that D-galacturonic acid reductase activity was enhanced in the transgenic lines relative to the controls, whereas activities of D-glucuronic acid reductase or L-galactose oxidase activities were no different from those in the control plants. To demonstrate fully that AKR2 itself was responsible for this activity, we immunopurified the protein from line 17 to electrophoretic homogeneity. The purified enzyme showed very high and specific NADPH-dependent reduction of D-galacturonic acid, with a value 250-fold higher per milligram of protein than that of the crude extract (Table 1). Hereafter we renamed *AKR2* as *GalUR*.

**Overexpression of *GalUR* increases vitamin C content in *A. thaliana*.** We next set out to determine whether transgenic *A. thaliana* lines ectopically expressing *GalUR* showed increased levels of ascorbic acid. The ascorbic acid content of three lines (3, 10, and 17) was two to three times higher than that of *A. thaliana* control plants (Fig. 2B). In addition, feeding the *A. thaliana* plants with D-galacturonic acid resulted in increased ascorbic acid content of the transgenic lines (10 and 17), whereas the level in the control *A. thaliana* plants remained unchanged (Fig. 2C).

**Vitamin C levels in strawberry fruit correlate with the expression of *GalUR*.** *GalUR* expression was specific to the receptacle tissue of strawberry fruit, both at the mRNA and protein levels, and its expression increased during ripening, being highest in fully mature red fruit (Fig. 3A–D). The amount of ascorbic acid in ripening strawberry fruit broadly correlates with the expression of *GalUR* (Fig. 3E). We also determined the presence of GalUR protein in ripe fruit of several *Fragaria* species: *F. chiloensis*, *F. virginiana*, and *F. moschata* (Fig. 3F). Amounts of GalUR varied among these species, and again the levels of ascorbic acid correlated with the expression levels (Fig. 3G). In *F. moschata* fruit, levels of both ascorbic acid and GalUR protein were very low, substantially different from those in other *Fragaria* species.

These results suggest that GalUR activity contributes to a substantial proportion of the ascorbic acid content of ripe strawberry fruit. If D-galacturonic acid is indeed an *in vivo* precursor in ascorbic acid biosynthesis, differences in ascorbic acid content may also be determined by the availability of the substrate. A number of transgenic strawberry

**Table 1. Enzyme activities of extracts of transgenic *A. thaliana* and the immunopurified enzyme**

| Substrate | WT | pSOV2 | Line 3 | Line 10 | Line 17 | Purified enzyme |
|---|---|---|---|---|---|---|
| D-Galacturonic acid | 0.10 ± 0.01 | 0.12 ± 0.02 | 1.12 ± 0.22 | 1.85 ± 0.27 | 4.80 ± 0.41 | 1,213.00 ± 68.02 |
| D-Glucuronic acid | 0.05 ± 0.01 | 0.03 ± 0.01 | 0.06 ± 0.03 | 0.20 ± 0.18 | 0.05 ± 0.01 | 15.45 ± 8.06 |
| L-Galactose | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.08 ± 0.02 | 0.10 ± 0.01 | 0.10 ± 0.01 | 13.42 ± 9.33 |

One unit of enzyme activity is defined as the number of nanomoles of NADPH oxidized (for D-galacturonic and D-glucuronic acids) or $NAD^+$ reduced (for L-galactose) per minute per milligram of protein. Protein concentrations were measured by the Bradford method[31]. Values given are ± s.e.

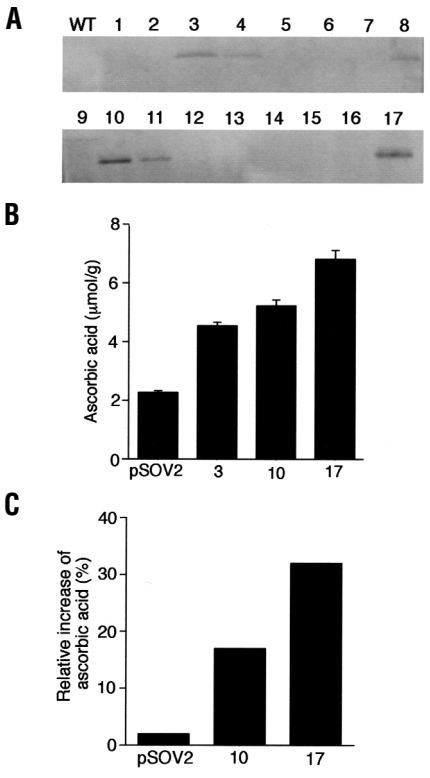


**Figure 2.** Overexpression of *GalUR* in *A. thaliana* increases the ascorbic acid content. (A) Immunoblot of extracts (10 μg total protein) prepared from young seedlings of 17 independent *A. thaliana* transgenic lines transformed with the *GalUR* coding sequence under the control of the 35SCaMV constitutive promoter. (B) Ascorbic acid content in the extracts of control (pSOV2) and three homozygous *A. thaliana* transgenic lines, numbers 3, 10, and 17. (C) Increase in whole-plant ascorbic acid content in 20-day-old *A. thaliana* control (pSOV2) and transgenic plants overexpressing *GalUR* (lines 10 and 17) after 48 h of supplementation with 30 mM D-galacturonic delivered in the hydroponic medium.

plants that incorporate an antisense sequence of a strawberry pectate lyase gene under the control of the 35S promoter have been generated[18]. In these antisense lines, the pectate lyase gene expression in ripe fruit is reduced, and fruit firmness is increased as result of a lower pectin solubilization[18]. When we measured the ascorbic acid content in the ripe fruit of these transgenic strawberry lines, we found that the ascorbic acid content was substantially lower than in the fruit of the untransformed control plants (Fig. 3H).

## Discussion

Radiotracer studies have frequently been used to identify intermediates and steps in metabolic pathways. However, the application of this approach to strawberry fruit has produced seemingly contradictory results. In one study, when labeled glucose was administered through the cut stem to strawberry fruit in the green-white stage of ripening, the L-galactose pathway was favored[8]. A detailed analysis of the literature indicates that specific ascorbic acid labeling by [$^{14}$C]glucose, administered either through the stem or by injection into the receptacle, decreases as treated fruit changes from white-pink to red stage[19]. By contrast, in a study in which D-galacturonic was administered to the cut stems of ripening berries, it was metabolized to L-ascorbic acid[10], indicating that both pathways function in strawberry fruit.

In this work we have isolated the gene encoding D-galacturonate reductase, which catalyzes a key step in this pathway (Fig. 1). We have shown that the expression of this gene correlates with *in vivo* ascorbic acid content both in ripening strawberry fruit and in transgenic *A. thaliana* plants. During the latest stages of fruit ripening, the cell walls are remobilized and hydrolytic pectin compounds are produced[20]. This process guarantees the availability of D-galacturonic acid. We speculate that the two pathways also function in other plants and that their relative contribution to vitamin C content is developmentally regulated. In photosynthesizing plant cells, the L-galactose pathway prevails and contributes to maintain the cells' oxidation-reduction state. This is supported by the identification of an *A. thaliana* mutant affected in this pathway that contains only 25% as much ascorbic acid as the wild type[21]. In addition, the ascorbic acid content in *A. thaliana* plants with antisense-suppressed L-galactose dehydrogenase reaches 45% as high as that of the wild type[22]. In contrast, evidence for the D-galacturonate pathway has been reported in *A. thaliana*: administration of the methyl ester of D-galacturonic acid to cultured *A. thaliana* cells substantially increases their ascorbic acid content[23].

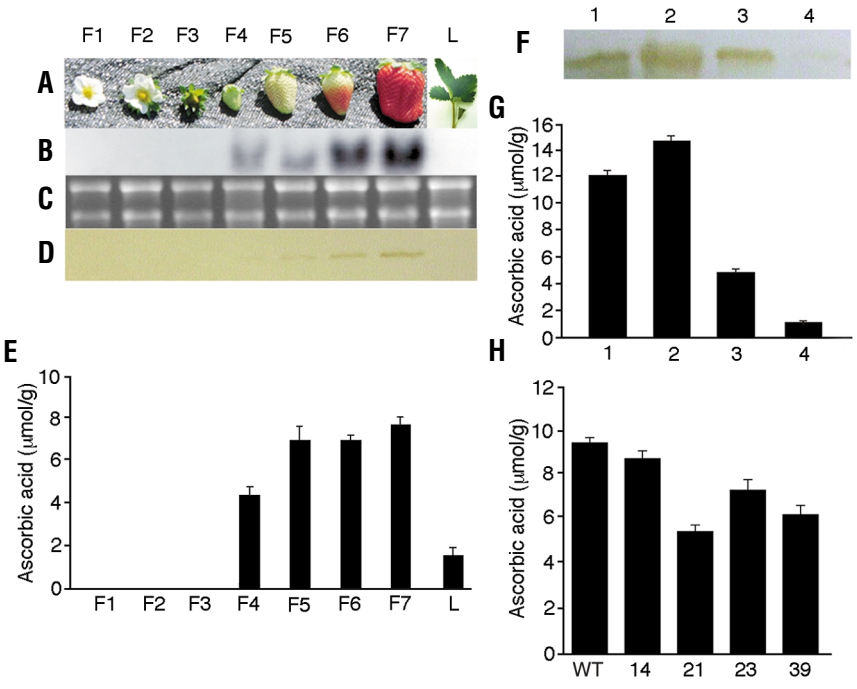


**Figure 3.** Ascorbic acid content correlates with the expression of *GalUR*. (A) Photographs of flowers and fruit at the developmental stages sampled: F1, young flower; F2, mature flower; F3, small green fruit; F4, green fruit; F5, white fruit; F6, intermediate fruit; F7, red fruit. (B) Northern blot of total RNA extracted from strawberry fruit at different ripening stages (F1–F7) and from leaves (L). (C) rRNA is shown as a loading control. (D) GalUR protein content, determined by immunoblot, at ripening stages F1–F7. (E) Ascorbic acid content of the tissues described in (A). (F) GalUR protein content, as determined by immunoblotting, of red-ripe fruit of *Fragaria* × *ananassa* (cv. Chandler) (1), *F. chiloensis* (2), *F. virginiana* (3), and *F. moschata* (4). (G) Ascorbic acid content of the same *Fragaria* species as in (F). (H) Ascorbic acid content in ripe fruit of wild-type (WT) and several transgenic strawberry plants overexpressing the antisense pectate lyase (lines 14, 21, 23, and 39).

In our study, we not only provide direct evidence for a previously hypothesized ascorbic acid biosynthetic pathway in strawberry fruit, but also show that overexpression of *GalUR* in *A. thaliana* results in a two- to threefold increase in vitamin C content. These results suggest both the feasibility of engineering increased vitamin C content and the broad function of the enzyme *GalUR*. Because the substrate of GalUR, D-galacturonic acid, is an abundant component of the cell walls of all plants, it constitutes a potentially universal substrate for the synthesis of this important compound. A previous report showed that the vitamin C content of lettuce can be increased up to sevenfold by overexpression of a L-gulono-γ-lactone oxidase gene from the rat[24]. It should be noted, however, that plants genetically modified with rat genes might not be appealing to consumers.

Vitamin C is the single most important specialty chemical manufactured in the world. Its industrial synthesis is a lengthy procedure involving microbial fermentation and a series of chemical steps[25]. The identification of the *GalUR* gene provides a new tool whose commercial application may have a substantial impact on the production of this highly valuable compound.

## Experimental protocol

**Growth of plants.** Strawberry plants were grown under field conditions in Huelva, in the southwest of Spain. The cultivar used was a commercial variety registered by the University of California (Davis) as *Chandler*. Fruit growth stages F1–F7 are depicted in Fig 3A. Fruit from other *Fragaria* species were provided by the *Fragaria* germ plasm collection from the Churriana Center (Málaga, Spain). *A. thaliana* (Columbia) plants were grown in a chamber under a 16 h/8 h cycle of 22 °C light/17 °C dark (light levels were 200 μmol $m^{-2}$ $s^{-1}$). Ascorbic acid content was measured in plants 18–20 days old. In the feeding experiments with D-galacturonic acid, the *A. thaliana* plants were grown in hydroponic Murashige and Skoog medium[26] under the same long day–high light regime.

**Immunoblotting.** GST-fused GalUR protein was obtained after cloning the 956 bp *GalUR* open reading frame (ORF) into a pGEX-KG plasmid[27] using added in-frame *Xba*I and *Hind*III restriction sites. The new plasmid pGEX-GalUR was transformed into XL1-Blue MRF' competent cells (Stratagene, La Jolla, CA). Protein was purified on a glutathione-agarose column (Sigma, St. Louis, MO) and eluted with 50 mM Tris and 2mM EDTA buffer (pH 8.0) containing 10 mM glutathione. The purified fusion protein was injected into New Zealand rabbits using standard protocols[28] and the antiserum was recovered. Proteins (20 μg) from the plant tissues were separated on a 10% (w/v) SDS-polyacrylamide gel and electroblotted. Polyclonal antibodies raised against the GST-GalUR recombinant protein were used for inmunoblotting, and blots were developed using peroxidase-conjugated anti-rabbit secondary antibodies following standard protocols.

**Preparation of transgenic plants.** A 956 bp fragment containing the complete ORF of the *GalUR* gene was amplified by PCR using a high-fidelity polymerase enzyme and oligonucleotides containing *Pst*I and *Hin*dIII sites. This product was subcloned in the pSOV2 binary vector[17], which contains the 35SCaMV promoter, after digestion with the *Pst*I and *Hin*dIII restriction enzymes. The new construct was transformed into *E. coli* cells and then delivered to strain GV3101 *Agrobacterium tumefaciens* by triparental mating. *A. thaliana* plants were transformed by vacuum infiltration[29]. Seeds were recovered from transformed plants, germinated, grown for 9–10 d, and sprayed with a 0.1 g/liter solution of phosphinotricin. After repeating the herbicide treatment four times, every 2 d, the seeds of the transgenic resistant plants were selected. Homozygous plants were identified among the T1 seedlings and used in subsequent experiments.

**Enzyme extraction, purification, and assay.** Crude extracts were prepared from 1 g of either ripe strawberry fruit, or seedlings of control lines (wild type and pSOV2) and transgenic lines 3, 10, and 17. In all cases, the tissue was macerated in liquid nitrogen and homogenized with 2 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 2 mM EDTA, 2 mM dithiothreitol, 20% (v/v) glycerol, and insoluble polyvinylpyrrolidone (0.1 g per gram fresh tissue). Extracts were filtered through Miracloth and centrifuged at 4 °C for 30 min at 6000 × *g*. The ectopically expressed GalUR protein in *A. thaliana* was purified by immunoaffinity chromatography using the Affi-GelHz Immunoaffinity kit (Bio-Rad). Ten grams of total plant tissue were ground in liquid nitrogen and homogenized in 20 ml of extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM DTT, and 0.2 g of polyvinylpyrrolidone per gram of plant tissue). Preparation of the affinity column with antibodies against GalUR was done according to the manufacturer's instruction manual. Final elution was done with 100 mM acetic acid. Each fraction was immunoblotted to follow the elution of GalUR, and the purity of the protein was confirmed by silver staining of the gels.

Enzyme activity was measured by the change in absorbance (340 nm) after the addition of the purified protein (4–6 μg/ml of reaction) to the assay medium. The medium contained 0.1 mM NADPH and 10 mM (30 mM in the assays of the crude extracts) D-galacturonic acid or D-glucuronic acid or 0.1 mM $NAD^+$ and 10 mM (30 mM in the assays of the crude extracts) L-galactose. The assay buffer consisted of 50 mM sodium phosphate, pH 7.2, 2 mM EDTA, and 2 mM DTT.

**Ascorbic acid measurements.** Ascorbic acid content was determined by the ascorbate oxidase assay[30]. Total ascorbic acid content was determined by measuring the absorbance at 265 nm after addition of 4 U of ascorbate oxidase (Sigma) to the reaction medium containing the plant extract and 100 mM potassium phosphate, pH 5.6. Plant extracts were obtained from tissue frozen in liquid nitrogen, macerated in 2 mM metaphosphoric acid containing 2% (w/v) EDTA. Absorbance values for the samples were compared to a standard curve created using known concentrations of ascorbic acid.

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*Competing interests statement*

*The authors declare competing financial interests: see the Nature Biotechnology website (http://www.nature.com/naturebiotechnology) for details.*

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