EXPRESSION PATTERN OF KEY VITAMIN C BIOSYNTHESIS GENES IN APPLE

H. ROKNI ZADEH*, J. KEULEMANS AND M. W. DAVEY
* Current address: Olive Research Group, Department of Molecular Biology, National Institute of Genetic Engineering & Biotechnology (NIGEB), Iran

Laboratory of Fruit Breeding and Biotechnology, Division of Crop Biotechnics, K.U.Leuven, William De Croylaan 42, B-3001 Heverlee, Belgium
E-mail: mark.davey@biw.kuleuven.be

INTRODUCTION

Vitamin C (L-AA, ascorbic acid) has major functions in oxidative stress defense (e.g. ozone, pathogens, wounding, etc.) because of its antioxidant properties, and this leads to improved fruit quality, storage and increased resistance to postharvest storage diseases. It also protects humans from various important diseases. Fruit is the best food source of vitamin C, but while apple is the most highly consumed fruit (in 13 European countries), it has only modest amounts of vitamin C (Davey *et al.* 2000). So because of these important functional and nutritional characteristics it is important to understand the mechanisms of vitamin C biosynthesis and regulation to help develop breeding strategies for increased fruit vitamin C content. The present work considers the expression profiles of six key vitamin C biosynthesis genes in apple and their relation to vitamin C tissue contents to understand more about its synthesis and regulation. In the long term we aim to have improved fruit quality and more efficient storage and postharvest diseases management.

MATERIAL AND METHODS

Plant Material

All leaf, stem and fruits tissues were collected from trees of the apple cultivar 'Golden Delicious', grown at the field station of Better3Fruit n.b. in Aarschot (52°N). Root material, was obtained from young trees grown in pots in the greenhouse. Three trees were hand-pollinated and fruit sampled at different development stages. Sampled fruit were transferred on ice to the laboratory and flesh (mesocarp) and peel tissues isolated, pooled and frozen in liquid nitrogen. Fully expanded young leaves were picked and immediately frozen in liquid nitrogen. For wounding experiments, young leaves were bruised with sterilized forceps whilst still on the tree, and harvested 24h later. Root samples were collected, washed in DEPC-treated water and frozen immediately in liquid nitrogen. All tissue samples were divided into two parts, half for RNA extraction and half for HPLC analysis and stored at -80 C.

RNA extraction and cDNA synthesis

The "pine tree" method was used for total RNA extraction (Gasic *et al.* 2004). Total RNA was quantified using a spectrophotometer at 260 and 280 nm and quality checked by running on 1% agarose gel to control integrity (Gasic *et al.* 2004). 1µg RNA was used to synthesize first-strand cDNA using Super-script™ II RT enzyme (Invitrogen) (Dreesen *et al.* 2007).

Bioinformatics studies

Sequence information on six key genes of L-AA biosynthesis in plants i.e. myo-inositol oxidase **(MIO),** GDP-Mannose-3', 5'-epimerase **(GME),** L-galactono-gamma-lactone dehydrogenase **(GLDH),** D-galacturonic acid reductase **(GalUA),** L-Galactose-1-Phosphate phosphatase **(GalPase),** L-Galactose dehydrogenase **(GalDH)** (the complete coding sequences (cds) of only **GalPase** and **GalDH** were present in databases) were collected and used as query for blasting (blastn) against *Malus* ESTs available in the public databases. The highest score ESTs or in some cases the contig compiled from different ESTs were selected for multiple alignment to sequences from other plant species to identify conserved regions, from which gene-specific primers were designed (using Primer3).

Semi-quantitative RT-PCR

cDNA was subject to PCR with primers specific for the candidate genes. For all PCR reactions 29 cycles were used, except for GME since due to its relatively high expression only 27 cycles was required. For the actin control, 32 cycles was used. Tm for all reaction was 55.6 C. For a negative control in subsequent PCR reactions, one RNA sample was performed without RT reaction. The *Arabidopsis* actin-2 gene was used as an internal control.

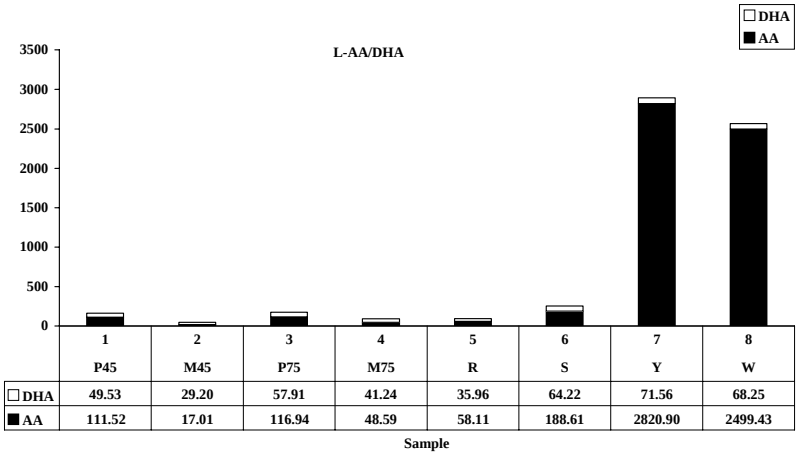
HPLC analysis

L-AA and its oxidised form, DHA, (dehydroascorbate) were measured by high performance liquid chromatography (HPLC) as described by Davey *et al.* 2004.

RESULTS

As expected, L-AA and GSH contents varied significantly between different tissues (Figure 1.a) (Davey *et al.* 2004), and our results are similar to those found in other plants. Total L-AA contents were greatest in leaves, with lower levels in stems and roots. L-AA level in fruit tissues was substantially lower than the concentrations in leaves, and concentrations remained relatively constant at all stages of development (Grantz *et al.* 1995). The proportion of DHA was higher in roots (60%) compared to leaves (2.5%) and stems (34%), while in fruit, L-AA level of peel was higher than the mesocarp, which again confirms our previous results from the analysis of 12 apple cultivars (Davey *et al.* 2004). Interestingly, we did not observe any increase in the contents of L-AA and its oxidized form under wounding stress – Fig. 1a.

a



	1	2	3	4	5	6	7	8
	P45	M45	P75	M75	R	S	Y	W
DHA	49.53	29.20	57.91	41.24	35.96	64.22	71.56	68.25
AA	111.52	17.01	116.94	48.59	58.11	188.61	2820.90	2499.43

Sample

b

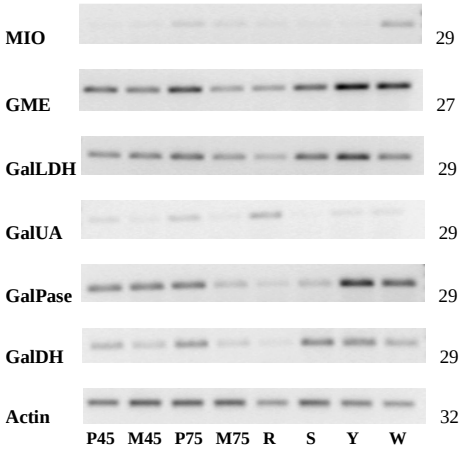


Figure 1. a) L-AA/DHA contents and b) candidate genes expression in different *Malus* fruit tissues.
P45 = Peel, young fruit 45 days after pollination (DAP), M45 = Mesocarp, young fruit 45 (DAP), P75 = Peel, fruit 75 (DAP), M75 = Mesocarp, fruit 75 (DAP), R = Root, S = Stem, Y = young leaf, W = Wounded leaf.

An equal level of actin expression was obtained among the different tissues, indicating that the PCR conditions were the same for all samples (Figure

1.b). GalLDH expression was dectected in all tissues studied (as L-AA is present in all plant tissues) and was much higher in L-AA-rich photosynthetic tissues (leaf and stem) than in L-AA-poor tissue (root) and its expression in peel was higher than in the mesocarp. However there was no correlation between its GalDH expression and L-AA contents in wounded leaf. These results are similar to the GalLDH expression patterns found in a similar study in melon (Pateraki *et al.* 2004). Previous results from our lab indicated that mature apple fruit tissues are incapable of L-AA biosyntheses and may be dependent on phloem transport but that there is probably biosynthesis in the young fruit during the first stages of growth (Davey *et al* 2004, Razavi *et al.* 2005). Our results also demonstrated the expression of the main L-AA biosynthesis genes in young fruit, we do not have data in expression levels in mature fruit.

GalDH was constitutively expressed in plant tissues regardless of L-AA concentration, while GalPase seems to be well correlated with endogenous L-AA contents. GME had a high expression in all tissues studied, and there was no change in response to wounding. Similar results were reported in rice (Watanabe *et al.* 2006). Interestingly MIO was expressed significantly under stress conditions (here wounded leaf), and recently MIO expression was recently observed to be induced by hypoxia stress in tomato (Kanellis - personal communication). GalUA expression showed an increase during ripening (similar results reported in strawberry by Agius *et al.* 2003) but no significant expression in leaf, and very interestingly most transcripts were in root.

Overall, the results obtained in apple (our study), melon, tomato and rice lead us to conclude that the regulation of L-AA level in response to wounding stress is probably not dependent on *in vivo* induction of GME, GLDH, GalUA, GalPase and GalDH, but may well depend on induction of MIO expression.

CONCLUSIONS

Although several studies on L-AA content and distribution in apple have been reported, this is the first study linking a transcriptional approach with HPLC analysis of L-AA levels. Overall, there appears to be a good correlation between the expression levels of our amplified fragments and the measured L-AA contents of the different tissues. However, before definitive results can be obtained, the amplified PCR fragments should be sequenced and the full lenght cDNA isolated using either RACE or long RT-PCR to confirm cDNA identity. A multidisciplinary approch including transcription expression, HPLC analysis, substrates uptake and localisation as well as microscopic studies can help to have more clear insight into the L-AA biosynthesis and regulation.

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