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Engineering ascorbic acid biosynthetic pathway in *Arabidopsis* leaves by single and double gene transformation

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Abstract

Six genes, which encode enzymes involved in ascorbic acid (AsA) biosynthesis, including guanosine diphosphate (GDP)-mannose pyrophosphorylase (GMP), GDP-mannose-3',5'-epimerase (GME), GDP-galactose guanylyltransferase (GGT), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH) and L-galactono-1,4-lactone dehydrogenase (GLDH) were transformed into *Arabidopsis thaliana*, to evaluate the contribution of each gene to AsA accumulation. Additionally, two combinations, *GGT-GPP* and *GGT-GLDH*, were co-transformed into *Arabidopsis* with a reliable double-gene transformation system. AsA content of *GGT* transgenic lines was 2.9-fold higher as compared to the control, and co-transformation led up to 4.1-fold AsA enhancement. These results provided further evidence that GGT is the key enzyme in plant AsA biosynthesis.

Additional key words: GDP-L-galactose guanyltransferase, transgenic plants, vitamin C.

Introduction

L-ascorbic acid (AsA, vitamin C) is one of the most well-known water-soluble metabolites produced by plants. Together with other antioxidants, including α-tocopherol and glutathione, AsA acts as a key factor for photo-protection and reactive oxygen species clearance (Loewus 1999, Muller-Moule *et al*. 2004, Smirnoff 2000). It is also a cofactor for the synthesis of hormones, such as ethylene and gibberellins (Loewus and Loewus 1987), thus involves in flowering (Barth *et al*. 2006), fruit ripening and senescence (De Tullio *et al*. 2004). AsA is suggested to be important in cell division, expansion and elongation (Arrigoni and De Tullio 2000, Davey *et al*. 2000, Pastori *et al*. 2003, Smirnoff and Wheeler 2000). More recently, AsA biosynthetic metabolism has been elucidated.

The L-galactose pathway (Smirnoff-Wheeler pathway; Wheeler *et al*. 1998), is now regarded as the main AsA biosynthetic pathway in higher plants. The pathway begins with D-mannose-1-phosphate and takes six steps to yield the final product. All genes involved in these crucial steps had been cloned, including those encoding GDP-D-mannose pyrophosphorylase (GMP; Conklin *et al*. 1999), GDP-D-mannose-3',5'-epimerase (GME; Wolucka and Van Montagu 2003), GDP-L-galactose guanyltransferase (GGT; Dowdle *et al*. 2007, Laing *et al*. 2007, Linster *et al*. 2007), L-galactose-1-phosphate phosphatase (GPP; Laing *et al*. 2004), L-galactose dehydrogenase (GDH; Gatzek *et al*. 2002) and L-galactono-1,4-lactone dehydrogenase (GLDH; Imai *et al*. 1998). Other suggested routes through galacturonic acid (Loewus 1999, Agius *et al*. 2003), L-gulose (Wolucka and Van Montagu 2003, 2007) and *myo*-inositol (Lorence *et al*. 2004) were proved not to compensate for the low AsA contents in L-galactose pathway mutants

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Abbreviations: GDH - L-galactose dehydrogenase; GDP - guanosine diphosphate; GGT - GDP-galactose guanylyltransferase; GLDH - L-galactono-1,4-lactone dehydrogenase; GME - GDP-mannose-3',5'-epimerase; GMP - GDP-mannose pyrophosphorylase; GPP - L-galactose-1-phosphate phosphatase.

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(*e.g*. vtc1, vtc2; Conklin *et al*. 1999, Linster *et al*. 2007). Dowdle *et al*. (2007) suggested that the L-galactose pathway was the main pathway to AsA biosynthesis in *Arabidopsis* with the evidence that double mutants of the two *GGT* genes (At4g26850, At5g55120) were lethal.

Metabolic engineering of the L-galactose pathway has achieved a great breakthrough in the last decade. Overexpression of *Malpighia glabra GMP* in tobacco led to about 2-fold higher AsA content than in the wild type (Badejo *et al*. 2008). Bulley *et al*. (2009) pointed out that GGT was the rate-limiting step and stable overexpression of *GGT* in *Arabidopsis* caused 4-fold increase in AsA content. Tobacco transformation with kiwifruit *GME* and *GGT* gene separately caused 1.2-fold and 4.2-fold promotion in AsA content, and 8.6-fold when used together (Bulley *et al*. 2009). The overexpression of *GDH* in tobacco resulted in a 3-fold increase in the AsA activity, but no effect on AsA content (Gatzek *et al*. 2002). Overexpression of *GLDH* with a CaMV 35S promoter in tobacco suspension cells led to approximately 2-fold enhancement of total ascorbate content during stationary phase (Tokunaga *et al*. 2005). However, differences existed in gene source, promoter type and plant species engineered, which made difficult to compare the transformation efficiency systematically. Furthermore, few works has been done to transform two or more AsA biosynthesis genes at the same time.

In this work, we transformed all of the six genes referred to AsA biosynthetic pathway under the control of CaMV 35S promoter into *Arabidopsis thaliana* separately, indicating that *GGT* and *GLDH* transgenic lines performed best in AsA accumulation. In further research we co-transformed two AsA biosynthesis genes at the same time. Two combinations, *GGT-GLDH* and *GGT-GPP* were transformed into *A. thaliana* with a double-gene transformation system. The results helped to evaluate each gene contribution and understand the cooperation between genes for further molecular breeding purpose.

Materials and methods

Seeds of wild-type *Arabidopsis thaliana* L. plants (Columbia ecotype) were sterilized with 5 % bleach and spread on 1/2 Murashige and Skoog (MS) medium and put into 4 °C for 3 d. They were germinated under 16-h photoperiod with irradiance of 120 μmol m^{-2} s^{-1}, temperature of 20 °C and relative air humidity of 60 % for 7 d, and then transferred to wet soil.

Total RNA was isolated from leaves. The cDNAs of *At-GMP*, *GME*, *GGT*, *GPP*, *GDH*, *GLDH* genes (acc. Nos. At2g39770, At5g28840, At4g26850, At3g02870, AT4g33670 and At3g47930) were prepared by *TaKaRa* (Shiga**,** Japan) one-step real time polymerase chain reaction (RT-PCR) kit (*AMV*). The primers used for cloning were listed in Table 1. The *At-GPP* cDNAs were pointed-mutated at *Xba*I site, while *At-GLDH* cDNAs were pointed-mutated at *Xba*I and *Bst*EII site. All of the amplified cDNA fragments with an introduced *Nco*I/*Bgl*II/*Spe*I site at the start codon (ATG) and an introduced *Bst*EII site after the stop codon were digested and sub-cloned into corresponding site of the vector pCAMBIA1304.

For the double-gene transformation in one vector, the expression cassettes of *GPP* and *GLDH* were amplified by PCR between the *Xba*I site in multiple cloning sites (MCS) and an introduced *Spe*I site after the NOS terminator. The fragments were sub-cloned into *Xba*I site of another single-gene vector (Fig. 1). Insertion order was tested by PCR. The p1304::target gene vectors were sequencing by gene primers to prove the cloning sequences in accord with the reported sequences in gene bank. Similarly, more cassettes could be inserted so that a multi-gene vector was obtained finally.

A hygromycin resistant gene is contained inside the T-DNA for the selection of transformants in p1304. The p1304::target gene constructions were transferred into *Agrobacterium tumefaciens* C58C1 (pGV3101; rifampicin resistant) by triparental mating. The floral-dip method was used to conduct transformation of *A. thaliana* (Zhang *et al*. 2006). T_0 seeds were screened on 1/2 MS basal plates supplemented with 50 μg cm^{-3} hygromycin. Then, 30 ~ 50 independent positive lines were transferred to soil and confirmed by PCR. For the screening of co-expression in transgenic plants, both of the genes were tested. Screening of T_1 was kept performing to find transgenic lines which accorded with Mendel's Law. And T_2 seeds were checked for homozygosis and genetic stability. Homozygous T_4 seeds were used for further analyses.

Genomic DNA was isolated from leaves of 4-week-old transgenic homozygotes, WT control and p1304 control lines by CTAB method (Ausubel *et al.* 1995). The presence of the transferred genes was detected by PCR to amplify target gene-Nos sequence.

Plants (wild type and transgenic strains) were cultivated for 5 weeks. Total RNA of each line was isolated from leaves and genomic DNA was removed by treatment with RQ1-RNase free DNase (*Promega*, Madison, WI). RNA (500 ng) was reverse transcribed to generate cDNA by *TaKaRa PrimeScript® RT* reagent kit. An aliquot of cDNA corresponding to 10 ng of total RNA was used in each real-time PCR assay (SYBR® *Premix Ex TaqTM* kit (*TaKaRa*) in a total volume of 0.025 cm^3. The results were normalized against ubiquitin RNA. Sequences of the primers used in the real-time PCR

assays are listed in Table 1.

For each transgenic line, 50 mg of leaves from 5-week-old plants were harvested simultaneously and ground in liquid nitrogen, mixed with 1cm^3 0.1 % oxalic acid in dim light, and placed on ice for 1 h. The samples were centrifuged at 10 000 *g* for 10 min and the supernatants were pooled and filtered for measurement. Standard L-AsA solutions (1 mg cm^{-3}) were freshly prepared prior to use. HPLC analysis of L-AsA was performed as previously described (Li *et al*. 2010).

Table 1. Primers used for PCR and RT-PCR. F - forward primer; R - reverse primer.

Gene	PCR primers	RT-PCR primers
GMP:	F 5'-AACCATGGGATCTCTCTCAAGGA-3' (NcoI)	F 5'-TCAGGCTGATCTCTTCCAATTTAC-3'
At2g39770	R 5'-AAGGTCACCTTAAGAATTACGAAAGC-3' (BstEII)	R 5'-TGATGCTCTAACTCCTTTTCCTTG-3'
GME:	F 5- CCAGATCTGGGAACTACCAATGGAAC-3' (BglII)	F 5'-AGGTGTTCGTGGTCGTAACTCA-3'
At5g28840	R 5'-AAGGATCCCTCACTCTTTTCCATCAGCC-3' (BstEII)	R 5'-ATCGCTTCCCTTTGCTTTCTC-3'
GGT:	F 5'-CCACTAGTATGTTGAAAATCAAAAGAG-3' (SpeI)	F 5'-TCGTTTCTGCTGTTTTGAGTGT-3'
At4g26850	R 5'-AAGGATCCCCTGAAGGACAAGGC-3' (BstEII)	R 5'-TCCCGTGTATAGCCGTGATTC-3'
GPP:	F 5'-ACAGATCTATGGCGGACAATGATTCTCTTGAT-3' (BglII)	F 5'-GTCCCTGTTGTTGGAGTTGTTTAT-3'
At3g02870	R 5'-TTGGTCACCTCATGCCCCTGTAAGCCG-3' (BstEII)	R 5'-CGCTTTGAGCTGACACTTTGAT-3'
GDH:	F 5'-CCAGATCTTCACACATGACGAAAATAGAGCTTCG-3' (BglII)	F 5'-GCAAATCAAAGGGCAAGAAGA-3'
AT4g33670	R 5'-ACGGTCACCTTAGTTCTGATGGATTCCACT-3' (BstEII)	R 5'-GCTCATCCCAACCAACACC-3'
GLDH:	F 5'-AACCATGGACATTTCCCTCAGG-3' (NcoI)	F 5'-GAGCAGCAGATTGGTGGTATTATTC-3'
At3g47930	R 5'-CAGGTCACCAAATGTTTTAAGCAGT-3' (BstEII)	R 5'-ATTGTTCCCTTCGCAGGAGTAA-3'
GLDHXbaI	F 5'-GATAAAGTTCTTGAGGTGGATAAAG-3'	
	R 5'-TTTATCCACCTCAAGAACTTTATCC-3'	
GLDHBstEI	F 5'-TGTTGCTGAGGTCACTCTCCAAT-3'	
I	R 5'-ATTGGAGAGTGACCTCAGCAACAA -3'	
1304F:	5'-CTATGACCATGATTACGAATTCGAG-3'	
NOSR:	5'-AGACTAGTCCAATATATCCTGTCAAACA-3' (SpeI)	
Ubiquitin		F 5'-TGCGCTGCCAGATAATACACTATT-3'
		R 5'-TGCTGCCCAACATCAGGTT-3'

Results

According to the standard curve of L-AsA, WT control lines contained 0.358 ± 0.086 mg(AsA) g^{-1}(f.m.). Overexpressing *At-GMP*, *GME*, *GGT*, *GPP*, *GDH* and *GLDH* increased AsA content by 1.3-, 1.4-, 2.9-, 1.5-, 1.2- and 1.8-fold, respectively. The transcription levels of the genes rose in various degrees (Table 2). The experiments were done with at least three independent lines of each transformant and each test was repeated three times.

Table 2. AsA content and relative content and AsA biosynthetic gene transcription level in single gene transgenic lines. Transcription levels of the six genes and AsA content were checked in p1304 control lines and assigned the value of 1. No significant differences were observed between WT and p1304 in gene transcription levels. The results were checked in at least three independent lines of each transformant when the T_4 samples were 5 weeks old. Means ± SE, $n \geq 3$.

	AsA [mg g^{-1}(f.m.)]	AsA [% of WT]	Transcription [% of WT]
WT control	0.358±0.086	100	100
p1304	0.348±0.115	105	100
GMP	0.508±0.084	130	750± 84
GME	0.497±0.095	140	219± 50
GGT	1.041±0.275	290	320± 80
GPP	0.559±0.102	150	1500±128
GDH	0.468±0.081	120	4200±277
GLDH	0.647±0.143	180	270± 73

To transform two or more genes at the same time, the isocaudarner method was introduced. The geneA expression cassette with an *Xba*I site before promoter and an introduced *Spe*I site after Nos terminator were sub-cloned into the only *Xba*I site of geneB-p1304. Both *Xba*I and *Spe*I site vanished after they were ligated, while *Xba*I-*Xba*I ligation resulted in a residual *Xba*I site, thus allowed another expression cassette to be inserted. The primer before the MCS site was used to test whether the expression cassette was inserted in the right order. In our work, vectors containing *GGT-GPP* and *GGT-GLDH* were constructed. Restriction digestion showed that there were only one *Xba*I site and no *Spe*I sites left regardless of the original *Spe*I site in *GGT*-p1304.

Plasmid containing two expression cassettes of the cDNAs encoding genes of AsA biosynthetic pathway enzymes *GGT-GPP* and *GGT-GLDH* were introduced into *A. thaliana via Agrobacterium tumefaciens* C58C1

separately. Hygromycin gene was used for genetically engineered strains screening. Exogenous genes were further examined by PCR of gene-Nos segments. More than 30 in molecular analysis positive, hygromycin resistant plant lines were assembled for homozygosis test. All the transgenic lines showed no difference from wild type in phenotype. Five independent lines of each transformant were gained for real-time PCR and HPLC analyses.

Real-time PCR showed that both WT control and p1304 control presented in a low transcription level (Table 3). *GGT* level in CF20, CD28 and CD53 were even below the level of control on average, suggesting the silence of endogenous gene. The best *GGT-GPP* transgenic line possessed 4.1-fold higher AsA content [1.425 ± 0.088 mg(AsA) g^{-1}(f.m.)] than WT control. Transformation of *GGT* and *GLDH* resulted in a peak AsA content of 1.244 ± 0.386 mg(AsA) g^{-1}(f.m.), which was 3.6-fold higher than control.

Pearson's correlations were calculated to reveal the relationship between transcription of genes involved in AsA biosynthesis and AsA content. The result showed that both *GGT* and *GLDH* levels appeared highly correlated with AsA content in GGT-GLDH lines (r^2 = 0.8168 and 0.7819, respectively, n = 6, P < 0.01). *GGT* transcription also appeared highly correlated to the AsA content (r^2 = 0.7897, n = 6, P < 0.01), while *GPP* level seemed to be low correlated with AsA content (r^2 = 0.4724, n = 6).

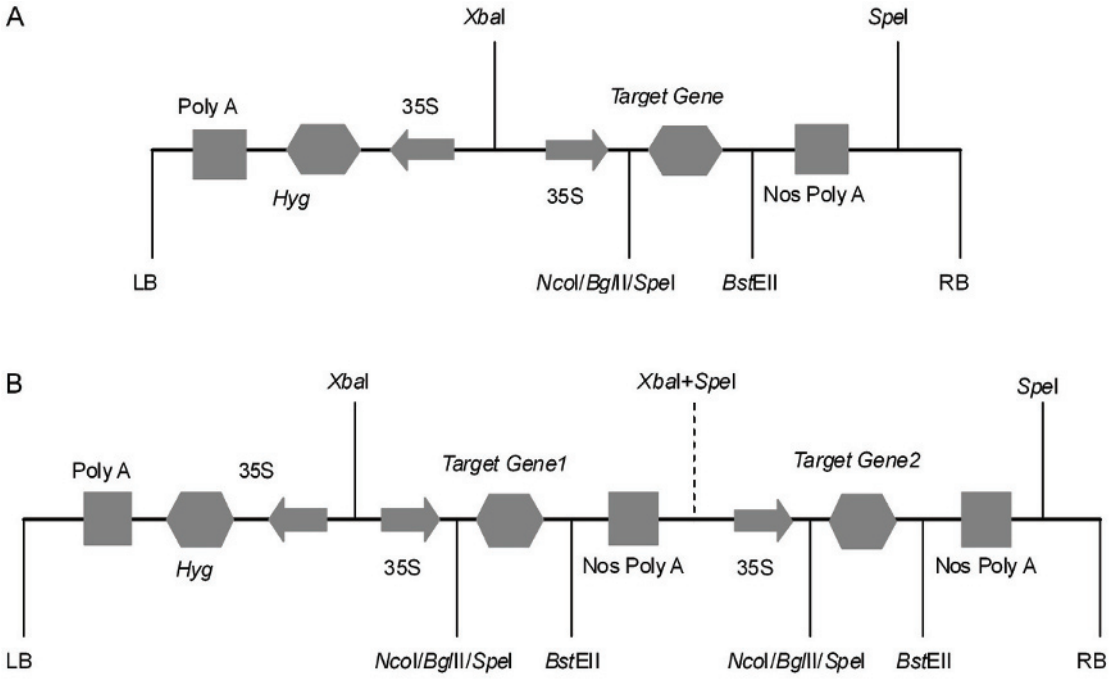

Fig. 1. Represent of double-gene vectors construction. The expression cassette of target gene 1 was amplified by PCR between the *Xba*I site in MCS and an introduced *Spe*I site after the NOS terminator (*A*), and then sub-cloned into *Xba*I site of target gene2-p1304 vector (*B*).

Table 3. Relative gene expression and AsA accumulation in *GGT-GLDH* and *GGT-GPP* transgenic lines. Gene transcription in WT control was assigned the value of 1. Means ± SE, n = 3.

	GGT-GLDH AsA [mg g^{-1}(f.m.)]	*GGT* transcription [% of WT]	*GLDH* transcription [% of WT]	*GGT-GPP* AsA [mg g^{-1}(f.m.)]	*GGT* transcription [% of WT]	*GPP* transcription [% of WT]
WT	0.358±0.086	100±51	100±21	0.358±0.086	100±21	100±17
p1304	0.348±0.115	105± 6	132± 1	0.348±0.115	105± 6	131± 6
CF1	0.367±0.197	64± 4	962±17	1.223±0.625	270±26	681±44
CF4	0.661±0.129	110±17	566±40	0.316±0.274	330±26	409±25
CF6	0.937±0.046	174± 6	3127±25	1.425±0.088	540±12	1241±15
CF10	0.645±0.137	129± 6	800±21	1.200±0.074	470±40	1330±15
CF20	1.244±0.386	409±10	3200±21	0.776±0.225	81± 6	1425± 6

Discussion

Previous work collected a lot of information about the AsA biosynthetic pathway in plants, which provides a solid foundation for modern breeding aimed at changing the AsA content in eatable species (Ishikawa *et al*. 2006). However, to our knowledge, we firstly overexpressed all of the six genes which referred to AsA biosynthetic pathway with the control of the same promoter CaMV 35S in the same species, *A. thaliana*.

It was proved that GGT is a major control point of AsA biosynthesis (Bulley *et al*. 2009, Linster and Clarke 2008). Two *At*5g55120 T-DNA insertion mutants (vtc5-1 and vtc5-2) have 80 % of the wild-type ascorbate content. Double mutants showed growth arrested immediately upon germination and the cotyledons subsequently bleached (Dowdle *et al*. 2007). GGT catalyzes the conversion from GDP-L-galactose to L-galactose-1-P. As GDP-D-mannose and GDP-L-galactose are also the precursors in the synthesis of cell wall polysaccharides and/or protein glycosylation (Reuhs *et al*. 2004, Smirnoff and Wheeler 2000, Wolucka *et al*. 2001), the reaction is the first step in the L-galactose pathway. Thus GGT is the reasonable target for the manipulation of L-ascorbate synthesis. Transforming *GGT* in *A. thaliana* increased AsA by 2.9-fold, which was followed by *GLDH* and *GPP* transgenic lines (1.8-fold and 1.5-fold, respectively). Transformation of the other genes failed to lead significant difference in AsA content compared to the control. Our results provide new evidence that GGT acts as a key enzyme in plant AsA biosynthesis.

The products catalyzed by GMP and GME are required for cell wall formation and protein glycosylation. GME was proved to be a key intersection of ascorbate and non-cellulosic cell-wall biosynthesis pathways in tomato (Gilbert *et al*. 2009). Neither GMP nor GME seems to be the crucial regulatory control in AsA biosynthesis. Overexpression of acerola *GMP* resulted to 2- to 3-fold increase in the AsA content in transgenic tobacco (Badejo *et al*. 2008). However, the promoter was also from acerola. Transient expressing kiwifruit *GME* led to a 1.2-fold increase in AsA in tobacco leaves (Bulley *et al*. 2009), which is similar to our result. Another less effective enzyme GDH was also manipulated in tobacco. Overexpression and antisense suppression of *GDH* in tobacco and *A. thaliana,* respectively, did not alter the AsA pool size in their leaves (Gatzek *et al*. 2002). It suggests that GDH exerts little control over flux through the L-galactose pathway. *GLDH* and *GPP* single transgenic lines displayed the next best AsA enhancement. GLDH is the last enzyme in AsA metabolic net. L-galactose, the product of GPP, is the precursor of AsA in plants. Our results revealed their contribution to AsA accumulation and they were thus applied to the co-transformation experiments.

We also set up a reliable method for stable transformation of two or more genes at the same time. Two combinations, including *GGT*-*GLDH* and *GGT*-*GPP*, were introduced into *A. thaliana*, separately. The results showed the great effect of the *GGT* involved transformation. In *GGT*-*GPP* transgenic lines, AsA content was raised 4.1-fold, accompanied by *GGT* transcription uttermost enhancement of 5.4-fold. Meanwhile, in *GGT*-*GLDH* transgenic lines, AsA content and *GGT* transcription increased 3.6- and 4.1-fold compared to control, although only in some *GGT*-involved lines the levels of AsA were below that in control. Linear test showed AsA that content corresponded to *GGT* transcription ($dP < 0.01$). We also notice that the effect of co-transformation was better than that of transforming genes separately. AsA in *GGT*-*GPP* lines were significantly higher than in *GGT* lines ($P = 0.02$), while AsA in *GGT*-*GLDH* lines was only a bit higher than that in *GGT* lines ($P = 0.33$).

GLDH transcription also correlated with AsA content. Nevertheless, in *GDH*-*GLDH* lines, *GLDH* transcription had nothing to do with AsA content ($r^2 = 0.2182$, $P > 0.05$, data not shown). Interfering *GLDH* in tomato did not alter the L-ascorbate synthesis capacity in the transgenic plants (Alhagdow *et al*. 2007). Bartoli *et al*. (2005) reported that correlation could not be established between GLDH expression and AsA content in wheat leaves. On the other hand, the observation that a *GPP*-knockout mutant is only partially deficient in AsA as well as GPP activity suggests that GPP can be compensated by other enzymes in *A. thaliana* (Conklin *et al*. 2006). Taken together, it suggests that *GPP* and *GLDH* are still not the perfect candidates for the co-transformation.

For further study, genes from bypass synthesis, oxidation, recycle and transport pathway can also be concerned. The promising candidates include D-galacturonate reductase (GalUR; Agius *et al*. 2003), L-gulono-1,4-lactone oxidase (GulO; Jain and Nessler 2000), *myo*-inositol oxygenase (Lorence *et al*. 2004) and dehydroascorbate reductase (DHAR) (Wang *et al*. 2010). Additionally, the manipulation of other antioxidants, such as α-tocopheral could also affect ascorbate accumulation (Li *et al*. 2010). The 2,4-dichlorophenol (DCP) or pentachlorophenol (PCP) provoked oxidant burst and thus increased AsA content (Michałowicz *et al*. 2010). Developmental processes, stress responses and environmental conditions are all known to affect the AsA content of individual species. More explorations about plant AsA metabolism are needed for future development of the full potential of metabolic engineering.

It is concluded that all of the six genes, involved in AsA biosynthesis, have been transformed under the same genetic background into *A. thaliana*. *GGT* transgenic lines had the highest AsA accumulation with a 2.9- fold increase to the control, which was followed by *GLDH* and *GPP* lines. Moreover, with a double-gene transformation system, two combinations, including

GGT-GPP and *GGT-GLDH* have also been introduced into *A. thaliana*. *GGT*-involved transformations lead to 4.1-fold AsA enhancement compared to the control, and best correlation is observed between *GGT* transcription and AsA content. The results all highlight that GGT is the rate-limiting step in AsA biosynthesis. Further physiological experiments are imperative as gene transcription does not clearly represent the relevant enzyme activity. Co-transformation performs better than single-gene transformation to some extent. However, combinations of two or more genes which are more effective need to be tested. The co-transformation results offer an approach for breeding high nutritional quality and stress tolerance of crop plants.

References

Agius, F., Gonzalez-Lamothe, R., Caballero, J.L., Munoz-Blanco, J., Botella, M.A., Valpuesta, V.: Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. - Natur. Biotechnol. **21**: 177-181, 2003.

Alhagdow, M., Mounet, F., Gilbert, L., Nunes-Nesi, A., Garcia, V., Just, D., Petit, J., Beauvoit, B., Fernie, A.R., Rothan, C., Baldet, P.: Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase affects plant and fruit development in tomato. - Plant Physiol. **145**: 1408-1422, 2007.

Arrigoni, O., De Tullio, M.: The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. - J. Plant Physiol. **157**: 481-488, 2000.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., Struhl, K.: Short Protocols in Molecular Biology. 3rd Ed. - John Wiley&Son, New York 1995.

Badejo, A., Tanaka, N., Esaka, M.: Analysis of GDP-D-mannose pyrophosphorylase gene promoter from acerola (*Malpighia glabra*) and increase in ascorbate content of transgenic tobacco expressing the acerola gene. - Plant Cell Physiol. **49**: 126-132, 2008.

Barth, C., De Tullio, M., Conklin, P.: The role of ascorbic acid in the control of flowering time and the onset of senescence. - J. exp. Bot. **57**: 1657-1665, 2006.

Bartoli, C., Guiamet, J., Kiddle, G., Pastori, G., Di Cagno, R., Theodoulou, F., Foyer, C.: Ascorbate content of wheat leaves is not determined by maximal L-galactono-1,4-lactone dehydrogenase (GalLDH) activity under drought stress. - Plant Cell Environ. **28**: 1073-1081, 2005.

Bulley, S., Rassam, M., Hoser, D., Otto, W., Schunemann, N., Wright, M., MacRae, E., Gleave, A., Laing, W.: Gene expression studies in kiwifruit and gene over-expression in *Arabidopsis* indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. - J. exp. Bot. **60**: 765-778, 2009.

Conklin, P., Gatzek, S., Wheeler, G., Dowdle, J., Raymond, M., Rolinski, S., Isupov, M., Littlechild, J., Smirnoff, N.: *Arabidopsis thaliana* VTC4 encodes L-galactose-1-phosphatase, a plant ascorbic acid biosynthetic enzyme. - J. biol. Chem. **281**: 15662-15670, 2006.

Conklin, P., Norris, S., Wheeler, G., Williams, E., Smirnoff, N., Last, R.: Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. - Proc. nat. Acad. Sci. USA **96**: 4198-4203, 1999.

Davey, M., Van Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I., Strain, J., Favell, D., Fletcher, J.: Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. - J. Sci. Food Agr. **80**: 825-860, 2000.

De Tullio, M., Liso, R., Arrigoni, O.: Ascorbic acid oxidase: an enzyme in search of a role. - Biol. Plant. **48**: 161-166, 2004.

Dowdle, J., Ishikawa, T., Gatzek, S., Rolinski, S., Smirnoff, N.: Two genes in *Arabidopsis thaliana* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. - Plant J. **52**: 673-689, 2007.

Gatzek, S., Wheeler, G., Smirnoff, N.: Antisense suppression of l-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated l-galactose synthesis. - Plant J. **30**: 541-553, 2002.

Gilbert, L., Alhagdow, M., Nunes-Nesi, A., Quemener, B., Guillon, F., Bouchet, B., Faurobert, M., Gouble, B., Page, D., Garcia, V., Petit, J., Stevens, R., Causse, M., Fernie, A., Lahaye, M., Rothan, C., Baldet, P.: GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. - Plant J. **60**: 499-508, 2009.

Imai, T., Karita, S., Shiratori, G., Hattori, M., Nunome, T., Oba, K., Hirai, M.: L-galactono-gamma-lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis. - Plant Cell Physiol. **39**: 1350-1358, 1998.

Ishikawa, T., Dowdle, J., Smirnoff, N.: Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. - Physiol. Plant. **126**: 343-355, 2006.

Jain, A., Nessler, C.: Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. - Mol. Breed. **6**: 73-78, 2000.

Laing, W., Bulley, S., Wright, M., Cooney, J., Jensen, D., Barraclough, D., MacRae, E.: A highly specific L-galactose-1-phosphate phosphatase on the path to ascorbate biosynthesis. - Proc. nat. Acad. Sci. USA **101**: 16976-16981, 2004.

Laing, W., Wright, M., Cooney, J., Bulley, S.: The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. - Proc. nat. Acad. Sci. USA **104**: 9534-9539, 2007.

Li, Y., Zhou, Y., Wang, Z., Sun, X., Tang, K.: Engineering tocopherol biosynthetic pathway in *Arabidopsis* leaves and its effect on antioxidant metabolism. - Plant Sci. **178**: 312-320, 2010.

Linster, C., Clarke, S.: L-Ascorbate biosynthesis in higher plants: the role of VTC2. - Trends Plant Sci. **13**: 567-573, 2008.

Linster, C., Gomez, T., Christensen, K., Adler, L., Young, B., Brenner, C., Clarke, S.: *Arabidopsis* VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. - J. biol. Chem. **282**: 18879-18885, 2007.

Loewus, F.: Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. - Phytochemistry **52**: 193-210, 1999.

Loewus, F., Loewus, M.: Biosynthesis and Metabolism of Ascorbic-Acid in Plants. - Crc Critical Rev. in Plant Sci. **5**:101-119, 1987.

Lorence, A., Chevone, B., Mendes, P., Nessler, C.: *myo*-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. - Plant Physiol. **134**: 1200-1205, 2004.

Muller-Moule, P., Golan, T., Niyogi, K.: Ascorbate-deficient mutants of *Arabidopsis* grow in high light despite chronic photooxidative stress. - Plant Physiol. **134**: 1163-1172, 2004.

Michałowicz, J., Urbanek, H., Bukowska, B., Duda, W.: The effect of 2,4-dichlorophenol and pentachlorophenol on antioxidant system in the leaves of *Phalaris arudinacea*. - Biol. Plant. **54**: 597-600, 2010.

Pastori, G., Kiddle, G., Antoniw, J., Bernard, S., Veljovic-Jovanovic, S., Verrier, P.J., Noctor, G., Foyer, C.H.: Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. - Cell **15**: 939-951, 2003.

Reuhs, B., Glenn, J., Stephens, S., Kim, J., Christie, D., Glushka, J., Zablackis, E., Albersheim, P., Darvill, A., O'Neill, M.: L-Galactose replaces l-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose-deficient mur1 *Arabidopsis* mutant. - Planta **219**: 147-157, 2004.

Smirnoff, N.: Ascorbate biosynthesis and function in photoprotection. - Phil. Trans. roy. Soc London B Biol. Sci. **355**: 1455-1464, 2000.

Smirnoff, N., Wheeler, G.: Ascorbic acid in plants: biosynthesis and function. - Crit. Rev. Biochem. mol. Biol. **35**: 291-314, 2000.

Tokunaga, T., Miyahara, K., Tabata, K., Esaka, M.: Generation and properties of ascorbic acid-overproducing transgenic tobacco cells expressing sense RNA for l-galactono-1,4-lactone dehydrogenase. - Planta **220**: 854-863, 2005.

Wang, Z., Xiao, Y., Chen, W., Tang, K., Zhang, L.: Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in *Arabidopsis*. - J. Integr. Plant Biol. **52**: 400-409, 2010.

Wheeler, G., Jones, M., Smirnoff, N.: The biosynthetic pathway of vitamin C in higher plants. - Nature **393**: 365-369, 1998.

Wolucka, B., Persiau, G., Van Doorsselaere, J., Davey, M., Demol, H., Van de Kerckhove, J., Van Montagu, M., Zabeau, M., Boerjan, W.: Partial purification and identification of GDP-mannose 3",5"-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway. - Proc. nat. Acad. Sci. USA **98**: 14843-14848, 2001.

Wolucka, B., Van Montagu, M.: GDP-mannose 3',5'-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. - J. biol. Chem. **278**: 47483-47490, 2003.

Wolucka, B., Van Montagu, M.: The VTC2 cycle and the *de novo* biosynthesis pathways for vitamin C in plants: An opinion. - Phytochemistry **68**: 2602-2613, 2007.

Zhang, X., Henriques, R., Lin, S., Niu, Q., Chua, N.: *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. - Nat. Protoc. **1**: 641-646, 2006.