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Gene expression of ascorbic acid-related enzymes in tobacco

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Abstract

GDP-D-mannose pyrophosphorylase (GMPase) and L-galactono-1, 4-lactone dehydrogenase (GalLDH) are key enzymes in L-ascorbic acid (AsA) biosynthesis of plants, and a full-length cDNA for GMPase was isolated from tobacco using PCR. Additionally, expression of GMPase, GalLDH and other AsA-related enzymes was examined in tobacco tissues and cultured BY-2 cells, and the relationship between their expression patterns and AsA content is discussed. It was found that the expression of GalLDH and GMPase mRNAs was markedly suppressed by loading AsA, suggesting that AsA concentration in the cells may regulate AsA biosynthesis. Moreover, the expression of GMPase and GalLDH mRNAs in tobacco leaf also suggested that AsA biosynthesis may be induced by light.

Keywords: Tobacco (*Nicotiana tabacum* L.); Ascorbic acid; GDP-D-Mannose pyrophosphorylase; L-Galactono-1,4-lactone dehydrogenase; Ascorbate oxidase; Ascorbate peroxidase; Solanaceae

1. Introduction

The biosynthetic pathway to L-ascorbic acid (AsA) in plants is poorly understood, and recently, possible alternate routes for its biosynthesis within plant hexose metabolism have been proposed (Davey et al., 1999). It seems that while in plants its biosynthesis of AsA in plants may be accomplished through multiple pathways. GDP-D-mannose pyrophosphorylase (GMPase: EC 2.7.7.22) is an enzyme in AsA catalyzing the initial step biosynthesis. However, GMPase which results in the production of GDP-D-mannose, is also used for cell wall carbohydrate biosynthesis and protein glycosylation. In another study, Conklin et al. (1997, 1999) analyzed an AsA-deficient mutant of *Arabidopsis thaliana*, *vtc1*, which was isolated as an ozone-sensitive mutant, and demonstrated that the VTC1 locus encodes GMPase. Furthermore, Keller et al. (1999) showed that the antisense inhibition of GMPase led to a decline in the mannose content of the cell wall, and also reduced the AsA levels in leaves of transgenic potato plants. L-Galactono-1,4-lactone dehydrogenase (GalLDH: EC 1.3.2.3) is the final enzyme in AsA biosynthesis in plants, and catalyzes the oxidation of L-galactono-1,4-lactone (GalL) to AsA. Recently, we reported that antisense suppression of GalLDH markedly reduced AsA levels in transformed tobacco BY-2 cultured cells, and this decline in AsA levels suppressed further cell division and cell growth (Tabata et al., 2001). Furthermore, AsA-deficient BY-2 cells were abnormal in both phenotype and structure. Hence, we proposed that AsA functions as a regulator of the cell cycle and as a supporter of cell structure (Tabata et al., 2001). This is because ascorbate oxidase (AAO: EC 1.10.3.3) catalyzes the oxidation of AsA to monodehydroascorbate, and functions as an important factor of the cell elongation/expansion (Kato and Esaka, 1996, 1999, 2000). Additionally, ascorbate peroxidase (APX: EC 1.11.1.11) plays a role in eliminating H_2O_2 generated during photosynthesis and oxidative stresses (Asada, 1997).

In this paper, the gene expression of two AsA-biosynthetic enzymes, GMPase and GalLDH, and three AsA-oxidizing enzymes, AAO, stromal APX (sAPX) and cytosolic APX (cAPX), was investigated in both cultured cells and tissues of tobacco. Finally, we discuss possible relationships between AsA content and the expression of these AsA-related enzymes.

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2. Results and discussion

The redox system of AsA is involved in regulation of cell division and elongation in plants (Liso et al., 1984; Citterio et al., 1994; De Pinto et al., 1999). Thus, we investigated the possible relationship between cell growth, AsA content and gene expression of AsA-related enzymes, GMPase, GalLDH, AAO, cAPX and sAPX, during suspension culturing of tobacco BY-2 cells. In this regard, a full-length GMPase cDNA was obtained from tobacco BY-2 cells with an open reading frame of 1,083 bp encoding a protein of 361 amino acids. The deduced amino acid sequence showed a high level identity with other GMPase amino acid sequences known from potato (97%) and *A. thaliana* (89%) (data not shown). Additionally, the amino acid sequence of GalLDH, another AsA-biosynthetic enzyme, was also highly conserved in various plant species (Tabata et al., 2001). As shown in Fig. 1 A, the AsA levels markedly increased in the cells for the first 1–2 days, after which they progressively decreased, concomitant with an increase in cell growth. In tobacco BY-2 cells, mitotic index at markedly increased within 1–2 days with an increase in AsA content (De Pinto et al., 1999). Thus, there is the possibility that an increase in AsA content may stimulate cell division to promote cell growth. Indeed, quiescent-center cells, which are mainly halted in the G1 phase, are induced to enter the S phase by addition of AsA (Liso et al., 1988; Innocenti et al., 1990). We had also suggested previously that in synchronized tobacco BY-2 cells the AsA redox system may regulate cell division (Kato and Esaka, 1999; Tabata et al., 2001).

GalLDH and AAO mRNAs also increased for the first 1–2 days, and then decreased, indicating that their expression patterns were similar to the changes in AsA level (Fig. 1B). It would be interesting to determine whether the large amounts of AsA produced by high expression of GalLDH stimulate cell division, and AAO may also be involved in cell elongation/expansion accompanied by cell division. On the other hand, the level of GMPase mRNA increased in the cells during 3–4 days, indicating that the increase occurs after an increase in AsA accountable levels (Fig. 1B).

That is the AsA levels did not increase further in the GMPase expression levels increased (Fig. 1A). However, GMPase also provides GDP-D-mannose used in protein glycosylation and for the biosynthesis of cell wall polysaccharides as well as for AsA biosynthesis (Keller et al., 1999; Reiter, 1998). Nevertheless, the pattern of change in GMPase mRNA was similar to that of total sugar content (Fig. 1C). The marked growth of tobacco BY-2 cells for 3–4 days requires a large amount of GDP-D-mannose to form cell walls during culturing. Consequently, the higher expression of GMPase for 3–4 days may be required to provide cell wall sugars, but not to biosynthesize AsA. On the other hand, sAPX and cAPX mRNAs appear to be expressed constantly during the culture.

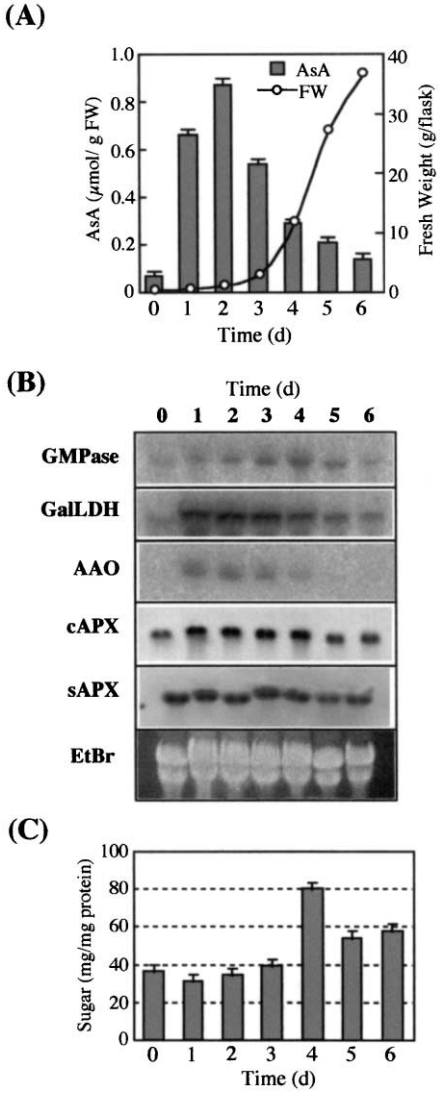

Fig. 1. Changes in AsA content and AsA-related enzyme expression during tobacco BY-2 suspension-culture. (A) Changes in AsA content and fresh weight of cells. (B) Northern blot analysis of AsA-related enzymes, GMPase, GalLDH, AAO, cAPX and sAPX. Total RNA (each 10 μg) was isolated from tobacco BY-2 cells cultured for 0, 1, 2, 3, 4, 5 and 6 days. Total RNA (each 10 μg) was loaded on agarose gel, and stained by ethidium bromide (EtBr). (C) Change in sugar content. The values are the mean ± SE of three experiments.

Arrigoni et al. (1997) showed that adding exogenous AsA or GalL raised the AsA levels and that increasing amounts of AsA stimulated both root and hypocotyl growth in *Lupinus albus*. By contrast, an addition of dehydroascorbate (DHA) resulted in a rapid increase in AsA content, but inhibited cell growth in cultured cells and tissues of *Lupinus albus* (De Pinto et al., 1999; Paciolla et al., 2001). Thus, we next focused on AsA turnover in tobacco BY-2 cells, with changes in AsA levels and AsA-related enzyme expression (following addition of

GalL or AsA) being examined. To do this, tobacco BY-2 cells were grown for 4 days then transferred to fresh medium containing 50 mM GalL or 50 mM AsA, and incubated for 3 or 6 h. After the cells were collected, the AsA content and mRNA expression of AsA-related enzymes were determined. As shown in Fig. 2A, AsA and GalL loading resulted in a minimal increase in AsA levels in the cells at 3 and 6 h, a result which differs from that reported by Arrigoni et al. (1997). Moreover, following incubation for 3 h, the levels of GMPase, GalLDH and AAO mRNAs were similar to those of the control (Fig. 2B). On the other hand, following incubation for 6 h, the GMPase and GalLDH mRNAs markedly decreased (Fig. 2B). Thus, there is a possibility that AsA biosynthesis may be 'feedback' regulated by the increased AsA concentration in the cells.

Recently, the control of AsA biosynthesis and turnover was investigated by using isotope-labeled AsA in pea seedlings (Pallanca and Smirnoff, 2000). The absolute turnover rate was higher in AsA-loading, suggesting the existence of a constant pool for AsA. Furthermore, it has been reported that the apparent rate of AsA synthesis was decreased by high AsA levels and that the effect was appreciable even after short loading period (Potters et al., 2000). Thus, it seems that a low level of AsA or GalL can stimulate cell progression, whereas a high level greater than the pool size may suppress the uptake and biosynthesis of AsA in order to maintain the intercellular AsA-DHA redox. In addition, AAO mRNA in the cells also decreased by AsA loading (Fig. 2B). Thus, the balance of AsA-DHA redox may regulate the cell cycle (Liso et al., 1984; Pallanca and Smirnoff, 2000) and, the loading of AsA may break this balance. Moreover, the AsA loading may markedly stimulate cell division, whereas expression of AAO, which is involved in cell elongation/expansion, may be suppressed. The cAPX mRNA was unchanged by AsA and GalL loading whereas sAPX mRNA was decreased by loading (Fig. 2B). This result supports the report that AsA loading had no effect on APX, monodehydroascorbate reductase, DHA reductase or glutathione reductase activity (Pallanca and Smirnoff, 2000).

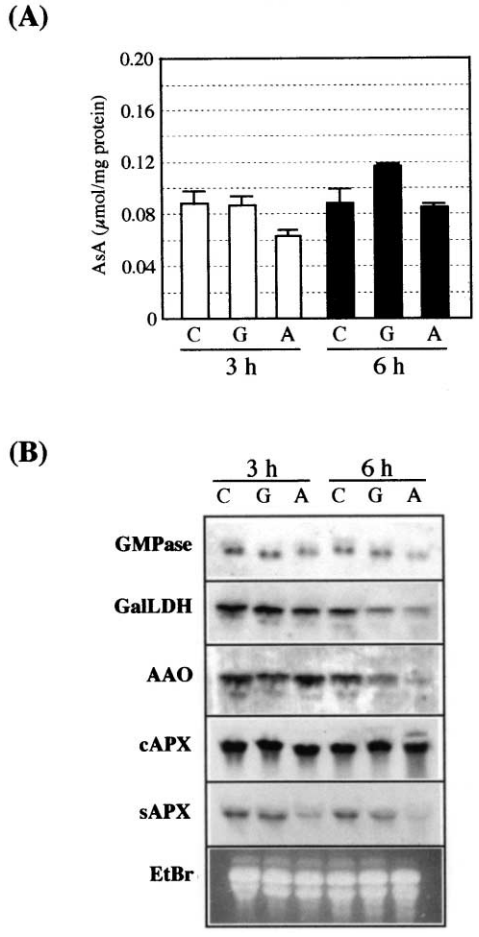

Fig. 2. The effect of GalL and AsA loading on AsA biosynthesis in tobacco BY-2 cells. The cells cultured for 4 days were transferred to fresh medium in the absence (C; control) or the presence of 50 mM GalL (G) or 50 mM AsA (A), and were incubated for 3 or 6 h at 28 °C in dark. (A) Change in AsA content. The values are the mean ± SE of three experiments. (B) Northern blot analysis of AsA-related enzymes. Total RNA (each 10 μg) was loaded on agarose gel, and stained by ethidium bromide (EtBr).

AsA is also required for the photosynthesis system as an antioxidant scavenging the active oxygen. Thus, we investigated the effect of light on the expression of AsA-related enzymes in tobacco leaf. No irradiation in the half of leaf was carried out by covering half using an aluminum wrap, and uncovered the other half side of leaf (a control) which was constantly irradiated (Fig. 3 A). After 5 days, the covered half was exposed to light for 3 h. AsA content is lower in the covered then the exposed half, and which then increased after removing of wrap (Fig. 3B), suggesting that AsA biosynthesis may be controlled by light. The cAPX mRNA was also markedly suppressed in the covered half, and recovered by re-irradiation (Fig. 3C). On the other hand, the sAPX mRNA decreased in the covered half, and was undetected in both sides of re-irradiated leaf. Recently, Yoshimura et al. (2000) demonstrated that the expression of cAPX was induced by high light stress in Spinach seedling. Thus, cAPX has light-dependent expression to eliminate H_2O_2 abundantly generated by light. Interestingly, GMPase, GalLDH and AAO mRNAs also markedly decreased in the non-irradiated half, and were significantly increased by re-irradiation (Fig. 3C). Indeed, the changes in GMPase and GalLDH mRNAs almost coincide with that in AsA content (Fig. 3B). Grace and Logan (1996) showed that AsA content in leaf was increased by high light. In *Arabidopsis*, high light is required to stabilize AsA and to induce expression of cAPX (Karpinski et al., 1997). Perhaps, the increase in AsA content by light could be explained by up-regulation of GMPase and GalLDH

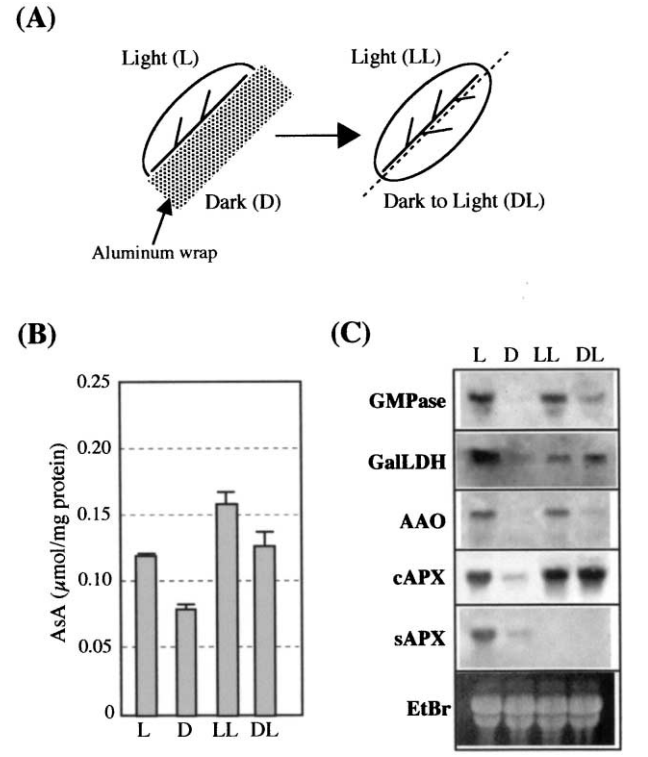

Fig. 3. Effect of light on expression of AsA-related enzymes in leaves. (A) Illustration for experimental method. The half side of tobacco leaf was covered using aluminum wrap and grown at 28 °C in light / dark (16/8 h). After 5 days, the covered half was exposed to light by removing wrap and incubated for 3 h. (L; un-covered half grown for 5 d in light, D; wrap-covered half grown for 5 days in dark, LL; uncovered half grown for 5 days in light and incubated for 3 h in the light, DL; covered half grown in dark for 5 days, and incubated for 3 h in light after removing the wrap) (B) Change in AsA content. The values are the mean±SE of three experiments. (C) Northern blot analysis of AsA-related enzymes, GMPase, GalLDH, AAO, cAPX and sAPX. Total RNA (each 10 μg) was loaded on agarose gel, and stained by ethidium bromide (EtBr).

expression. Thus, light may regulate leaf AsA biosynthesis, which is required for photoprotection. Veljovic-Jovanovic et al. (2001) recently suggested that AsA plays a key role in maintaining homeostasis in the antioxidative system using *Arabidopsis vtc-1*, an AsA-deficient mutant. We thus support the proposal that AsA-related enzymes such as AAO, sAPX and cAPX must play some role in protection system from oxidative stresses. However, lack of irradiation may also induce senescence accompanied by decline of enzyme expression. Thus, it cannot be ruled out that these changes resulted from senescence during non-irradiation and regrowth during re-irradiation. Further studies will be required to clarify the influence of light on the biosynthesis of AsA.

In this study, we suggested that AsA-biosynthetic enzymes, GMPase and GalLDH, may regulate AsA biosynthesis, and their expression may be "feedback" regulated by AsA pool in the cells. Furthermore, we propose that AsA biosynthesis in leaf may be regulated by light. AsA controls cell division/elongation and also play an important role in photoprotection system. However, the detailed mechanism controlling AsA levels in cells remains unclear. Further analyses of the relationship between AsA level and AsA-related enzymes will be required to clarify regulation mechanism of AsA in plants.

3. Experimental

3.1. Plant material

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow No.2) cells were cultured in modified Linsmaier and Skoog medium with 2.5 mM K_2HPO_4, 3 mM thiamine HCl, 3% (w/v) sucrose and 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D), according to the method of Nagata et al. (1992). Tobacco (*Nicotiana tabacum* L. cv. SR-1) plants were grown at 28 °C in light/dark (16/8 h) in the greenhouse.

3.2. cDNA cloning for GMPase from tobacco BY-2 cells

Tobacco GMPase cDNA was cloned by RT (reverse transcription)–PCR method using total RNA, which was prepared from tobacco BY-2 cells cultured for 5 days by modified SDS-phenol method (Chirgwin et al., 1979). Oligonucleotide primers (Fw; 5′-GTCCCAAAGCCACTCGTC-3′, Rv; 5′-AACAGTAGAGTGCCAGCC-3′) were designed based on amino acid sequences from potato (Keller et al., 1999) and *Arabidopsis thaliana* GMPase (accession number: AF076484). A full-length GMPase cDNA (accession number: AB066279) was obtained by 5′- and 3′-RACE (rapid amplification of cDNA ends) methods using Marathon cDNA amplification kit (Clontech). The cAPX and sAPX cDNA fragments were obtained by RT-PCR using gene specific primers (cAPX-Fw: 5′ - GAGCAGTTTCCTATCCTC - 3′, cAPX - Rv: 5′ - CGTCTAATAACAGACGCC - 3′, sAPX - Fw: 5′ - GACAAGTATGCTAATGTG - 3′, sAPX - Rv: 5′-CTCCTTCATTAGGCTACC-3′) designed, based on tobacco sAPX (accession number, AB022274) and cAPX (accession number, D85912).

3.3. Northern blot analysis

Total RNA for northern blot analysis was isolated by modified ATA (aurintricarboxylic acid) method (Verwoerd et al., 1989). RNA (10 μg) was subjected to electrophoresis on a 1% (w/v) agarose gel containing 16.7% (v/v) formaldehyde and 1 × MOPS (20 mM (N-morpholino)propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), and transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech). The membrane was

pre-hybridized at 65 °C for 2 h in 50% (v/v) formamide, 5×SSC (750 mM sodium chloride, 75 mM sodium citrate, pH 7.0), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS and 2% (w/v) blocking reagent. The membrane was hybridized at 65 °C for 16 h in the presence of DIG (digoxigenin)-UTP-labeled RNA probes. DIG-labeled RNA probes were synthesized from tobacco BY-2 GMPase, GalLDH, AAO, sAPX and cAPX using DIG RNA labeling kit (SP6/T7) (Roche). The membrane was washed with 2×SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS at room temperature for 15 min and twice with 0.2×SSC (30 mM sodium chloride, 3 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS at 65 °C for 15 min. The detection of mRNA was followed with DIG-system (Roshe).

3.4. Measurements of AsA and sugar content

Frozen and powdered cells were suspended in cold 50 mM potassium phosphate (pH7.5) containing 1 mM EDTA. After centrifuging at 10,000 *g* for 10 min, the supernatant was used for the measurement of AsA and total sugar contents. AsA content was measured in crude extract by Ascorbic Acid Test (Merck) using RQ flex plus (Merck). The amount of sugar was determined according to the method of Hatanaka and Takahara (1980).

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