We investigated the alteration in L-ascorbate (AsA, reduced form) content and the expression pattern of its related genes during the phase transition in Oncidium orchid. During the vegetative growth, a high H₂O₂ level was associated with a high content of the reduced form of AsA. During the bolting period, the AsA content and H₂O₂ level were greatly reduced in parallel with increased expression of OgLEAFY, the gene encoding a key transcription factor integrating different flowering-inducing pathways. This observation suggests that reduced AsA content, due to it having been consumed in scavenging H₂O₂, is a prerequisite for mediating the phase transition in Oncidium. A survey of the AsA biosynthetic pathway revealed that the gene expression and enzymatic activities of the products of relevant genes of the galacturonate (GalUA) pathway, such as polygalacturonase (OgPG), pectin methylesterase (OgPME) and galacturonate reductase (OgGalUAR), were markedly decreased during the bolting period, as compared with during the vegetative stage. However, the genes whose products were involved in the Smirnoff–Wheeler pathway retained a similar expression level in the two growth stages. The data suggested that reduced AsA content, due to it having been consumed in scavenging H₂O₂, is a prerequisite for mediating the phase transition in Oncidium. A survey of the AsA biosynthetic pathway revealed that the gene expression and enzymatic activities of the products of relevant genes of the galacturonate (GalUA) pathway, such as polygalacturonase (OgPG), pectin methylesterase (OgPME) and galacturonate reductase (Og GalUAR), were markedly decreased during the bolting period, as compared with during the vegetative stage. However, the genes whose products were involved in the Smirnoff–Wheeler pathway retained a similar expression level in the two growth stages. The data suggested that OgPME of the GalUA pathway was the pivotal gene in regulating AsA biosynthesis during the bolting period. Further elucidation by overexpressing OgPME in Arabidopsis demonstrated a considerable increase in AsA content, as well as a resulting delayed-flowering phenotype. Our results strongly imply that the reduced level of AsA, regulating bolting for phase transition, resulting in part from its consumption by scavenging H₂O₂, was mainly caused by the down-regulation of the GalUA pathway, not the Smirnoff–Wheeler pathway.

**Keywords:** L-Ascorbic acid (AsA) • Bolting period • GalUA pathway • H₂O₂ • Pectin methylesterase (PME) • Smirnoff–Wheeler pathway.

**Abbreviations:** AIR, alcohol-insoluble residue; AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, L-ascorbate; B, bolting period; CaMV, cauliflower mosaic virus; DAB, 3,3′-diaminobenzidine; DAS, days after sowing; DTT, dithiothreitol; GalDH, D-galactose dehydrogenase; GalL, L-galactono-1,4-lactone; GalLDH, GalL dehydrogenase; GalUA, D-galacturonic acid; GalUAR, galacturonate reductase; GMP, GDP-β-mannose pyrophosphorylase; PG, polygalacturonase; PME, pectin methylesterase; PVPP, polyvinylpolypyrrolidone; R, reproductive stage; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; TCA, tricarboxylic acid; V, vegetative stage.

**Introduction**

L-Ascorbate (AsA) is an important antioxidant to eliminate reactive oxygen species (ROS) produced by environmental stress or during plant growth and development. It is also a cofactor for many enzymes involved in the biosynthesis of phytohormones, anthocyanins and various secondary metabolites (Smith et al. 2007). AsA has recently been suggested to be important in regulation of developmental senescence, plant defense against pathogens and redox signaling across the cell membrane, and as the precursor of tartrate and oxalate (Pignocchi and Foyer 2003, Barth et al. 2004, Noctor 2006, Debolt et al. 2007). Recent evidence suggests that it may also play a role in floral induction (Barth et al. 2006).

VTC1 encodes a GDP-β-mannose pyrophosphorylase (GMP), which is an enzyme in the Smirnoff–Wheeler pathway for AsA biosynthesis in plants. The mutant vtcl of Arabidopsis, accumulating a low level of AsA and displaying an early flowering time, was considered to promote senescence during the long-day photoperiod (Conklin and Barth 2004). Interestingly, the opposite effect on delayed flowering
time was considered to be due to decreased activity of gibberellins during the short-day photoperiod (Veljovic-Jovanovic et al. 2001, Attolico and De Tullio 2006). Therefore, AsA has a significant effect on delaying flowering time, and alterations in AsA content mediate the expression of genes in all known pathways for flowering in Arabidopsis (Kotchoni et al. 2009). The expression of many genes in the circadian clock, photoperiodic pathway and autonomous pathway, such as LHY, TOC1, GI, CO and FLC, is significantly higher in vtc1 mutants than in wild-type plants under each photoperiod. Furthermore, β-glucuronidase (GUS) activity driven by the AtLEAFY promoter was decreased after treatment with l-galactono-1,4-lactone (GalL), the precursor of AsA (Attolico and De Tullio 2006). Therefore, AsA has a definitive relationship with flowering time. Surprisingly, H₂O₂ levels were not significantly elevated in vtc mutants as compared with the wild-type plants at flowering time (Kotchoni et al. 2009), so the early flowering phenotype in the vtc mutant is caused not just by elevated oxidative stress. Moreover, an AsA oxidase (AO) antisense plant displayed a higher level of AsA and a greater redox ratio than wild-type plants, and also a later flowering time (Yamamoto et al. 2005). In addition, some double mutants of Arabidopsis which were deficient in cytosolic and thylakoid AsA peroxidase (APX) exhibited early flowering under oxidative stress (Pnueli et al. 2003). Therefore, flowering time was proposed to be mediated by the AsA level and its redox state, and associated with the level of ROS. Although the effect of the causal relationship of AsA and ROS on flowering induction is unclear, the accumulation of ROS before flowering has been observed in morning glory (Pharbitis nil) (Hirai et al. 1995), wheat (Badiani et al. 1996) and Arabidopsis (Lokhande et al. 2003). Therefore, AsA levels or its redox state was suggested to be an internal signal to facilitate plants in responding to environmental signals by adjusting plant development, including the transition from the vegetative to the reproductive growth stage (Colville and Smirnoff 2008, Kotchoni et al. 2009).

Knowledge of AsA biosynthesis has remained incomplete. In addition to the major route of the Smirnoff–Wheeler pathway (Wheeler et al. 1998), other potential branch pathways have been discovered in plants, such as the galacturonate (GalUA) pathway (Agius et al. 2003), the gulose pathway (Wolucka and Van Montagu, 2003) and the myoinositol pathway (Lorence et al. 2004). l-Gall is a precursor of AsA biosynthesis. It is converted to AsA by Gall dehydrogenase (GalLDH), and GalLDH is the final step of both the Smirnoff–Wheeler and GalUA pathways (Wheeler et al. 1998). GalLDH, as well as other genes in the Smirnoff–Wheeler pathway, are induced by jasmonic acid through stimulation of H₂O₂ (Sasaki-Sekimoto et al. 2005, Wolucka et al. 2005). Recent results also revealed that the de novo rate of AsA synthesis increased as a result of treatment with glutathione (Pavet et al. 2005) or α-tocopherol (Kanwischer et al. 2005). Furthermore, d-GalUA produced from the degradation of the cell wall pectin was the beneficial precursor of AsA biosynthesis (Davey et al. 1999). Further evidence showed that the AsA level was increased in transgenic Arabidopsis because of the conversion of d-GalUA to galactonic acid by overexpression of GalUAR, cloned from strawberry (Agius et al. 2003). Accordingly, a GalUA pathway might exist in Arabidopsis and strawberry and act as an alternative route for AsA biosynthesis. The GalUA pathway was processed at the cell wall; both pectin methylesterase (PME) and polygalacturonase (PG) were considered to demethylate and digest pectin into d-GalUA for AsA biosynthesis (Smirnoff 2003). However, the involvement of the genes encoding these enzymes in AsA biosynthesis and the regulatory mechanism of the GalUA pathway are still unclear.

Our recent research into carbohydrate mobilization in Oncidium orchid revealed that the level of mannan, pectin and AsA in pseudobulbs varied during the vegetative stage and bolting period (Wang et al. 2008). In particular, the l-Gall level decreased 5-fold during the bolting period (Wang et al. 2003). Also, many specific genes related to carbohydrate mobilization and bolting were found to be preferentially expressed in the developing pseudobulb (Tan et al. 2005). The phase switch from the vegetative to the reproductive stage is specified by the bolting period at the pseudobulb base (Fig. 1A). The transition to bolting entails a radical change within the apical meristem of the axillary shoot apex and determines the inception of reproductive growth. The shoot apex that commits the apical meristem to bolting is collectively referred to as floral evocation. Once the shoot meristem becomes committed to the new developmental program (bolting period in Oncidium), it is considered to be florally determined or reproductive stage determined. The floral (bolting in Oncidium) stimulus to the axillary shoot meristem that alters the developmental fate of the cell of the shoot apex is still ambiguous in Oncidium. Our recent work revealed a greatly increased AsA content and H₂O₂ level in pseudobulbs at the vegetative stage but the levels rapidly decreased during bolting. We were interested in the physiological effect on the bolting mechanism and growth of the floral stalk in Oncidium. The investigation into the relevant AsA biosynthetic genes in Oncidium showed that the expression of genes of the GalUA pathway, an alternative pathway for AsA synthesis, was associated with the H₂O₂ level. Many lines of evidence indicated that the GalUA pathway was predominant in regulating AsA content to control the phase switch. Therefore, in this study, we addressed the alteration in AsA content and the physiological significance of the GalUA pathway in the bolting mechanism of Oncidium.

## Results

As shown in Fig. 1A, the developmental stages of Oncidium are delineated by a vegetative stage (V), bolting period (B)
and a reproductive stage (R), in parallel with pseudobulb growth. During the bolting period, so-called floral induction in Oncidium, an axillary bud at the second node develops into a juvenile inflorescence stalk. The phase change is thus switched on from V to R. The inflorescence stalk then develops quickly into a mature inflorescence and many flowers. The level of AsA, H$_2$O$_2$ and pectin in pseudobulb tissues diminished during the phase transition. We addressed the regulatory mechanism of bolting from the pseudobulb in this study.

The marked decrease in AsA content and H$_2$O$_2$ level in the pseudobulb during the bolting period

The endogenous AsA and H$_2$O$_2$ levels were biochemically quantified and monitored by histochemical staining in pseudobulb tissues of Oncidium at three different developmental states: V, B and R. The concentration of the reduced form of AsA at V and B was found to be 2.61 and 1.4 µmol g FW$^{-1}$, respectively (Fig. 1B), indicating that the reduced form of AsA at V was 1.9-fold that at B. At R, the content of the reduced form of AsA increased a little, to 1.55 µmol g FW$^{-1}$. Notably, the ratio of the reduced to oxidized form of AsA changed from 12 at V to 3.92 at B, and back to 12 at R. This finding suggests that a reduced form of AsA was largely consumed during B. Meanwhile, the level of H$_2$O$_2$ was 175 µM g FW$^{-1}$ at V, 145.3 µM g FW$^{-1}$ at B and 96.29 µM g FW$^{-1}$ at R (Fig. 1B). The decrease in AsA content in parallel with that of the H$_2$O$_2$ level suggests that AsA is actively carrying out the antioxidation function in scavenging ROS. Consumption of AsA in tissues is due to APX (in Fig. 3B-8, OgAPX) activity that utilizes AsA to remove H$_2$O$_2$ molecules in the pseudobulb. The histograms of H$_2$O$_2$ staining by 3,3′-diaminobenzidine (DAB) and AsA staining by AgNO$_3$ confirm the biochemical measurements, showing...
theat H$_2$O$_2$ and AsA greatly accumulated at V and strikingly decreased around bolting (Fig. 1B).

Furthermore, the expression profile of OgLEAFY during various developmental stages of the pseudobulb was investigated because its function is linked directly to floral induction in planta. OgLEAFY was inactive in both the axillary bud and inflorescence bud at V (Fig. 2A). Once bolting occurred, it was actively expressed in juvenile inflorescence at B and lasted for the whole of R (Fig. 2A). Therefore, this indicated that OgLEAFY expression was functionally involved in the development of bolting and suggested that low AsA content at B was associated with OgLEAFY expression and triggered bolting in the pseudobulb.

To understand further the effect of AsA content on bolting and OgLEAFY expression, juvenile inflorescences of Oncidium plants at B were treated with 0.1 M AsA solution twice each day (as described in Materials and Methods). The expression of OgLEAFY was repressed (Fig. 2B) and bolting was later than that of the control plants (Fig. 2C, D). In contrast, H$_2$O$_2$ treatment not only enhanced OgLEAFY expression (Fig. 2B) but also sped up early bolting (Fig. 2C) and fast-growing inflorescence (Fig. 2D). When H$_2$O$_2$ was applied after AsA treatment, the expression of OgLEAFY was active as in normal growth (Fig. 2B, A/H), but when AsA was applied after H$_2$O$_2$, the expression of OgLEAFY was repressed (Fig. 2B, H/A). These data, obtained by artificially altering the level of AsA, support the expression of OgLEAFY occurring with a low content of AsA but repression occurring with a high content of AsA. Moreover, the H$_2$O$_2$ level can counteract the endogenous AsA level to control the expression level of OgLEAFY. In conclusion, a low level of AsA mediates bolting emergence and determines the phase change from vegetative to reproductive growth.

Reduction of AsA content during the bolting period is due to the down-regulation of the genes of the GalUA pathway but not the Smirnoff–Wheeler pathway

The Smirnoff–Wheeler and GalUA pathways are two definite routes for AsA biosynthesis in plants (Smirnoff 2003). We investigated relevant genes in the Smirnoff–Wheeler
pathway, such as OgGMP and OgGalDH, as well as those in the GalUA pathway, such as OgPG, OgPME and OgGalUAR, for their expression pattern during V and B in Oncidium. As shown in Fig. 3A, the expression of OgGMP and OgGalDH did not differ significantly between V and B. However, the expression of OgPG, OgPME and OgGalUAR was less active at B than at V. OgPME seemed to be the critical gene in regulating AsA content, because its expression level was strikingly decreased at B (Fig. 3A). Concomitantly, the expression of OgGalLDH, the final step integrating the two AsA biosynthetic routes to form AsA, was reduced at B (Fig. 3A). The enormously reduced transcriptional activity of the components of the GalUA pathway at B implied that the pathway could cause a decreased AsA level in Oncidium.

Our previous observation indicated that the H$_2$O$_2$ level was high in the pseudobulb at V and was decreased at B (Fig. 1). To unravel the effect of H$_2$O$_2$ related to the regulation of the GalUA pathway, Oncidium plants at B were sprayed with H$_2$O$_2$ solution (1 mM), and the expression patterns of the genes of both the GalUA and Smirnoff–Wheeler pathways were monitored 2 h after H$_2$O$_2$ treatment. The expression of genes involved in both the GalUA and Smirnoff–Wheeler pathways was enhanced on H$_2$O$_2$ stimulation (Fig. 3A). These data first show that the GalUA pathway is enhanced by H$_2$O$_2$ stimulation.

Further investigation of the enzymatic activities of the above-mentioned AsA biosynthetic genes showed that they were largely coincident with the transcriptional levels. The activities of OgGalDH and OgGMP in the Smirnoff–Wheeler pathway were equal at V and B (Fig. 3B), whereas the activities of OgPME and OgGalUAR in the GalUA pathway were greatly reduced at B (Fig. 3B). The activity of GalUA oxidase, which converts D-GalUA to galactaric acid, competing for the same substrate with GalUAR, was higher at B. However, enzymes that consume AsA (i.e. OgAPX and OgAO) showed higher activities at B than at V (Fig. 3B). This evidence suggests that the decrease in AsA content at B resulted wholly from the down-regulation of the expression of genes of the GalUA pathway and the increased activities of AsA catabolic enzymes such as OgAPX and OgAO. Thus, the GalUA pathway rather than the Smirnoff–Wheeler pathway is predominant in regulating the AsA content at B in Oncidium.

**Pectin accumulation and high PME activity show the availability of the GalUA pathway to carry out AsA biosynthesis at the vegetative stage in Oncidium**

Pectin, abundant in primary cell walls of developing cells, is considered the precursor of the GalUA pathway. To understand the role of the GalUA pathway in AsA biosynthesis at V and B, we assayed the change in pectin content and PME activity in the pseudobulb at V and B. Pectin content was decreased, from 42 mg g FW$^{-1}$ at V to 18 mg g FW$^{-1}$ at B (Fig. 4A). Histochemical staining by ruthenium red revealed abundant formation of demethylated pectin in tissues at V but little formation at B (Fig. 4B). This indicated that the demethylation reaction was performed by OgPME in the pseudobulb at V. In contrast, the level of methylated pectin was found to be greater at B than at V on staining pseudobulb tissues with hydroxylamine-FeCl$_3$ (Fig. 4B). Meanwhile, a high OgPME activity was detected only at V (Fig. 4B). The higher OgPME activity and plentiful pectin at V, both of which corresponded to abundant AsA biosynthesis, revealed that pectin was largely utilized to synthesize AsA at V.
These findings, together with the observation of a reduced level of OgPME transcription at B (Fig. 3A, B), also suggest that this enzyme plays an important role in regulating the GalUA pathway for AsA biosynthesis between V and B. In summary, the GalUA pathway, which utilizes demethylated pectin as a substrate, could be an important route to supply AsA for the vegetative growth of *Oncidium*. Overexpressing OgPME in Arabidopsis demonstrates the elevated AsA level delaying flowering time and a significant effect of the GalUA pathway on flowering

Although PME was recognized as an intermediate key enzyme functioning in the GalUA pathway (Smirnoff 2003), its physiological significance in AsA synthesis is still ambiguous. Therefore, we generated transgenic Arabidopsis overexpressing OgPME to investigate the functional role of PME in AsA biosynthesis and related effects. Transgenic plants were grown under a long-day photoperiod for 14–35 days after sowing (DAS) and confirmed by reverse transcription–PCR (RT–PCR) and enzymatic activity analysis (Fig. 5). One of the independent lines, named OgPMEOX, was selected for functional characterization from 12 transgenic lines. As shown in Fig. 6A, AsA content in transgenic lines (4.5 µmol, on average) was consistently higher than in wild-type plants (2.3 µmol, on average). In addition, transgenic lines showed a delay in flowering time. Transgenic lines flowered at 27.7 DAS with 10.6 leaves as compared with wild-type plants, which flowered at 21.3 DAS with 8.2 leaves (Fig. 7A, B). Moreover, the ratio of the reduced to oxidized form of AsA was steady (2.0–2.5) in transgenic lines but fluctuated (1.3–5.3) in wild-type plants (Fig. 6B). Notably, the redox ratio of wild-type plants was high (5.3) at 14 DAS but was strikingly decreased (1.3) at 21 DAS (Fig. 6B). This implied that an increase of the oxidized form of AsA or a reduction of the reduced form of AsA occurring in the wild type at 21 DAS was necessary for flowering induction in wild-type Arabidopsis. These results showed that overexpressing OgPME in Arabidopsis could increase the AsA content and maintain a steady ratio of the AsA redox state, consequently delaying flowering time. This finding strongly implies that PME expression in the GalUA pathway is an effective step to regulate AsA synthesis and can affect floral induction and phase transition.

**Discussion**

The phase transition in *Oncidium* orchid is switched on from the point of onset of bolting, which arises from a tiny inflorescent bud at the base of the pseudobulb. The developmental program that commits to the induction of bolting and inflorescence growth is an interesting aspect of *Oncidium*.  

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**Fig. 4** Pectin content and composition in the pseudobulb varied with PME activity. (A) Variation of pectin content in the growth stages V, B and R. Vertical bars represent the standard deviation of the mean (n = 5). (B) PME activity staining (upper) and histogram staining of transverse sections of pseudobulbs. Demethylated pectin was examined by ruthenium red staining (filled triangle) and methylated pectin by hydroxylamine-FeCl₃ staining (open triangle). The black scale represents 50 µm.

**Fig. 5** Overexpression of PME in Arabidopsis. OgPME overexpressed in transgenic Arabidopsis (T₅) was detected by RT–PCR, and activity staining was carried out after different growth durations. WT, wild type; OX, transgenic Arabidopsis.
When the pseudobulb grows at the vegetative stage (V), the organ accumulates a large quantity of carbohydrates and nutrients to supply the floral development during the bolting period (B) and the reproductive stage (R). The marked reduction in AsA content associated with a sharp decrease in H$_2$O$_2$ level was a regular event at B (Fig. 1B). Meanwhile, OgLEAFY was subsequently activated (Fig. 2A). The actual function of such a high AsA content in the developing pseudobulb is unknown, because this aspect of AsA metabolism has not been thoroughly investigated. However, the finding implies some physiological significance associated with the growth and development of Oncidium. ROS, including H$_2$O$_2$, hydroxyl ion and superoxide, are commonly generated during the active growth and developmental state and under environmental stress. AsA, as an effective antioxidant, can prevent cells from being damaged by ROS by counteracting the toxicity of H$_2$O$_2$. In our experiments in Oncidium, the active expression of AsA biosynthetic genes, such as those in the GalUA pathway, is induced or enhanced by a high concentration of H$_2$O$_2$ (Fig. 3A). In association with the active function of OgAPX, AsA is used up for the elimination of H$_2$O$_2$ during B (Fig. 3B). Therefore, AsA production could be a defense mechanism and have an antioxidative effect against the H$_2$O$_2$ outburst during the active metabolism state in the Oncidium pseudobulb. The reduction in AsA level at B is attributed in part to its consumption in the scavenging reaction of OgAPX against H$_2$O$_2$ and largely comes from the decreasing yield of the GalUA pathway (Fig. 3A, B).

The AsA-deficient vtc1 mutant of Arabidopsis, characterized by a lower content of AsA as compared with that of the wild-type plants, showed an early flowering time when grown over long days (Attolico and De Tullio 2006). It was identified as the mutation in GMP of the Smirnoff–Wheeler pathway (Veljovic-Jovanovic et al. 2001, Conklin and Barth 2004). In addition, an early flowering time was also observed in tobacco plants overexpressing AO, which could reduce...
the redox ratio of AsA in plants (Pignocchi et al. 2006). In Oncidium, we showed that the inflorescence bud (bolting) emerged in parallel with a marked decrease in endogenous AsA level and redox ratio (Fig. 1A). Further support for the role of AsA in regulating Oncidium bolting was found in Oncidium plants sprayed with AsA, which were late in terms of flowering time as compared with plants sprayed with water (Fig. 2B–D). These observations clearly indicated that the AsA content, or AsA-dependent redox ratio, was involved in regulating the flowering time in planta.

The AsA biosynthetic pathway was recently fully elucidated through an amalgamation of biochemical, genetic and transgenic approaches. The Smirnoff–Wheeler and GalUA pathways are two general routes utilized in most plants. In general, the Smirnoff–Wheeler pathway is the main one in plant tissues, especially in ripening fruit of blackcurrant (Ribes nigrum) (Hancock et al. 2007). However, the GalUA pathway is considered an alternative pathway in biosynthesizing AsA (Agius et al. 2003). The pathway was first reported in the biosynthesis of AsA in strawberry (Fragaria ×ananassa) fruit, occurring through D-GalUA, a principal component of cell wall pectin. The expression of PME was associated with the production of D-GalUA, and that of GalUAR affected the yield of AsA content. Both genes were considered critical in biosynthesizing AsA in the GalUA pathway (Smirnoff 2003). Our present observations of the expression profile of the GalUA pathway during V and B seems to agree with previous reports. The low transcriptional levels and enzymatic activities of OgPME and OgGalUAR in the GalUA pathway found during B may cause a low level of AsA production (Fig. 3A, B). Together with the fact that AsA was also consumed in the elimination of H₂O₂, these findings may explain the marked decrease in AsA content in the pseudobulb at B. This evidence supported the GalUA pathway rather than the Smirnoff–Wheeler pathway as the dominant pathway in regulating the AsA level during flowering induction in Oncidium.

OgPME is the enzyme responsible for the demethylation of pectin in the cell wall and directed the production of D-GalUA (Smirnoff, 2003). The functional activity of OgPME was able to affect the AsA yield of the GalUA pathway. Our data showed that OgPME was actively expressed in the pseudobulb at V but not at B (Fig. 3A, B). Also, enzymic staining and staining of the histograms demonstrated that cell wall pectin was being demethylated at V (Fig. 4B), and its content became less at B compared with at V (Fig. 4A). This indicates that there is abundant demethylated pectin produced by PME for AsA biosynthesis at V. This observation, in association with the expression pattern of OgGalUAR (Fig. 2), indicated that the GalUA pathway was a critical pathway in regulating the AsA level to affect bolting.

Furthermore, methanol was produced from demethylation of pectin by PME in the developing cell such as that at V in Oncidium. The role of PME in methanol production in tomato fruit was examined by relating the tissue methanol content to the PME enzymatic activity in wild-type Rutgers and isogenic PME antisense fruits with lowered PME activity (Frenkel et al. 1998). The microarray data revealed that AsA-related genes, such as PME, PG, and DHAR (dehydroascorbate reductase), were inducible by methanol stimulation (Downie et al. 2004). Moreover, H₂O₂ was produced when methanol was metabolized in plants (Fall and Benson 1996). H₂O₂ was recognized as an important signal molecule to increase the expression of AsA-related genes in the process (Wolucka et al. 2005). The expression levels of genes in two routes of AsA biosynthesis in Oncidium were also increased by H₂O₂ treatment (Fig. 3A). Therefore, the increasing expression level of AsA-related genes and amount of AsA were associated with the higher PME activity at V. Accordingly, the elevated AsA amount in transgenic Arabidopsis overexpressing OgPME was caused not just by conversion of carbohydrate but also by other associated physiological processes.

Other evidence to support the physiological significance of the GalUA pathway in the bolting of Oncidium is the observation of transgenic Arabidopsis overexpressing OgPME. Transgenic plants, overproducing AsA by 10–50% throughout all growth stages compared with wild-type plants, displayed a mean flowering delay of almost 7 d (Figs. 6, 7). The steady state of the AsA redox ratio (2.0–2.5) in transgenic plants as compared with the wild type (1.5–5.3) (Fig. 6B) indicated that OgPME has a critical function in regulating not only AsA content but also AsA redox state.

In summary, we have analyzed the gene regulatory profile related to AsA biosynthesis during V and B in Oncidium orchid. First, oxidative stress occurred in association with a high content of AsA at V of Oncidium. Secondly, the marked decline in AsA level, resulting from both its consumption by the OgAPX reaction to scavenge H₂O₂ and down-regulation of genes of the GalUA pathway, caused the expression of the OgLEAFY gene and bolting. Thirdly, OgPME has a critical function in the GalUA pathway for AsA biosynthesis. Thus, the GalUA pathway is the dominant route in regulating the AsA level to effect bolting in Oncidium orchid. However, the pathway choice for AsA biosynthesis in different developmental stages seems to be a complex genetic network and is plant dependent. The mechanism of coordination of the AsA biosynthetic pathway to adapt the physiological condition in plants is worthy of further investigation.

Materials and Methods

Plant materials

Oncidium ‘Gower Ramsey’ plants were obtained from Shih-Dong orchid nursery in Taiwan. The plants were grown in 30 cm diameter pots under growth conditions of 25–32°C.
and a 14 h photoperiod in a greenhouse. Total pectin, AsA content, enzyme activity and mRNA expression analysis were carried out with use of the pseudobulbs of different developmental stages. Arabidopsis thaliana ecotype Col-0 and transgenic lines overexpressing OgPME, driven by the cauliflower mosaic virus (CaMV) 35S promoter, were grown at 23 ± 2°C under long-day conditions (16 h light/8 h dark).

**Treatment of Oncidium orchids with AsA and H₂O₂**

To monitor the OgLEAFY gene expression related to the AsA level, Oncidium orchids growing during the bolting period were used for exogenous application of AsA and H₂O₂, and the treated tissues were harvested for total RNA extraction. Four groups of plants, 10 pots for each group, were subjected to careful spraying of chemical solution onto the juvenile inflorescent bud tissues as follows: A, spraying with 30 ml of 10.1 M AsA, and sampling for RNA extraction at 4 h after treatment; H, spraying with 30 ml of 1 M H₂O₂, and sampling for RNA extraction after treatment; A/H, spraying with 30 ml of 0.1 M AsA and, after a 4 h interval, followed by spraying with 30 ml of 1% H₂O₂, then sampling for RNA extraction after 4 h; H/A, spraying with 30 ml of 1% H₂O₂, and, after a 4 h interval, followed by spraying with 30 ml of 0.1 M AsA, then sampling for RNA extraction after 4 h.

To survey the bolting process related to the AsA level and H₂O₂, the orchids from 30 pots growing during the bolting period were used for the treatment. Each group of 10 pots was sprayed with 30 ml of 0.1 M AsA, 1% H₂O₂ and H₂O (control), respectively, on the juvenile inflorescent bud tissues once a day for 1 month. The phenotypic trait of the treated plants was carefully observed and photographed.

**AsA and pectin measurement**

The extraction and measurement of AsA were performed as described (Gillespie and Ainsworth 2007) with slight modification. Oncidium pseudobulbs and Arabidopsis plants at various growth stages were homogenized under liquid nitrogen and then mixed well with 1 ml of 6% trichloroacetic acid (TCA). The supernatant obtained from a 15 min centrifugation at 4°C, 6,000 × g was used as a substrate to measure the total, reduced and oxidized forms of AsA. For the assay of total AsA, the substrate was mixed with 10 mM dithiothreitol (DTT) to reduce the pool of the oxidized form of AsA. After incubation at room temperature for 10 min, 0.5% N-ethylmaleimide (NEM) was added to the mixture to remove the excess DTT. For the assay of reduced AsA, the substrate was supplemented with deionized water only. The mixture was supplemented with reaction buffer (10% TCA, 43% H₃PO₄, 4% α-α′-bipyridyl and 200 µl of 3% FeCl₃) and incubated at 37°C for 1 h. The amount of total and reduced AsA was determined from the A₂₅₀, and the amount of the oxidized form of AsA was calculated from the difference between the total pool and the reduced pool.

The extraction and measurement of pectin was carried out as described previously (Wang et al. 2008). In brief, the pseudobulb was ground in 80% ethanol (5 ml g⁻¹ of tissue) and then boiled for 40 min. After filtering, the residue was washed with 80% ethanol and dried to obtain alcohol-insoluble residues (AIRs). Starch was removed from the AIRs by suspending them in 90% dimethylsulfoxide for 16 h at 20°C and centrifuging at 20,000 × g for 20 min. Pectic polysaccharide was extracted from the starch-free AIRs by stirring in 0.5% ammonium oxalate solution (25 ml g⁻¹ of AIRs) at 80°C for 1 h and centrifuged at 20,000 × g for 20 min. The supernatant was collected, and ethanol was added to five times the volume of the extract to precipitate pectic polysaccharides. The fibrous precipitate was collected by filtration through four layers of miracloth, vacuum dried and weighed.

**AsA and pectin staining**

Histochemical localization of AsA in Oncidium pseudobulb tissue was carried out as described by Tedone et al. (2004) with some modification. Briefly, pseudobulbs from different growth stages were hand sliced into sections of approximately 2 mm. After washing with deionized water, the samples were further fixed and stained in 5% AgNO₃ dissolved in 70% ethanol and 10% glacial acetic acid at 4°C in the dark for up to 24 h. The reaction was stopped by washing the tissue in ethanolic ammonium hydroxide for 15 min. The tissue was finally transferred to 70% (v/v) ethanol and stored at 4°C prior to photography.

For pectin staining, blocks of Oncidium pseudobulbs were initially fixed in FAA (50% ethanol, 5% acetic acid and 10% formaldehyde) for at least 2 h. Sections were stained with 0.02% ruthenium red and further immersed in 70% ethanol for 2–5 min to detect unmethylsterified (acidic) pectin (Sabba and Lulai 2002). To detect methylesterified pectin, tissue sections were incubated with hydroxylamine–ferric chloride solution (14% hydroxylamine and 14% hydroxylamine hydrochloride) for 10 min (Reeve 1959). After draining off the solution, sections were incubated with 33% HCl for 5 min. The sections were finally mounted in a mixture of 10% ferric chloride in 0.1 N HCl. The demethylated and methylated pectin were observed as a red and brownish-red color, respectively, under standard light microscopy, and photographed.

**H₂O₂ measurement and staining**

The concentration of H₂O₂ in pseudobulb tissue was measured following the method described (Maxwell et al. 1999) with some modification. Pseudobulb tissue at different stages was ground to a powder in liquid nitrogen and further homogenized with 100% methanol. The supernatant was obtained from a 20 min centrifugation at 5,000 × g, 4°C and immediately frozen in liquid nitrogen until further analysis. The samples were thawed prior to the fluorescence detection,
and 2,7-dichlorofluorescein diacetate (H$_2$DCF-D) was added to the extract at a final concentration of 5 µM. Fluorescence was measured by use of a Hitachi F2000 fluorescence spectrophotometer (Tokyo, Japan) with excitation and emission wavelengths set at 488 and 525 nm, respectively.

The histogram staining of H$_2$O$_2$ was performed according to the method described by Thordal-Christensen et al. (1997) with the following modifications. The basal portion of pseudobulbs from different growth stages was hand sliced into sections of approximately 2 mm and incubated in 1% DAB-HCl, pH 3.8, in the dark at room temperature for 24 h. After removing the chlorophyll by boiling in ethanol (96%, v/v) for 10 min, the H$_2$O$_2$ showed as a reddish-brown spot by instant polymerization of DAB.

**Enzyme assays**

PME, PG, GalUA, GalUAO, GMP, GalDH, GalLDH, APX and AO activities were assayed following the method described by Nakano and Asada (1981), Marolda and Valvano (1993), Oba et al. (1995), Kato and Esaka (1996), Agius et al. (2003), Fachin et al. (2004), Mieda et al. (2004), Jiang et al. (2005) and Cantu et al. (2006), respectively, with some modifications. Pseudobulbs for the PME activity assay were ground in extraction buffer (0.1 M citrate, 0.1 M sodium citrate, 1 M Na$_2$HPO$_4$ and 1 M NaCl, pH 5.0), those for PG activity assay were ground in extraction buffer (1 M NaCl and 0.2 M Na$_2$HPO$_4$ in 1 M citrate buffer, pH 4.0) and those for APX activity assays were ground in extraction buffer (2.5 ml of 25 mM potassium phosphate buffer, pH 7.2) containing 2% polyvinylpyrrolidone (PVPP), 0.4 mM EDTA and 1 mM AsA. For the other activity assays, the pseudobulbs were ground in extraction buffer (50 mM sodium phosphate buffer, pH 7.2, 2 mM EDTA, 2 mM DTT, 20% glycerol and PVPP). The supernatant obtained after a 30 min centrifugation at 4°C at 6,000×g was used as the crude enzyme. For PME assay, the crude protein was mixed with reaction buffer (0.1% esterified pectin in 0.2 M Na$_2$HPO$_4$ buffer, pH 6.3). After overnight incubation at 37°C, 0.05% ruthenium red was added and mixed for incubation for 10 min. Then 0.6 M CaCl$_2$ was added to precipitate the demethylated pectin. The mixture was centrifuged at 14,000×g for 15 min to remove the precipitate. The absorbance of the supernatants of the sample were measured at 534 nm. For PG assay, the crude protein was mixed with reaction buffer (1% cyanoaceticamide in 0.1 M borate buffer, pH 7.0) for 5 min. PG activity was determined from the increase in A$_{235}$ of 2-cyanoacetamide by production of galacturonic acid. One unit of PG was defined as the activity that produced 1 µmol of galacturonic acid min$^{-1}$ g FW$^{-1}$. For GalUA assay, the crude protein was mixed with reaction buffer (0.1 mM NADPH and 0.1 mM galacturonic acid in 50 mM sodium phosphate buffer, pH 7.2) for 1 min. GalUA activity was determined from the increase in A$_{290}$ by the production of NADP$^+$. One unit of GalUA reductase was defined as the activity that oxidized 1 µM NADPH min$^{-1}$ mg$^{-1}$ total protein. For GalUOA assay, the crude protein was mixed with reaction buffer (0.1 mM galacturonic acid and 1% 2-cyanoacetamide in 50 mM sodium phosphate buffer, pH 7.2) for 1 min. GalUOA activity was determined from the decrease in A$_{276}$ of 2-cyanoacetamide by the oxidation of galacturonic acid. One unit of GalUA oxidase was defined as the activity that decreased 1 ng GalUA min$^{-1}$ mg$^{-1}$ total protein. For GMP assay, the crude protein was mixed with reaction buffer (1 mM MgCl$_2$, 0.4 mM glucose, 0.1 mM ADP, 0.1 mM GDP-mannose in 50 mM Tris–HCl buffer, pH 7.0). The reaction was started by serially adding 12 U of hexokinase, 3 U of glucose-6-phosphate dehydrogenase and 1 mM sodium pyrophosphate. GMP activity was monitored by measuring the A$_{340}$ by the formation of NADH. One unit of GMP was defined as that which reduced 1 µM NADP$^+$ min$^{-1}$ mg$^{-1}$ total protein. For GalDH assay, the crude protein was mixed with reaction buffer (0.1 mM NAD$^+$ and 0.15 mM L-galactose in 50 mM sodium phosphate buffer, pH 7.2). GalDH activity was determined from the increase in A$_{340}$ by the formation of NADH. One unit of GalDH was defined as the activity that reduced 1 mM NAD$^+$ min$^{-1}$ mg$^{-1}$ total protein. For the GalLDH assay, the crude protein was mixed with reaction buffer (0.2% cytochrome c and 4.2 mM L-galactono-1,4-lactone in 0.01 M potassium phosphate buffer, pH 7.8). GalLDH activity was determined from the increase in A$_{290}$ by the reduction of cytochrome c. One unit of GalLDH was defined as the activity that oxidized 1 µmol of L-galactono-1,4-lactone min$^{-1}$ mg$^{-1}$ total protein. For APX activity, the crude protein was mixed with reaction buffer (25 mM potassium phosphate buffer, pH 7.0, 0.25 mM AsA, 0.4 mM EDTA-4H and 0.1 mM H$_2$O$_2$). APX activity was determined from the decrease in A$_{290}$ by the oxidation of AsA. One unit of APX was defined as the activity that consumed 1 µmol AsA min$^{-1}$ mg$^{-1}$ total protein. For AO activity, the crude protein was mixed with reaction buffer (50 mM potassium phosphate buffer, pH 5.3, and 1 mM AsA). AO activity was determined from the decrease in A$_{265}$ by the oxidation of AsA. One unit of AO was defined as the activity that consumed 1 µmol AsA min$^{-1}$ mg$^{-1}$ total protein.

The gel activity of PME was measured following the method described by Bosch et al. (2005). Acidic continuous native PAGE was performed at 4°C and the gel was incubated in substrate buffer (0.5% esterified pectin in 0.1 M citrate and 0.2 M Na$_2$HPO$_4$, pH 6.3) for 90 min at 37°C. The gel was briefly rinsed with water and stained with 0.02% (w/v) ruthenium red.

**Gene cloning by RT–PCR and RACE**

Total RNA for one-step reverse transcription–PCR (RT–PCR) analysis was extracted from Oncidium pseudobulbs and wild-type or transgenic Arabidopsis. A 1 µg aliquot of total
RNA was used as the template in RT–PCR with the following forward and reverse primers: for OgPME (ACJ38539),
PME-F-5′-AGGCGGCTGCATTCTGACTG-3′/PME-R-5′-ACGGCGGTGGCGGCAGAGGA-3′; for OgGalUAR (ABV24998),
GalUAR-F-5′-TCACTGGGTAAACACATATAGC-3′/GalUAR-R-5′-ACGGCGTCGCTATGCGACT-3′; for OgGalDH (ACJ38540),
GalUDH-F-5′-TTCCAGCGCTGCCGCTCCA-3′/GalUDH-R-5′-CATCAGTCCAAAATAAGGAGCA-3′; for OgGMP (FJ618566),
GMP-F-5′-GAAAACAAGATAAAATATAGC-3′/GMP-R-5′-ACGGCGGTGGCGGCAGAGGA-3′; for OgPG (FJ618567),
PG-F-5′-ACGCGGCGTGCGCAGAGGA-3′/PG-R-5′-ACCGCGTCGCTATGCGACT-3′; and for OgLEAFY (FJ618567),
LEAFY-F-5′-ACGGCGCTGCATATGCAGACT-3′/LEAFY-R-5′-ACGGCGTCGCTATGCGACT-3′. A one-step RT–PCR kit
(TAKARA BIO INC., Shiga, Japan) was used for processing all samples. The template was reverse transcribed at 50°C
for 30 min and denatured at 94°C for 2 min, followed by 12–15 cycles of amplification (94°C for 30 s for denaturation, 47–66°C for 30 s for annealing, depending on the genes, 72°C for 30 s for elongation) and by extension at 72°C for 10 min. The RT–PCR to amplify 18S rRNA using speciﬁc primers was performed as described above for the internal control. The ampliﬁed DNA fragments of each candidate gene were cloned in pGEM-Teasy vector (Promega, Madison, WI, USA) and the sequence was identiﬁed. The whole gene was completed by the rapid ampliﬁcation of cDNA ends (RACE) method (SMART™ RACE cDNA Ampliﬁcation Kit, Clontech, Mountain View, CA, USA). The conﬁrmed gene sequence was deposited in Genbank with the accession number assigned.

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References


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