國立台灣大學植物科學研究所

Institute of Plant Biology, National Taiwan University

博士論文

Doctoral Dissertation

指導教授:葉開溫博士 **(Dr. Kai-Wun Yeh)**

維他命C調控文心蘭南茜品系開花誘導之研究

The ascorbate level mediates the floral initiation of *Oncidium* Gower ramsey

研究生: 沈金輝 **(Chin-Hui Shen)**

中華民國九十九年一月

January, 2010

國立臺灣大學博士學位論文
口試委員會審定書

維他命C調控文心蘭南茜品系開花誘導之研究

The ascorbate level mediates the

floral initiation of *Oncidium* Gower ramsey

本論文係沈金輝君（F92B42021）在國立臺灣大學植物科學研究所完成之博士學位論文，於民國九十九年一月二十九日承下列考試委員審查通過及口試及格，特此證明

口試委員：

高雄大學生物科技研究所 教授 陳文輝 博士	陳文輝
中興大學生物科技學研究所 教授 楊長賢 博士	楊長賢
台灣大學農藝學系暨研究所 教授 高景輝 博士	高景輝
台灣大學生物產業機電工程學系 教授 方煒 博士	方煒
台灣大學園藝系助理教授 張耀乾 博士	張耀乾
台灣大學植物科學研究所 教授 林讚標 博士	林讚標
台灣大學植物科學研究所 教授 葉開溫 博士	葉開溫

（指導教授）

所長 葉開溫 （簽名）

致謝

本論文得以完成首先要感謝我的指導教授葉開溫老師，他賦予我精彩的研究主題，提供學生充足的資源並且不懈地鞭策學生，為學生矢志學習之典範。更感謝口試委員陳文輝教授、楊長賢教授、高景輝教授、方煒教授、張耀乾教授、林讚標教授對於學生論文細心的指正，極具遠瞻的見解與犀利的批判都成為學生受益無窮的歷練。

這七年於植科所求學的時光受惠眾多師長與同儕的扶持，感謝九樓的老師們在研究與做人處事上的孜孜教誨，在每次的討論之中都為學生灌注一股新活力。感謝已畢業在外活躍的學長與朋友黃斌、黃英豪、炤哥和科明，亦師亦友的關心著我的一切。好老哥譚軍，在我懵懵懂懂的的第一年給予我很多支持。也感謝在天國的 Shripathi 教給我的生化知識，以及歷年來的乖學弟妹們包容我緩傲癡忘的個性。我歷年來的好室友們也謝謝你們一起共分擔與分享的酸甜苦。

家人不計地陪伴與傾聽讓我無後顧之憂的勇往直前，你們的付出是最多的，卻也是我虧欠最多，我的絲絲溫暖皆出於你們的呵護，我的壘壘蜜果當與你們共享。陪伴我八年多的樂音天使，這些年有你才有我，謝謝你的坦白與過往的美好。

最後我要謝謝 22 歲的自己，一開始你站在這條路的起點是既興奮又期待，不計較終點的高度是否陡峭地難以克服，不在乎盡頭的緯度是否嚴峻地難以生存，29 歲的我，現在收到人生中重要的一份禮物。

Table of Contents

LIST OF TABLES

List of Figures

LIST OF Appendixes

摘要:

維他命C是植物對抗環境逆境時所需的抗氧化劑，亦是多種植物荷爾蒙生合成酵素的輔助因子(cofactor)，近年來的研究發現植物內生性維他命C的含量多寡具有顯著調控植物花期的功能。文心蘭在營養生長階段，假球莖會累積大量的維他命C，但在進入生殖生長階段時，其含量會明顯地下降。利用0.1M維他命C每日施加於文心蘭假球莖基部的花莖芽，結果發現可有效抑制開花相關基因*LEAFY*的表現量，並明顯延緩花莖生長，因此可知維他命C在文心蘭開花機制中扮演著抑制者的角色。維他命C含量生合成路徑如Smirnoff-Wheeler路徑與半乳醣醛酸路徑(galacturonate pathway)的基因在營養生長階段都有明顯的表現量，然而當進入生殖生長階段時，僅半乳醣醛酸路徑上的基因表現量有明顯的下降，分析其酵素活性亦呈現相同結果。再進一步轉殖半乳醣醛酸路徑中的果膠甲酯酶(pectin methylesterase)於阿拉伯芥中，發現轉殖植物亦會累積維他命C並且延遲開花。由此可知半乳糖醛酸路徑同時調控植物維他命C含量與開花生理。甲醇是半乳糖醛酸路徑的副產物，處理50mM甲醇於文心蘭擬原球體（protocorm like body，PLB）中發現可提高維他命C相關基因的表現與維他命C含量。利用羥胺(hydroxylamine)與DPI分別抑制代謝甲醇產生過氧化氫的乙醇氧化酶(alcohol oxidase)與NADPH氧化酶，發現原本受甲醇誘導的維他命C相關基因表現量也會受到抑制，因此認為過氧化氫為植物誘導維他命C相關基因表現的重要次級傳遞訊息者（secondary messenger)。綜合這些結果可知文心蘭營養生長階段因具有較高活性的果膠甲酯酶可產生甲醇並進一步產生過氧化氫而誘導維他命C相關基因表現，此訊息傳遞之生理意義在於緩和該階段因為生長而累積的氧化逆境。而當植物細胞壁的果膠含量因為細胞延長而逐漸減少時，果膠甲酯酶活性降低，進而降低維他命C含量而促進開花基因的表現量，而使植物進入生殖生長階段。本論文主要在解析維他命C在文心蘭開花生理中扮演負調控的角色，而果膠甲酯酶則為調控文心蘭不同生長階段維他命C含量重要的基因。

Abstract:

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 hydrogen peroxide (H_2O_2) level was associated with a high content of the reduced form of AsA. At the bolting period, the AsA content and H_2O_2 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 the reduced AsA content, resulted from the consumption of H_2O_2, is a prerequisite for mediating the phase transition in *Oncidium*. A survey of the AsA biosynthetic pathway revealed that the gene expressions and enzymatic activities of the relevant genes in the galacturonate (GalUA) pathway were markedly decreased at the bolting period, as compared with at the vegetative stage. However, the genes involved in the Smirnoff-Wheeler pathway retained a similar expression level in the two growth stages.

Moreover, MeOH produced form the demethylation of pectin by pectin methylesterase (*OgPME*) also could trigger the synthesis of H_2O_2 and was effective in enhancing the expression of AsA-biosynthetic genes. It 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. We proposed a model in controlling the phase transition by ascorbate homeostasis which is associated with the functional role of PME in *Oncidium*.

1. Chapter I.

Decreased L-Ascorbate Content Mediating Bolting is Mainly Regulated by the Galacturonate Pathway in *Oncidium*

1.1 Introduction:

In plants, ascorbate (AsA) is highly abundant and accumulates in intracellular concentrations of 2-25 mM (Davey et al., 2000). Ascorbate 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 (Barth et al., 2004; Noctor, 2006; Pignocchi et al., 2006; Debolt et al., 2007). Recent evidence suggests that it may also play a role in floral induction (Barth et al., 2006).

1.1.1 Ascorbate level influences the flowering time.

The mutant *vtc1* 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). *VTC1* encodes a GDP-D-mannose pyrophosphorylase (GMP; EC: 2.7.7-), which is an enzyme in the Smirnoff-Wheeler pathway for AsA biosynthesis in plants. 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) (Appendix 1). 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 definite relationship with flowering time. Surprisingly, H_2O_2 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; EC: 1.11.1.11) 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).

1.1.2 The biosynthesis of ascorbate in *planta*

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 *myo*-inositol pathway (Lorence et al., 2004) (Appendix 2). L-GalL is a

precursor of AsA biosynthesis. It is converted to AsA by GalL dehydrogenase (GalLDH; EC:1.3.2.3), 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 H_2O_2 (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; EC: 3.1.1.11) and polygalacturonase (PG; EC 3.2.1.15) were considered to demethylate and digest pectin into 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.

1.1.3The current work on the regulatory mechanism of flowering time in *Oncidium*

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) (Appendix 3). 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 phase 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. It is considered to be florally determined or reproductive stage determined when the shoot meristem grows to the new developmental program (bolting period in *Oncidium*). 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*.

In this work, we discovered the increasing AsA content and H_2O_2 level in pseudobulbs at the vegetative stage, and the levels rapidly decreased during bolting. We are interested in the physiological effect of AsA level on the bolting mechanism and on the growth of the floral stalk in *Oncidium*. The investigation into the relevant AsA biosynthetic genes in *Oncidium* showed that the gene expression of the GalUA pathway, an alternative pathway for AsA synthesis, was associated with the H_2O_2 level. Several lines of evidence indicated that the GalUA pathway was predominant in regulating AsA content to control the phase switch in *Oncidium*.

1.1.4The delineation of the development stages of *Oncidium*

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.

1.2 Results:

1.2.1 The marked decrease in AsA content and H_2O_2 level in the pseudobulb during the bolting period

The endogenous AsA and H_2O_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^{-1} FW, respectively (Fig. 1 B), 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^{-1} FW. 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_2O_2 was 175 μM g^{-1} FW at V, 145.3 μM g^{-1} FW at B and 96.29 μM g^{-1} FW at R (Fig. 1 B). The decrease in AsA content in parallel with that of the H_2O_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. 4B-8, OgAPX) activity that utilizes AsA to remove H_2O_2 molecules in the pseudobulb. The histograms of H_2O_2 staining by 3, 3'-diaminobenzidene (DAB) and AsA staining by silver nitrate ($AgNO_3$) confirm the biochemical measurements, showing that biochemical measurements and AsA greatly accumulated at V and strikingly decreased around bolting (Fig. 1 B). 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. 2 A). 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**.** 2 B) and bolting was later than that of the control plants (Fig**.** 2 C, D). In contrast, H_2O_2 treatment not only enhanced *OgLEAFY* expression (Fig**.** 2 B) but also sped up early bolting (Fig**.** 2 C) and fast-growing inflorescence (Fig**.** 2 D). When H_2O_2 was applied after AsA treatment, the expression of *OgLEAFY* was active as in normal growth (Fig**.** 2 B, A/H), but when AsA was applied after H_2O_2, the expression of *OgLEAFY* was repressed (Fig**.** 2 B, H/A). These data, obtained by artificially altering the level of AsA, supports that the expression of *OgLEAFY* occurs with a low content of AsA but the repression occurs with high a content of AsA. Moreover, the H_2O_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.

1.2.2 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. 3, 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, 3). Concomitantly, the expression of *OgGalLDH*, the final step integrating the two AsA biosynthetic routes to form AsA, was reduced at B (Fig. 3). 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_2O_2 level was high in the pseudobulb at V and was decreased at B (Fig. 1A).To unravel the effect of H_2O_2 related to the regulation of the GalUA pathway, *Oncidium* plants at B were sprayed with H_2O_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_2O_2 treatment. The expression of genes involved in both the GalUA and Smirnoff-Wheeler pathways was enhanced on H_2O_2 stimulation (Fig. 3).These data first show that the GalUA pathway is enhanced by H_2O_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. 4**)**, whereas the activities of OgPME and OgGalUAR in the GalUA pathway were greatly reduced at B (Fig. 4). 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. 4). 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.*

1.2.3 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^{-1} FW at V to 18 mg g^{-1} FW at B (Fig. 5A). Histochemical staining by ruthenium red revealed abundant formation of demethylated pectin in tissues at V but little formation at B (Fig. 5B). 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. 5B). Meanwhile, a high OgPME activity was detected only at V (Fig. 5B). 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. 3, 4), 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.*

1.2.4 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. 6). One of the independent lines, named *OgPMEOX*, was selected for functional characterization from 12 transgenic plants. As shown in Fig. 7, 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 27.7DAS with 10.6 leaves as compared with wild-type plants, which flowered at 21.3 DAS with 8.2 leaves (Fig. 8A, 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. 7B). 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. 8B). 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 21DAS 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 fl oral induction and phase transition.

1.3 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* When the pseudobulb grows at the vegetative stage (V), the organ accumulates a large quantity of carbohydrates and

nutrients to supply the floral development at the bolting period (B) and reproductive stage (R). The marked reduction in AsA content associated with a sharp decrease in H_2O_2 level was a regular event at bolting period (Fig. 1 B). Meanwhile, *OgLEAFY* was subsequently activated (Fig. 2 A). 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_2O_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_2O_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_2O_2 (Fig. 3). In association with the active function of *OgAPX*, AsA is used up for the elimination of H_2O_2 at bolting period (Fig. 4). Therefore, AsA production could be a defense mechanism and have an antioxidant effect against the H_2O_2 outburst during the active metabolism state in the *Oncidium* pseudobulb. The reduction in AsA level at bolting period is attributed in part to its consumption in the scavenging reaction of OgAPX against H_2O_2 and largely comes from the decreasing yield of the GalUA pathway (Fig. 1A, 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. 1 A). 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. 2 B-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 at vegetative stage and bolting period seems to agree with previous reports. The low transcriptional levels and enzymatic activities of OgPME and OgGalUAR in the GalUA pathway found at bolting period may cause a low level of AsA production (Fig. 3, 4). Together with the fact that AsA was also consumed in the elimination of H_2O_2, these findings may explain the marked decrease in AsA content in the pseudobulb at bolting period. 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 vegetative stage but not at bolting period (Fig. 3, 4). Also, enzymatic staining and staining of the histograms demonstrated that cell wall pectin was being demethylated at V (Fig. 5B), and its content became less at bolting period compared with at vegetative stage (Fig. 5A). This indicates that there is abundant demethylated pectin produced by PME for AsA biosynthesis at vegetative stage. 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 vegetative stage 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, H_2O_2 was produced when methanol was metabolized in plants (Fall and Benson, 1996). H_2O_2 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 H_2O_2 treatment (Fig. 3). Therefore, the increasing expression level of AsA-related genes and amount of AsA were associated with the higher PME activity at vegetative stage. Accordingly, the elevated AsA amount in transgenic *Arabidopsis* overexpressing *OgPME* was caused not only 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 (Fig. 7, 8). 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. 7B) indicated that *OgPME* has a critical function in regulating not only AsA content but also AsA redox state.

2. Chapter II

The signal network of ascorbate homeostasis associated with the development and growth in *Oncidium*.

2.1 Introduction:

In chapter I, ascorbate presents a negative regulator on floral initiation in *Oncidium* and brings our interest on the physiological role of the high ascorbate level existing in vegetative stage. Pectin methylesterase (PME) is a crucial gene in galacturonate pathway for AsA biosynthesis, and methanol is subject to be produced, when pectin is demethylated by PME. Moreover, hydrogen peroxide which generated from the step of methanol oxidization could enhance the expression level of genes in Smirnoff-Wheeler and galacturonate (GalUA) pathways. In this chapter, we propose a working model of the signaling network for ascorbate homeostasis in association with the apoplastic factors, such as methanol and oligogalacturonide during the growth and development of *Oncidium* pseudobulb cells.

2.1.1The role of reactive oxygen species in plants

The production of reactive oxygen species (ROS) is an unavoidable consequence in plants, and it was generated not only from the unexpectedly environmental stresses but also from several physiological processes, including photosynthesis, respiration and cell enlargement (Foyer and Noctor, 2005). In recent years, it has become apparent that ROS play an important signaling role in plants controlling processes such as growth, development, response to biotic and abiotic environmental stimuli, and programmed cell death (Noctor, 2006). Although various antioxidants and defense genes induced to eliminate the oxidative intensity in chloroplasts and

mitochondria have been investigated, the apoplastic ROS metabolism and its functional roles are still unclear. Unlike the cytoplasm, the apoplast is abundant in ascorbate but deficient in NAD(P)H and glutathione and contains a very active ascorbate oxidase (Pastori et al., 2003). Thus apoplastic ascorbate is apt to be oxidized and provides a low redox buffering capacity (Foyer and Noctor, 2005). However, a low antioxidant buffering in apoplast could allow oxidative signals to accumulate and further trigger serial signaling events in the cell wall, such as production of oligogalacturonide generated during the breakdown of pectin (Ridley et al., 2001). It is likely that, methanol is markedly released from the pectin demethylation which is associated with the damage of cell wall by wounding or alternation on cell wall structure during the growth and development (Micheli, 2001; Pelloux et al., 2007). Methanol accumulates in the intercellular air space or in the liquid pool at night when the stomata close. Therefore, hydrogen peroxide would be produced in apoplast, while methanol is oxidized by alcohol oxidase to prevent cell damage (Hanson and Roje, 2001).

Methanol could increase the biomass of several C3 plant because of reducing the photorespiration (Fall and Benson, 1996). Thus the effect of methanol on plant growth is decided on its dosage and treated species. The main principle depicted here is that,

we used optimal methanol dosage to investigate its influence on the ascorbate homeostasis in *Oncidium* and revealed a signaling mechanism of hydrogen peroxide under methanol stimulation (Shen and Yeh, 2009). In addition to that, the transgenic Arabidopsis overexpressing *OgPME (PECTIN METHYLESTERASE)* displayed a delayed flowering time is associated with the higher ascorbate level than wild-type plant (Shen et al., 2009). Therefore, methanol is potential to affect the phase transition because the elevated ascorbate level through the hydrogen peroxide signaling network.

2.1.2 The proposed ascorbate homeostasis in pseudobulb cell of *Oncidium* orchid in three developmental stages.

In Fig.9, we proposed a model in controlling the phase transition by ascorbate homeostasis at different growth stages in *Oncidium*. At the vegetative stage, abundant methyl-pectin in the pseudobulb cell provides rich substrate for PME to produce methanol (Wang et al., 2008). The gene expression and enzyme activity of polygalacturonase (PG) were both active under methanol stimulation in *Oncidium* (Shen and Yeh, 2009). Therefore the hydrogen peroxide released from the cell wall was not only from the oxidation of methanol but also from the OGA-triggered network. Furthermore, the hydrogen peroxide is also released from the active photosynthesis in chloroplast and respiration

in mitochondria through the enlargement of pseudobulb cell at vegetative stage. Thus, the level of ascorbate increases in response to the reduction of oxidative intensity at the enlarged pseudobulb cell. During the vegetative stage, the elevated level of hydrogen peroxide is important and essential for inducing the expression level of ascorbate-related genes, including Smirnoff-Wheeler pathway and galacturonate pathway biosynthesis such as *ASCORBATE PEROXIDASE*, *ASCORBATE OXIDASE* and *MONODEHYDROASCORBATE REDUCTASE* in defense system (Shen et al., 2009). Subsequently, the expression level of *OgLEAFY* which controls the development of inflorescence is down-regulated by high ascorbate level. *Oncidium* containing higher concentration of endogenous ascorbate displayed a longer vegetative stage (LV) (Fig.10). Likewise the exogenous ascorbate treatment would abolish the bolting of *Oncidium*. Thus, increased ascorbate at vegetative stage not only functions on scavenging the hydrogen peroxide but also limits the phase transition (Barth et al., 2006; Kotchoni et al., 2009). At the bolting period, the ascorbate level in pseudobulb is obviously lower than that at vegetative stage. Consequently, the level of gene expression and activity of PME markedly decreased suggest that it is a pivotal gene in regulating AsA biosynthesis at the bolting period. Similarly, pectin amount is also less at the bolting period and proposed that hydrogen peroxide generated from the digestion and demethylation of pectin in cell wall is not sufficient to elicit the

expressions of ascorbate-related genes. As a result, the cell wall would be more rigid by conjugating with calcium and terminated the production of methanol from pectin (Micheli, 2001). Therefore, the repression on floral-initiated genes is abolished as a cause of decreased ascorbate level.

2.2 Discussion:

Although the involvement and regulation of hydrogen peroxide on the ascorbate homeostasis have been characterized (Apel and Hirt, 2004), there is still ambiguous on the mechanism of redox ratio of ascorbate and ROS controlling in flowering time. High levels of ABA in *vtc1*, which is ascorbate deficient mutant, presumably caused by the up-regulation of NCED involving in the ABA biosynthesis (Pignocchi et al., 2006). The late-flowering phenotype of *vtc1* in short day photoperiod was associated with the increasing ABA that is known to act antagonistically to GA (Barth et al., 2006; Finkelstein, 2006). This result is opposite to the recent results which revealed that the flowering time of *vtc* all displayed early flowering time in long and short day photoperiod (Kotchoni et al., 2009). Furthermore, the transgenic tobacco overexpressing ascorbate oxidase displayed early flowering time and figured out the role of dehydroascorbate or oxidized ascorbate, a positive regulator for flowering time. In *Oncidium* bolting period, the decrease of whole ascorbate amount and increase of

oxidized ascorbate are crucial events for the success of phase transition. Therefore, the mechanism of reduced form ascorbate on flowering could be a complicated network involved in the action of phytohormones and metabolites, and it is worthy to investigate the functional roles of redox ratio regulated by endo/exogenous factors on the flowering time.

3. Chapter III

Hydrogen peroxide mediates the expression of ascorbate-related genes in response to methanol stimulation in *Oncidium*

3.1 Introduction:

3.1.1The potential influence of methanol on the growth of *Oncidium*

In chapter II, we proposed a hypothesis of ascorbate (AsA) homeostasis in different growth stages in *Oncidium* and figure out the signaling role of hydrogen peroxide. To further investigate the relationship of AsA level and pectin (or its derivates) in vegetative stage of *Oncidium*, we monitor the expression levels of AsA-related genes under methanol stimulation. In this chapter, we determined the optimal concentration of MeOH effective in activating AsA-related genes and regulating the AsA reduction or oxidation. Our results suggest that H_2O_2, a byproduct of MeOH oxidation, is a secondary signal in regulating associated gene expression in the MeOH-induced network. The role of methanol in plant physiology was also discussed in this chapter.

3.1.2The extended function of methanol in plant

Methanol (MeOH) is a volatile organic waste product, originating from the demethylation of pectin by pectin methylesterase (PME) for tightening of the cell wall, especially throughout the early stage of leaf expansion (Fall and Benson, 1996). Some MeOH emissions have also been observed during changes in cell wall construction during the development of roots and fruits (Fall and Benson, 1996). Additionally, MeOH might be produced and emitted in large quantities by mechanical wounding or under various stresses (Fukui and Doskey, 1998; Penuelas et al., 2005; von Dahl et al., 2006; Pelloux et al., 2007) (Appendix 4). Methanol accumulates in the intercellular air space or in the liquid pool at night, when the stomata closes, and is rapidly converted to formaldehyde, formic acid and CO_2 to prevent damage by alcohol oxidase

Although the metabolism of MeOH is not completely understood in plants, its

contribution to plant physiology is highlighted by its use in C_3 plants for photosynthetic productivity (Nonomura and Benson, 1992). Methanol influences C_3 plant growth under foliar spray or irrigation (Ramírez et al., 2006), but has no effect on C_4 plants. Foliar application of MeOH causes an increase of fresh and dry weight in *Arabidopsis* and tobacco, whereas MeOH irrigation significantly delays the growth of *Arabidopsis*, tobacco and tomato (Ramírez et al., 2006). The growth promotion by foliar application was ascribed to the increased carbon fixation due to detoxification from photorespiration. Radiotracer ^{14}C and ^{13}C NMR studies revealed that MeOH is metabolized by alcohol oxidase to formaldehyde and formic acid, which are further converted to serine, methionine, purine and thymidylate (Gout et al., 2000). The CO_2 produced from the oxidization of MeOH is utilized within the Calvin-Benson cycle for glucose metabolism (Hanson and Roje, 2001).

Recently, a global gene expression profile resulting from 10% MeOH stimulation in *Arabidopsis* leaves was reported (Downie et al., 2004). Most of the genes induced by MeOH function in detoxification and stress responses. After 1 h of MeOH treatment, the genes with the highest up-regulation are associated with metabolism, cell communication/signal transduction processes, defense, and RNA processing, but none are involved in photosynthesis. At 24- and 72-h MeOH treatment, the genes with the highest up-regulation are related to anthocyanin and flavonoid metabolism. Additionally, genes encoding detoxification proteins, including cytochrome P450s (EC: 1.14.15.6), glucosyl transferase (EC: 2.4.1.-) and ascorbate peroxidase (APX), were induced by MeOH. Altogether, these data revealed that detoxification and signaling pathways are predominantly activated in plants exposed to methanol.

The modulation of gene expression by chemically inducible systems has attracted interest recently for its potential impact on both fundamental and applied plant science (Caddick et al., 1998; von Dahl et al., 2006). Although many reports

have described effects of MeOH on metabolism and biochemistry, information on the regulatory mechanisms of gene expression and MeOH-induced signal transduction is still limited.

3.2 Results

3.2.1 Exogenous application of methanol stimulates AsA biosynthesis in *Oncidium* PLB cultures

To study the effect of the MeOH dosage on AsA biosynthesis in *Oncidium*, 10-500 mM MeOH was applied exogenously to *Oncidium* PLB culture. The endogenous AsA level in tissues was measured at 6, 12, 24 and 30 h after MeOH application. As shown in Fig. 11, application of MeOH resulted in varied AsA levels in the PLB cultures. In general, the AsA level preferentially decreased during the first 6 h of incubation then showed an irreversible response to various concentrations of MeOH. Notably, the PLB culture was lethally affected by 500 mM MeOH (Fig. 12), and the AsA level was markedly decreased. Upon treatment with 50 mM MeOH, the AsA level of the *Oncidium* PLB culture increased following 24 h of inoculation. Thus, a 50 mM MeOH concentration was concluded to be appropriate for signaling AsA biosynthesis in *Oncidium*.

3.2.2Characterization of AsA induction by MeOH stimulation

To unravel the mechanism of AsA induction after 50 mM MeOH stimulation, several AsA-inducing compounds (Davey et al., 1999), such as D-galacturonate (D-GalUA) and L-galactose (L-Gal), were applied to the PLB culture and their effects were compared (Fig. 13, 14). The AsA levels in the PLB culture increased by MeOH (50 mM), D-GalUA (50 mM), and L-Gal (50 mM) treatment (Fig. 13); however, treatment of MeOH alone significantly decreased the AsA level during the first 6 h of

inoculation. Interestingly, assays of the AsA redox state (reduced form AsA / oxidized form AsA) in the PLB culture showed a similar pattern to that of AsA level (Fig. 14). The distinct variation of the AsA profile with MeOH application suggests that MeOH is deleterious to *Oncidium* cells. The level of H_2O_2 significantly increased from 27.8 to 39.1 μM with MeOH application during the first 6 h of treatment (Table 1), whereas no significant effects were observed by the other chemicals, such as L-Gal and D-GalUA. Detoxification of MeOH during the first 6 h of treatment is important for the up-regulation of AsA-related genes. Moreover, MeOH has a distinct effect of H_2O_2 generation when applying to *Oncidium* culture.

3.2.3 Methanol enhances AsA levels by up-regulating AsA-biosynthesis and defense genes

Since the application of 50 mM MeOH to *Oncidium* PLB culture was effective in elevating the AsA level (Fig. 13), we investigated the effect of 50 mM MeOH on the expression level of AsA-biosynthetic genes in the GalUA pathway, such as polygalacturonase (*OgPG*), pectin methylesterase (*OgPME*) and galacturonate reductase (*OgGalUAR*; EC 1.1.1.19), as well as those in the Smirnoff-Wheeler pathway, such as GDP-D-mannose pyrophosphorylase (*OgGMP*) and galactose dehydrogenase (*OgGalDH*; EC:1.1.1.122). The RT-PCR data showed that *OgPG* and *OgPME*, which are involved in pectin degradation, were both up-regulated after 6 h of MeOH treatment. However, the expression of *OgPME* was decreased at 24 h. In contrast, no further changes in the expression level of *OgGalUAR* by MeOH treatment were observed (Fig. 15). On the other hand, both *OgGMP* and *OgGalDH* of the Smirnoff-Wheeler pathway were up-regulated during the first 6 h of MeOH treatment, effects lasting for 30 h (Fig. 15). This is similar to the effect by L-Gal stimulation, which acts as a carbon source, similar to D-GalUA in AsA-biosynthetic

routes (Fig. 15; Davey et al., 1999). Finally, the expression of galactono-1, 4-lactone dehydrogenase (*OgGalLDH*), an integrator of the AsA biosynthetic pathway, displayed an enhanced level upon MeOH treatment (Fig. 15). In addition, the levels of defense genes, including ascorbate peroxidase (*OgAPX*) and monodehydroascorbate reductase (*OgMDHAR*; EC: 1.6.5.4), were also increased at 6-24 h after MeOH treatment (Fig. 15). Taken together, a 50 mM MeOH treatment was effective to enhance the expression level of most AsA-related genes in the GalUA pathway, Smirnoff-Wheeler pathway and defense system.

To further understand the proteins associated with the AsA-related genes under 50 mM MeOH stimulation, their enzymatic activities were assayed. As shown in Fig. 16, the activities of OgPG, OgMDHAR, OgAPX and OgSOD were specifically enhanced from 6 to 12 h upon MeOH treatment, whereas other enzymes, such as OgGalUAR, OgGMP, OgGalDH, OgGalLDH, were not significantly enhanced by MeOH stimulation, even though they were enhanced in RNA levels. These results indicated that mRNA levels of many of these genes are not correlated with enzymatic activities, which may be related to post-translational modifications.

The pectin content of the *Oncidium* PLB culture was decreased in 50 mM MeOH treatment, but not in L-Gal or D-GalUA treatment (Table 1). The degradation appeared to result mainly from the elevated activity of OgPG under MeOH stimulation (Fig. 16). In conclusion, the AsA level was elevated in *Oncidium* PLB culture by MeOH stimulation, primarily because of the enhanced expression level and enzymatic activity of OgPG. Although the mRNA levels of a number of AsA-biosynthetic genes were certainly induced and enhanced, their functional contribution in AsA biosynthesis is unclear due to the absence of increased enzymatic activity with MeOH treatment.

3.2.4 Hydrogen peroxide production in *Oncidium* PLB cultures through the

activation of alcohol oxidase and NADPH oxidase under MeOH stimulation

Plant cells are able to convert MeOH to formaldehyde and H_2O_2 by alcohol oxidase (Gout et al., 2000). The H_2O_2 level was elevated from 27.8 to 39.1 μM in *Oncidium* PLB cultures in response to exogenous application of MeOH, but not D-GalUA and L-Gal, during the first 6 h of treatment (Table 1). To unravel the source of H_2O_2 production, we applied hydroxylamine (1 mM) and DPI (5 mM), inhibitors of alcohol oxidase and NADPH oxidase respectively, with MeOH in PLB cultures, and monitored the H_2O_2 levels. In *Oncidium* PLB cultures incubated with 50 mM MeOH, an early H_2O_2 burst (~45 μM) was detected during the first 30 min, followed by a subsequent decrease in accumulation (~40 μM) that lasted for another 6 h (Fig. 17). However, this H_2O_2 burst was attenuated by incubation with 50 mM MeOH combined with 1 mM hydroxylamine or 5 mM DPI. The DPI inhibitor was more effective in blocking H_2O_2 generation than the alcohol oxidase inhibitor. NADPH oxidase could play a more significant role in the systemic production of H_2O_2 than alcohol oxidase does. Therefore, the stimulation of the H_2O_2 level by MeOH in the *Oncidium* culture occurs directly, through MeOH metabolism (or detoxification) by alcohol oxidase activity, and indirectly, through the subsequent induction of NADPH oxidase activity to amplify H_2O_2 production. Moreover, the early oxidative peak of the H_2O_2 level in the *Oncidium* culture could be largely due to the conversion of MeOH by alcohol oxidase, and the later H_2O_2 burst could result primarily from NADPH oxidase activation (Fig. 17).

3.2.5 The up-regulation of AsA-related genes stimulated by MeOH is through

H_2O_2 signal transduction

To confirm the potential signaling effects of H_2O_2 on the expression of AsA-related genes, we investigated the expression of AsA-related genes under the application of the inhibitors alone or with MeOH. As shown in Fig. 18, the expression of AsA-related genes did not change after 6 h with 1 mM hydroxylamine or 5 mM DPI treatment. However, the expressional levels of AsA-related genes were lower with MeOH combined with hydroxylamine or DPI than with MeOH alone. Hydroxylamine and DPI inhibited H_2O_2 production (Fig. 17), consequently reducing the MeOH effect on the expression of AsA-related genes in *Oncidium* PLB cultures (Fig. 18). The results suggest that H_2O_2 signaling is critical in up-regulating the expression of AsA-related genes.

3.3 Discussion

Methanol (MeOH) is known as a deleterious waste product derived from pectin demethylation during the cell wall reconstruction process in plants. Its effects on plant growth in *Vigna radiata* were reported 20 years ago (Bhattacharya et al., 1985). Although the effects of MeOH on plant physiology and gene expression have been investigated (Gout et al., 2000; Galbally and Kirstine, 2002; Downie et al., 2004), its mechanism of signal transduction mechanism has not been elucidated. As previously reported in *Arabidopsis*, AsA-biosynthetic genes in the Smirnoff-Wheeler pathway and AsA-recycling and pectin degradation genes were all stimulated by an appropriate concentration of MeOH (Downie et al., 2004; Ramírez et al., 2006), but genes related to photosynthesis were not responsive to MeOH application (Downie et al., 2004).

In the present study, exogenous application of MeOH (50 mM) to *Oncidium* PLB cultures increased the AsA level by 30% (Fig. 11-14), and AsA-related genes were markedly up-regulated (Fig. 15). Moreover, the H_2O_2 level was elevated after 30 min

treatment and maintained for at least 6 h (Fig. 17). By adding hydroxylamine or DPI compounds with MeOH into the *Oncidium* PLB cultures, the activities of alcohol oxidase and NADPH oxidase were inhibited. Accordingly, H_2O_2 production was markedly decreased in PLB cultures by 8% to 20% (Fig. 17). In addition, NADPH oxidase was more effective than alcohol oxidase in producing H_2O_2, because the inhibition of NADPH oxidase activity by DPI had a greater effect on the H_2O_2 level (-20%) than inhibition of alcohol oxidase activity (-8%) (Fig. 17). Thus, the H_2O_2 level was enhanced by MeOH stimulation through two steps: MeOH oxidation by alcohol oxidase and systemic amplification by NADPH oxidase. In addition, the diminished H_2O_2 level with inhibition of alcohol oxidase and NADPH oxidase caused a reduced expression of AsA-related genes (Fig. 17). In conclusion, the results strongly suggest that H_2O_2 acts as a signaling messenger to regulate AsA-related gene expression under MeOH stimulation.

Hydrogen peroxide is a reactive oxygen species (ROS) produced by plants under stress conditions (Mittler et al., 2004). Induced H_2O_2 can act as a local signal for hypersensitive cell death and as a diffusible signal for the induction of defense genes in adjacent cells (Alvarez et al., 1998). The functional roles are complicated and diversified. Therefore, the induction of the plant defense system is tightly controlled for its production and scavenging. In several model systems of plants, the oxidative burst and accumulation of H_2O_2 appear to be mediated by the activation of a membrane-bound NADPH oxidase complex (Zhang et al., 2007; Königshofer et al., 2008; Wen et al., 2008). In the *Oncidium* system, H_2O_2 induction has been identified as a signal to induce AsA-related genes at vegetative stage (Shen et al., 2009). The enzymatic activities (but not the mRNA levels) of some AsA-biosynthetic genes, such as OgGMP, OgGalDH, OgGalLDH and OgGalUAR, were not enhanced by MeOH stimulation, indicating regulation based on post-translational modifications. However,

the AsA level is eventually increased in response to MeOH. These enzymatic activities are not critical for AsA synthesis in AsA-biosynthetic pathway experiencing the effects of MeOH. On the other hand, the increased activity of OgPG is necessary for AsA production as well as the OGA product, a ligand to induce H_2O_2 generation after MeOH treatment. In addition, AsA-recycling enzymes, OgAPX and OgMDHAR, are essential for scavenging ROS. OgSOD can function in dismutating superoxide (which was produced by NADPH oxidase) into H_2O_2 (Mittler et al., 2004). Their increased activity is indeed beneficial to plant cells undergoing MeOH stimulation.

Both methyl-galacturonate (MeGalUA) and D-GalUA are key intermediates in the GalUA pathway (Fig. 15), one of the AsA-biosynthetic routes *in planta* (Smirnoff, 2003). D-GalUA is converted from MeGalUA by pectin methylesterase in plant cells, with MeOH being produced as a byproduct. MeGalUA, an upstream intermediate in the GalUA pathway, was more effective in enhancing the AsA level in *Arabidopsis* cultures than D-GalUA, a downstream intermediate in the GalUA pathway (Davey et al., 1999). In our study, the application of D-GalUA in *Oncidium* PLB cultures led to increased AsA levels (Fig. 13,14), but had no effect on the expression of AsA-related genes, such as *OgGalUAR* and *OgGMP* (Fig. 15). A possible explanation for the contrasting MeGalUA and D-GalUA effects is that degradation of MeGalUA to D-GalUA can produce MeOH and induce AsA-related gene expression, whereas D-GalUA acts only as a carbon source in AsA biosynthesis. Therefore, the MeOH effect derived from MeGalUA conversion is critical for AsA biosynthesis.

We present a model for the enhanced expression of AsA-related genes in *Oncidium* PLB cultures in response to MeOH stimulation (Fig. 19). Methanol is generated from pectin degradation of the plant cell wall through the activation of PME during cell wall extension (Fall and Benson, 1996). The production of poisonous MeOH induces rapid detoxification into formaldehyde and H_2O_2 by alcohol oxidase.

The H_2O_2 molecules initially generated from MeOH oxidation may also have a feedback mechanism to enhance PG and PME expression (Bergey et al., 1999). As a consequence, the levels of pectin component are decreased in response to the MeOH effect (Table 1). In the subsequent step of pectin degradation by PG, an oligogalacturonic acid (OGA) byproduct can also act as a ligand to enhance NADPH oxidase activity (Ridley et al., 2001). Moreover, OGA fragments produced from cell walls could increase the expression of many defense genes through H_2O_2 signal transduction (Ridley et al., 2001; Aziz et al., 2004). Eventually, more H_2O_2 was produced to induce the network of the AsA-biosynthetic genes, by Smirnoff-Wheeler and GalUA pathways, and defense genes, such as APX and MDHAR (Fig. 19).

4. CONCLUSION:

In the present work, we find that the phase transition of *Oncidium* was associated with the ascorbate level, which is mediated by pectin metabolism. The high ascorbate level represses the gene expression level of *OgLEAFY* and plays a negative regulator on floral initiation and development of floral stalk. Moreover, pectin is an optimal source for ascorbate level because not only GalUA pathway is a crucial route of ascorbate biosynthesis at vegetative stage of *Oncidium* but also methanol produced from the demethylation of pectin could be converted to hydrogen peroxide and enhanced the expression levels of ascorbate-related genes. In summary, we have analyzed the gene regulatory profile related to AsA biosynthesis at vegetative stage and bolting period in *Oncidium* orchid. Firstly, oxidative stress occurred in association with a high content of AsA at vegetative stage of *Oncidium.* Secondly, the marked decline in AsA level, resulting from both its consumption by the OgAPX reaction to scavenge H_2O_2 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. Furthermore, we demonstrate that H_2O_2 is a secondary messenger for inducing AsA-related gene expression in *Oncidium* PLB cultures in response to MeOH produced form the GalUA pathway. The induced gene cascade from MeOH stimulation to AsA-related gene expression in plants is a defensive response to MeOH toxification, involving H_2O_2 as a critical transduction signal in this complicated genetic network.

5. Future prospect:

Oncidium is a high economical ornamental crop in Taiwan, and it is important to develop a technology to regulate the flowering time to meet the market requirement. The carbohydrate mobilization in *Oncidium* pseudobulb was recognized as the key process during the phase transition, and the decrease of ascorbate content was the threshold to initiate the reproductive growth. Therefore, the goal of the future prospect is to unravel the mechanism of ascorbate level in regulating flowering time process and the signal transduction to flowering identity genes. Moreover, the mechanism of ascorbate biosynthesis affected by environmental factors in *Oncidium* is also a target subject to investigate during different growth and development stages of *Oncidium*. *Oncidium* is a pectin-rich plant and suitable for investigating the physiological role of alternative ascorbate biosynthesis. The pathway choice for AsA biosynthesis in different developmental stages seems to be a complex genetic network and is plant-dependent. Thus, the coordinate regulation of the AsA biosynthetic pathways in adapting the physiological condition is worthy of further investigation.

6. Materials and Methods:

6.1 Plant materials

(1) *Oncidium*

Oncidium Gower ramsey plants were obtained from Shih-Dong orchid nursery (旭東蘭園) in Taiwan. The orchids for analyzing the H_2O_2 and AsA level were grown in 30 cm diameter pots under growth conditions of 25-32 ° C (April to June) or 25°C (September to November). The orchids for analyzing the effect of H_2O_2 and AsA on flowering time were grown under growth temperature conditions about 25°C or lower. It is because that the warm growth temperature could promote the growth of floral stalk and cause the incredible results.

(2) *Arabidopsis*

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).

6.2 Treatment of Oncidium orchids with AsA and H_2O_2

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 H_2O_2, 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 0.1 M AsA, and sampling for RNA extraction at 4 h after treatment. The fresh ascorbate was prone to spray without light and solved in 0.1M potassium phosphate buffer (pH 6.0); H, spraying with 30 mL of 1% H_2O_2, and sampling for RNA extraction after treatment. The fresh H_2O_2 was prepared and spray without light and solved in ddH_2O; 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% H_2O_2, then sampling for RNA extraction after 4 h; H/A, spraying with 30 mL of 1% H_2O_2 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 H_2O_2, 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% H_2O_2 and H_2O (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.

6.3 Ascorbate measurement

Preparation:

Equipment: spectrophotometer, centrifuge (4°C), oven (37°C), mortar and pestle.

Material: liquid nitrogen, 1.5mL and 2mL microcentrifuge tubes, 96well microplate.

Reagents:

1. Fresh 1M ascorbate (AsA) and dithiothreitol (DTT)

Procedures:

1. Homogenize tissue (0.5g) by pre-chilled mortar and pestle with liquid nitrogen.
2. Mix 1mL 6% trichloroacetate (TCA) well and transfer the mixture to a 2mL microcentrifuge tubes.
3. Vortex well for 10 second and keep the mixtures on the ice.
4. Delaminate by centrifugating with 13000rpm for 5min at 4°C and transfer the supernatants to the new 1.5mL microcentrifuge tubes.
5. Transfer 200 μL supernatant to new 2 mL microcentrifuge tubes labeled total AsA.
6. Transfer 200 μL supernatant to new 2 mL microcentrifuge tubes labeled reduced AsA.
7. Prepare 200 μL 6% TCA as blank and 200 μL AsA standards (0.15-1 mM) in the 2mL microcentrifuge tubes.
8. Add 100 μL 75 mM sodium phosphate buffer (pH7.0) to all above microcentrifuge tubes.
9. Add 100 μL 10 mM DTT to the total AsA microcentrifuge tubes and incubate at room temperature for 10 min. DTT would reduce the oxidized AsA.
10. Add 100 μL 0.5% *N*-ethylmaleimide (NEM) to the total AsA tubes for removing the excess DTT and incubate for 30 second.
11. Add 200 μL ddH_2O to the reduced AsA microcentrifuge tubes.
12. Add 500 μL 10%TCA, 400 μL 43% phosphoric acid (H_3PO_4), 400 μL 4% α-α'-bipyridyl and 200 μL 3% ferric chloride ($FeCl_3$) to all microcentrifuge tubes.
13. Incubate the microcentrifuge tubes at 37°C for 1hr.
14. Load 200 μL samples, blank and standards in a 96-well microplate and measure the absorbance at 525nm.
15. Calculate a linear regression curve from the A_{525} of the AsA standards.
16. Concentration of total AsA and reduced AsA of samples can be estimated through the linear regression equation.
17. Oxidized AsA can be estimated by subtracting the reduced portion from the

total ascorbate pool

Principles:

This protocol describes a microplate-adapted colorimetric ascorbate assay, in which ferric ion (Fe^{3+}) is reduced by ascorbate to the ferrous ion (Fe^{2+}). The ferrous ion reacts with α-α'-bipyridyl to form a complex with characteristic absorbance at 525 nm. With the DTT reduction of any dehydroascorbate (DHA) in a sample, total ascorbate can be assayed using the α-α'-bipyridyl method, and DHA can be estimated by subtracting the reduced portion from the total ascorbate pool.

Notes:

1. Reagents would work at room temperature for avoid the precipitation of α-α'-bipyridyl and storage them at 4°C.
2. All sample and standards were prone to keep on ice and avoid the light.
3. 10% TCA, 400 μL 43% phosphoric acid (H_3PO_4), 400 μL 4% α-α'-bipyridyl and 200 μL 3% ferric chloride ($FeCl_3$) could be mixed in order before use.
4. α-α'-bipyridyl is prepared in or higher 4% phosphoric acid.
5. Mix the 3% ferric acid after solving 4% α-α'-bipyridyl completely to avoid precipitation of 4% α-α'-bipyridyl.

Reference:

Gillespie, K.M. and Ainsworth, E.A. (2007). Measurement of reduced, oxidized and total ascorbate content in plants. Nat. Protoc. **2:**871-874.

6.4 Ascorbate staining

Preparation:

Equipments: microscope, vacuum dryer.

Materials: knifes (razor), tweezers, glass slides, 50mL microcentrifuge tubes.

Reagents:

1. Fix buffer: 5 g silver nitrate ($AgNO_3$) in 66 mL 95% ethanol, 5 mL glacial acetic acid and add ddH_2O water to 1 L.
2. Wash buffer: 95 mL 70% ethanol plus 5 mL ammonium hydroxide (NH_4OH) ACS reagent.

Procedures:

1. Samples were sliced to form 2 mm section.

2. Wash with ddH_2O two times.
3. Fixe and stain with 5% $AgNO_3$ Buffer.
4. Increase the efficiency of the fixation by evacuation for 2 min twice.
5. Keep sections in the dark at 4°C for up to 24 hr.
6. Wash the sections and stop the reaction by gently shocking in wash buffer for 5 min twice in dark.
7. Keep the sample in the 70% ethanol.
8. Photograph the sections.

Principles:

The AsA location using methanolic $AgNO_3$ exploits the ability of AsA to reduce Ag^+ at low temperature resulting in the formation of metallic silver deposits.

Notes:

1. Silver nitrate is toxic compound.
2. Monitor the evacuation of section and avoid the generation of bubbles rapidly.

Reference:

Tedone L, Hancock RD, Alberino S, Haupt S, and Viola R. (2004). Long-distance transport of L-ascorbic acid in potato. BMC Plant Biol. **4:** 16.

6.5 Hydrogen peroxide measurement

Preparation:

Equipments: spectrophotometer, centrifuge (4°C), mortar and pestle

Materials: 2 mL microcentrifuge tubes 96well microplate, liquid nitrogen

Reagents:

1. Buffer 1:10mM 3-amino-1.2.4-triazole in 50mM sodium phosphate buffer (pH 6.5).
2. Buffer 2: Titanium (IV) oxysulfate-sulfuric acid solution.
3. 30% (9.8M) H_2O_2.

Procedures:

1. Prepare the hydrogen peroxide standard (9.8 mM~9.8 nM) to confirm the efficiency of the buffers before assay.
2. Homogenize tissue (0.5 g) with liquid nitrogen in pre-chilled mortar and

pestle

3. Add 1.8 mL Buffer 1 to extract and transfer the mixture to the 2 mL microcentrifuge tubes.
4. The samples were centrifugated with 13000 rpm for 5 min at 4°C and transfer the supernatants to the new 1.5 mL microcentrifuge tubes.
5. Repeat the centrifugation.
6. Preparation 200 μL Buffer 2 to the new microcentrifuge tubes
7. Add 600 μL supernatant and H_2O_2 (9.8 mM~9.8 nM) to above microcentrifuge tubes and mix well.
8. All mixtures were centrifugated with 13000 rpm for 5 min at 4°C
9. Take 500 μL supernatant (yellow or light pink) to the new microcentrifuge tubes and discard the pellet (white).
10. The supernatants were centrifugated with 13000 rpm for 5 min at 4°C to remove excess Titanium chelate.
11. Load 200 μL samples, blank and standards in 96-well microplate and measure the absorbance at 410 nm.
12. Calculate a linear regression curve from the A_{410} of the H_2O_2 standards.
13. H_2O_2 amount in each sample can be estimated through the linear regression equation.

Principles:

Ti (IV) in sulfuric acid is present as $[Ti(OH)_2]^{2+}$ and $[Ti(OH)_3]^{+}$ colorless ions. However, it would be converted into $[Ti(O)_2OH]^{+}$ yellow-orange ion. This chelate is soluble in sulfuric acid and could be separated with excess Ti ion by a simple centrifugation. The limit of detection of hydrogen peroxide is 10 nM.

Notes:

1. The color is dark red when add excess H_2O_2 as standard, and the optimal standard concentration ranges from 9.8 mM~9.8 nM and display the colour in yellow.
2. (samples or standard mixing with Buffer1) / (Buffer 2) = 3:1 (v/v).

Reference:

Jana, S. and Choudhuri, M.A. (1982). Glycolate metabolism of three submersed aquatic angiosperms during ageing. Aquat. Bot., **12:** 345-354.

6.6 Hydrogen peroxide staining

Preparation:

Equipments: microscope, vacuum dryer, water bath.
Materials: knifes (razor), tweezers, glass slides, 50 mL microcentrifuge tubes.
Reagents:

1. DAB buffer (0.5 mg mL^{-1}): 50 mg 3,3'-diaminobenzidine in 100 mL 50 mM sodium phosphate buffer (pH 7.0).
2. Wash buffer: 95 mL 70% ethanol plus 5 mL NH_4OH ACS reagent.
3. Ethanol

Procedures:

4. Samples were sliced to form 2 mm section.
5. Sections were incubated in DAB buffer.
6. Increase the efficiency of the fixation by evacuation for 2 min twice.
7. Keep the sections in the dark at room temperature for 24 h.
8. Boil the sections with ethanol (96%, v/v) until remove the chlorophyll completely
9. Keep the sections in the 100% ethanol.
10. Photograph the sections.

Principles:

The staining was based on the instant polymerization of DAB (to form a reddish-brown complex which is stable in most solvents), as soon as it comes into contact with H_2O_2 in the presence of peroxidase.

Notes:

1. Keep the DAB buffer in the dark and storage at 4°C

Reference:

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. (1997), Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J. **11:**1187-1194.

6.7 Pectin measurement

Preparation:

Equipments: water bath, centrifuge, mortar and pestle.

Materials: liquid nitrogen, miracloth, spoons, 100mL beakers, 50mL microcentrifuge tubes.

Reagents:

1. Ethanol, acetone, dimethylsulfoxide (DMSO)
2. Ethanol-oxalic acid (pH3.3)

Procedures:

1. Homogenize tissue (2 g) with liquid nitrogen in pre-chilled mortar and pestle.
2. Mix well with 10 mL boiling 80% ethanol for 30 min.
3. Filter the mixture by miracloth to remove the free sugar (mono-or oligo-saccharide).
4. Wash the pellet with cold ethanol 3 times.
5. Repeat step 2 to step 4.
6. Dry the pellet by washing with100% acetone three times and collect the alcohol-insoluble residue (AIR).
7. Resuspend AIR with 4 mL 90% DMSO at room temperature for 16 hr to remove the starch.
8. Collect the starch-free AIR by centrifugating the mixture with 13000 rpm for 5min at 4°C.
9. Resuspend the pellet with 25 mL 0.5% ammonium oxalate (pH 3.3) at 80°C for 1 hr.
10. Delaminate by centrifugating with 13000 rpm for 20 min at 4°C.
11. Transfer the supernatant to a microcentrifuge tube and dilute with five fold volume 100% ethanol-oxalic acid mixture (pH3.3).
12. Collect the fibrous by filtering the pellet with miracloth.
13. Remove the excess ethanol from the pellet by evacuation.

14. The weight of pellet is the pectin amount (g).

Principles:

DMSO could gelatinize the fibrous starch and release the resistant starch from AIR. Moreover, Acidic ammonium oxalate was superior to hydrochloric acid or sodium polyphosphates in the extraction of pectin. Ammonium oxalate-oxalic acid forms calcium oxalate in isolated pectin and the complex can only be removed by extensive washing with acidified alcohol at a low pH condition.

Notes:

1. The time of incubation of AIR in DMSO would be elongated if the AIR is too sticky for the dissolution.
2. Ethanol-oxalic acid mixture would remove the calcium oxalate and prevent the overestimation of the pectin amount.

Reference:

Wang, C.Y., Chiou, C.Y., Wang, H.L., Ramanarayan, K., Venkatagiri, S., Tan, J., and Yeh, K.W. (2008). Carbohydrate mobilization and gene regulatory profile in the pseudobulb of *Oncidium* orchid during the flowering process. Planta. **227:** 1063-1077.

6.8 Pectin staining

Preparation:

Equipments: microscope.

Materials: knifes (razor), tweezers, glass slides, 50 mL microcentrifuge tubes.

Reagents:

1. Fix buffer: 50% ethanol, 5% acetic acid and 10% formaldehyde
2. Ruthenium red: soluble in ddH_2O and storage at 4°C in the dark.
3. Acidic ferric acid solution (fresh): 10% ferric chloride in 0.1 N hydrochloric acid.
4. HA-$FeCl_3$ solution: hydroxylamine-ferric chloride solution (14% hydroxylamine and 14% hydroxylamine hydrochloride)
5. Ethanol
6. Hydrochloric acid

Procedures:

1. Samples were sliced to form 2 mm section.
2. Fixe the sections with fix buffer in the dark overnight.
3. Wash the sections by 50% ethanol twice.
4. Sections were stained with 0.02% ruthenium red and further immersed in 70%

ethanol for 2-5 min.

5. The demethylated (acidic) pectin was present as red specks around the cell wall.
6. To detect methylesterified pectin, tissue sections were incubated in HA-$FeCl_3$ solution for 10 min.
7. After draining off the solution, sections were incubated with 33% hydrochloric acid (HCl) for 5 min. (In hood)
8. Spray the acid ferric acid solution on the surface of the sections to exposure the black specks in the cell wall.
9. Wash the sections by 50% ethanol.
10. The methylated pectin was present as brownish-red specks around the cell wall.
11. Photograph the sections under standard light microscopy.

Principles:

Ruthenium red could bind to free carboxyl groups of pectin. Pectin present two pattern of demethylation : Blockwise pattern by plant pectin methylesterase ; Radom pattern by sodium hydroxide treatment or microbial pectinesterase. Moreover, the reaction of the ester groups in pectin with alkaline hydroxylamine at room temperature produces hydroxamic acids. Pectin-hydroxamic acid forms with ferric ions an insoluble complex and present black speck in cell wall. Acetohydroxamic acid, produced from secondary acetyl groups of pectin, forms a soluble red complex and would release form the cell wall by washing with ethanol.

Notes:

1. Don't shake the sections which were sprayed with acidic ferric acid solution, because pectin-hydroxamic-ferric complex is apt to depart from the cell wall.

Reference:

Unmethylesterified (acidic) pectin: **Sabba, R.P. and Lulai, E.C.** (2002). Histological analysis of the maturation of native and wound periderm in potato (Solanum tuberosum L.) tuber. Ann. Bot. **90:** 1- 10.

Methylesterified pectin: **Reeve, R.M.** (1959). Histological and histochemical changes in developing and ripening peaches. II. The cell walls and pectin. Amer. J. Bot. **46:** 241- 248.

6.9 Total protein extraction

Preparation:

Equipments: centrifuge (4°C), mortar and pestle.

Materials: 2 mL/1.5 mL microcentrifuge tubes, 96well microplate, liquid nitrogen

Extraction buffer:

1. For PME activity assay: 0.1 M citrate, 0.1 M sodium citrate, 1 M sodium phosphate dibasic (Na_2HPO_4) and 1 M sodium chloride (pH 5.0).
2. For PG activity assay: 1 M sodium chloride (NaCl), and 0.2 M sodium phosphate dibasic in 1 M citrate (pH 4.0).
3. For APX activity assay: 2% polyvinylpolypyrrolidone (PVPP), 0.4 mM Ethylene diamine tetraacetic acid (EDTA) and 1 mM AsA in 25 mM potassium phosphate buffer (pH 7.8).
4. For L-galactose dehydrogenase; L-galactone1,4-lactone dehydrogenase; GDP-D-mannose pyrophosphorylase; galacturonate reductase; galacturonate oxidase (EC: 1.1.3-); ascorbate oxidase (EC: 1.10.3.3): 2% PVPP, 2 mM EDTA, 2 mM DTT, 20% glycerol in 50 mM sodium phosphate buffer (pH 7.2).

Procedures:

1. Homogenize tissue (0.1 g) with liquid nitrogen in pre-chilled mortar and pestle
2. Add 1mL suitable extraction buffer for different enzyme assay and mix well.
3. Transfer the slurry to the new microcentrifuge tubes.
4. Delaminate by centrifugating with 8000 rpm for 15 min at 4°C.
5. Transfer the supernatant to the new microcentrifuge tubes and centrifugate again to remove the residue.
5. The supernatant is crude protein and keep it on the ice for following analyses or storage it at -80°C for one week.

6.10 Quantitative analysis of protein concentration

4.10.1 Bio-Rad Protein Assay Kit

Preparation:

Equipment: spectrophotometer.

Material: 96 well microplate.

Reagents:

1. Bio-Rad Protein Assay kit
2. BSA(1 μg μL^{-1})

Procedures:

1. Mix dye and ddH_2O by 4:1 (v/v).
2. Add 1,2,4,8,16 μL bovine serum albumin (BSA) and 1 μL~5 μL crude proteins with above mixture to 200μL and incubate at room temperature for at least 5 min.
3. Measure the absorbance at 595 nm and calculate a linear regression curve from the A_{595} of the BSA standards.
4. Protein concentration of crude protein can be estimated through the linear regression equation.

Principles:

This assay is based on the method of Bradford. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine.

Notes:

Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hr.

4.10.2 Lowry Assay

Preparation:

Equipments: spectrophotometer, centrifuge.

Materials: 1.5/2 mL microcentrifuge tubes, 96 well microplate.

Reagents:

1. Buffer A : 2 g sodium carbonate (Na_2CO_3), 0.4 g sodium hydroxide (NaOH) and add ddH_2O to100 mL.
2. Buffer B: 0.5 g copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 0.1 g sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and add H_2O to 10 mL.
3. Buffer C: 50 mL Buffer A + 1 mL Buffer B.

Procedures:

1. Mix 5 μL crude protein with 500 μL 10% trichloroacetic acid (TCA)
2. Delaminate the mixture by centrifugating with 10000 rpm for 5 min at room temperature.
3. Remove the supernatant (TCA).
4. Add 200 μL 0.1M NaOH to the above samples and BSA standards.
5. Add 1 mL Buffer C and incubate at room temperature for 10min.
6. Add 80 μL Folin-Gocalteu phenol reagent (FCR) and incubate at the dark for 30 min.
7. The protein amount could be estimated through the linear regression equation from the A_{750} of the BSA standards.

Principle:

Notes: TCA is strong acid and should be operated with care.

6.11 Pectin methylesterase activity measurement

Preparation:

Equipments: spectrophotometer, oven (37°C), centrifuge.

Materials: 2 mL/1.5 mL microcentrifuge tube, 96 well microplate

Reagents:

1. Pectin substrate buffer : 0.1% (w/v) 92% esterified pectin in 0.2 M sodium phosphate dibasic (Na_2HPO_4) buffer, pH 6.3

Procedures:

1. Mix 100 μL exude protein from each samples or ddH_2O (standard) with 1 mL of pectin substrate buffer and incubate at 37°C overnight.
2. Add 0.2 mL 0.05% ruthenium red and incubate for 10 min.
3. Add 0.5 mL 0.6M calcium chloride ($CaCl_2$) to precipitate the demethylesterified pectin which is bound with ruthenium red.
4. Remove the precipitate completely by centrifuging with 13000 rpm for 15 min twice.
5. Load 200μL supernatant to the 96 well microplate.
6. Measure the absorbance at 534 nm and calculate the relative activities between the samples and blank.

7. Relative PME activity can be estimated through the decrease of the absorbance at 534 nm.

Principles:

The demethylesterified pectin generated by pectin methylesterase would bind to ruthenium red. Therefore the colour with red in supernatant would be diluted because of the production of red pellet.

Notes:

1. Commercial pectin is better than galacturonate as substrate for this assay; it is because commercial galacturonate is frequently demethylated.
2. Warm sodium phosphate buffer is helpful to dissolve the esterified pectin.

Reference:

Dedeurwaerder, S., Menu-Bouaouiche, L., Mareck, A., Lerouge, P., Guerineau, F. (2009). Activity of an atypical Arabidopsis thaliana pectin methylesterase. Planta, **229:** 311-321.

6.12 Polygalacturonase activity measurement

Preparation:

Equipments: spectrophotometer, oven (37°C), centrifuge.

Materials: 2 mL/1.5 mL microcentrifuge tube, 96 well microplate

Reagents:

1. Borate buffer (pH 9.2): 8 mL 0.05M Borax, 2 mL 0.2M Borate and 10 mL ddH_2O.
2. 0.2% pectin

Procedures:

1. Prepare galacturonate (1 nM to1 mM) as standards.
2. Mix 100 μL crude protein and standards with 200 μL of 0.2% pectin at 35°C for 1 hr.
3. Add 1 mL of 0.1 M borate buffer to stop the reaction.
4. Prepare another 100 μL crude protein mixing with 200 μL ddH_2O for analyzing the initial pectin concentration in each sample.
5. Add 200 μL of 1% cyanoacetamide to all above tubes and incubate in the dark for 5 min.
6. Boil the above mixtures for 10 min.
3. Until the mixture cool down to room temperature, load 200 μL samples, blank and standards in a 96-well microplate and measure the absorbance at 276 nm.
   A_{S60}: the absorbance of sample mixing with 0.2% pectin
   A_{Si}: the absorbance of sample mixing without 0.2% pectin
7. The real increase of absorbance is corrected by A_{Si} subtracting from A_{S60}.
8. The increase of galacturonate amount can be estimated through the linear regression equation from the A_{276} of the galacturonate standards.
9. One unit of PG was defined as the activity that produced 1 μmol of galacturonate $min^{-1}g^{-1}$ FW.

Principles:

The reducing group of galacturonate, which was released from the pectin digested by polygalacturonase, would be bound with 2-cyanoacetamide covalently.

Reference:

Gross, K.C. (1982). A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. Hort. Science **17:**6,933-934.

6.13 Galacturonate reductase activity measurement

Preparation:

Equipments: spectrophotometer, oven (37°C), centrifuge.

Materials: 2 mL/1.5 mL microcentrifuge tube.

Reagents:

1. Substrate buffer:30 mM D-galacturonic acid,2 mM EDTA, and 2 mM DTT in 50 mM sodium phosphate (pH 7.2)
2. Nicotinamide adenine dinucleotide phosphate (NADPH) buffer: 1 mM NADPH in 50 mM sodium phosphate buffer (pH 7.2)

Procedures:

1. Assay the NADPH standard (1 nM~1 mM) to confirm the efficiency of NADPH before the assay.
2. Incubate the NADPH standard with the substrate buffer and immediately measure the absorbance at 340 nm (A_{NADPH0}).
3. Incubate for 5 min and measure the absorbance at 340 nm to calculate the natural decadence of NADPH at 37°C for 5 min (A_{NADPH5}).
4. Mix 200 μL crude extracts with 400 μL substrate buffer at 37°C for 10 min.
5. Add the 400 μL NADPH buffer to above mixture and measured immediately the absorbance at 340 nm as A_0
6. Incubate the mixture at 37°C for 5 min and measured the absorbance at 340 nm as A_5.
7. The decrease of absorbance is the cause of the conversion of NADPH to $NADP^+$ when reducing galacturonate.
8. The real decreasing absorbance by the action of galacturonate reductase is corrected by following equation: A_0-A_5+ (A_{NADPH0}- A_{NADPH5}).
9. The conversion of NADPH amount can be estimated through the linear regression equation from the A_{340} of the NADPH standards.
10. One unit of galacturonate reductase was defined as the activity that oxidized 1 μM NADPH min^{-1} mg^{-1} protein.

Principles:

An absorbance spectrum of $NADP^+$ is at A_{260}, and that of NADPH is at A_{260} and A_{340}. NADPH is necessary in the reduction of galacturonate by galacturonate

reductase. Therefore the value at A_{340} decreases because that NADPH would release hydrogen ion and became $NADP^+$.

Notes:

There are several factors in the crude protein would interact with NADPH. Thus, the volume of the samples should be less than NADPH to maintain the excess NADPH for the assay.

Reference:

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

6.14 Galacturonate oxidase activity measurement

Preparation:

Equipments: spectrophotometer, centrifuge.

Materials: 2 mL/1.5 mL microcentrifuge tube

Reagents:

Substrate buffer: 3 mM galacturonate and 0.1 M potassium phosphate buffer (pH 7.4)

Procedures:

1. Prepare the galacturonate (1 nM to1 mM) as standards.
2. Mix 400 μL crude protein and standard with 500 μL substrate buffer at 37°C and measure the absorbance at 276 nm immediately (A_0).
3. Incubate these mixtures at 37°C for 60 min.
4. Add 2-cyanoacetamide to above tubes and measure the absorbance at 276 nm immediately (A_{60}).
5. The decrease of absorbance at 276 nm is the cause of the oxidization on the galacturonate.
6. The oxidized galacturonate amount can be estimated through the linear regression equation from the A_{276} of the galacturonate standards.
7. The optimal concentration of galacturonate standard ranges from1 nM to1 mM.
8. One unit of GalUA oxidase was defined as the activity that decreased 1 ng

GalUA min^{-1} mg^{-1} protein.

Principles:

Galacturonate would be interacted with galacturonate oxidase and reductase and provide an intractable result on the absorbance. The substrate buffer without NADPH could prevent the reduction of galacturonate reductase and has no influence on the action of galacturonate oxidase.

References:

Riov, J. (1975). Metabolism of uronic acids in plant tissues: partial purification and properties of uronic acid oxidase from *Citrus* leaves. Plant Physiol. **55:** 602-606.

6.15 GDP-mannose pyrophosphorylase activity measurement

Preparation:

Equipments: spectrophotometer, centrifuge, oven (37°C).

Materials: 1.5/2 mL microcentrifuge tubes

Reagents:

1. 2X reaction buffer: 20 mM sodium fluoride (NaF), 2 mM magnesium chloride
2. ($MgCl_2$), 0.8mM glucose, 0.2 mM adenosine diphosphate phosphate (ADP), 0.2 mM GDP-mannose and 100 mM Tris-HCl (pH 7.0).
3. ADP buffer: 0.1 mM ADP in 50 mM Tris-HCl (pH 7.0)

Procedures:

1. Mix the 250 μL crude protein with the 250 μL 2X reaction buffer and measure the absorbance at 340 nm immediately (A_0).
2. Add 5 μL hexokinase and incubate for 10 min at 37°C.
3. Add 5 μL glucose-6-phosphate dehydrogenase (G6PDH) and incubate for 10 min at 37°C.
4. Add the fresh 100 μL ADP buffer and measure the absorbance at 340 nm immediately (A_{ADP}).
5. Incubate these mixtures for 5 min at 37°C and measure the absorbance at 340 nm (A_{ADP5}).
6. The increase of absorbance at 340 nm is the cause of the generation of NADPH.
7. The amount of NADH produced by this enzymatic cascade can be estimated through the linear regression equation from the A_{340} of the NADPH standards.
8. The optimal concentration of galacturonate standard ranges from1 nM to1 mM.
9. One unit of GMP was defined as that which reduced 1 μM $NADP^+$ min^{-1} mg^{-1} protein.

Principles:

GDP-mannose and PPi are the substrates to product GTP by GDP-mannose pyrophosphorylase. GTP will be converted to ATP by nucleoside-diphosphate kinase (hexokinase). Finally, the ATP will be convert to NADPH from coupling reaction by adding G6PDHase.

Notes:

1. Flowchart:

GDPM+PPi->GTP+mannose-1-phosphate

GTP+ADP->ATP+GDP

ATP+glucose->G6P+ADP

G6P+NADP+H2O->6PG+NADPH

2. GTP, ATP and NADPH are sensitive molecules and decade quickly. Therefore, it is very important to confirm their efficiency before the assay
3. Fresh 2X reaction buffer is better for assay or storage the excess 2X reaction buffer at -80°C within one week.
4. It is important to adjust the A_0 of all samples, because the excess ADP will disarrange the reaction trend.

6.16 L-Galactono-1,4-lactone dehydrogenase activity measurement

Preparation:

Equipments: spectrophotometer, centrifuge.

Materials: 1.5/2 mL microcentrifuge tube.

Reagents:

1. Substrate buffer: cytochrome c (1.5 mg mL^{-1}) and L-galactono-1,4-lactone (4.2 mM) in 0.01 M potassium phosphate buffer (pH 7.8).
2. Reduced form cytochrome c standard: cytochrome (1 nM~1 mM) in 2 mM DTT in 0.01 M potassium phosphate buffer (pH 7.8).

Procedures:

1. Mix 500 μL crude protein with 500 μL substrate buffer and measure the absorbance at 550 nm immediately (A_0).
2. Incubate these mixtures for 5 min and measure the absorbance at 550 nm immediately (A5).
3. The amount of reduced form cytochrome c can be estimated through the linear regression equation from the A_{550} of the cytochrome c standards.
4. One unit of enzyme was defined as the amount that oxidized 1 μmol of L-galactono-1,4-lactone per minute, which was equivalent to the reduction of 2 μmol of cytochrome c.

Principles:

Cytochrome c has a sharp absorption band at 550 nm in the reduced state. In this assay, cytochrome c is reduced by the active L-Galactono-1,4-lactone dehydrogenase.

Notes:

Add the cytochrome c in the substrate buffer before the assay.

Reference:

Oba, K., Ishikawa, S., Nishikawa, M., Mizuno, H., and Yamamoto, T. (1995) Purification and properties of L-galactono-1,4-lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. J. Biochem. **117:**120-124.

6.17 Galactose dehydrogenase activity measurement

Preparation:

Equipment: spectrophotometer, centrifuge, oven (37°C)

Materials: 1.5/2 mL microcentrifuge tubes.

Reagents:

1. substrate buffer: 0.1 mM NAD^+, 0.6 mM L-galactose, 1 mM EDTA, 2 mM DTT and 100 mM Tris-HCl (pH 7.0)
2. The standard is 0.1~5 μM NADH in 50 mM sodium phosphate buffer (pH 7.2)

Procedures:

1. Mix 500 μL crude protein with 500 μL substrate buffer and measure the absorbance at 340 nm immediately (A_0).
2. Incubate the mixture for 5 min at 37°C and measure the absorbance at 340 nm (A_5).
3. The increase of absorbance is the cause of the reduction of NAD^+ to NADH when dehydrogenation of L-galactose by L-galactose dehydrogenase.
4. The NADH amount generation form the dehydrogenation of L-galactose can be estimated through the linear regression equation from the A340 of the NADH standards.
5. One unit of GalDH was defined as the activity that reduced 1 nM NAD^+ min^{-1} mg^{-1} protein.

Principles:

An absorbance spectrum of NAD^+ is at A_{260}, and that of NADH is at A_{260} and A_{340}. NAD^+ is necessary in the dehydrogenation of L-galactose by L-galactose dehydrogenase. Therefore the value of the absorbance at 340 nm increases because that NAD^+ would accept the hydrogen ion and became NADH.

Notes:

1. Storage the substrate buffer at -80°C

Reference:

Mieda, T., Yabuta, Y., Rapolu, M., Motoki, T., Takeda, T., Yoshimura, K., Ishikawa, T., and Shigeoka, S. (2004). Feedback inhibition of spinach L-galactose dehydrogenase by L-ascorbate. Plant Cell Physiol. **45:** 1271-1279.

6.18 Ascorbate oxidase activity analysis

Preparation:

Equipments: spectrophotometer, centrifuge, oven (37°C)

Materials: 1.5/2 mL microcentrifuge tubes.

Reagent:

1. Ascorbate: fresh ascorbate (1 nM~1 mM) in 0.1 M sodium phosphate buffer (pH 6.5)

Procedures:

1. The crude protein in supernatant (SS) is prepared for assay of soluble ascorbate oxidase activity.
2. Resuspend the pellet in 1 M NaCl in 0.1 M sodium phosphate buffer (pH 6.5),

and vortex for 10 min at 4°C.

3. Delaminate the mixture by centrifugating with 13000 rpm for 10 min at 4°C.
4. The supernatant (Si) is prepared for analyzing the ionically bound ascorbate oxidase.
5. Mix 200 μL crude protein (SS and Si) with 1.8 mL sodium phosphate buffer (pH 6.8).
6. Add 1 mL 0.1 mM AsA to above microcentrifuge tubes and measures the absorbance at 290 nm immediately (A_0).
7. Incubate the mixture for 5 min at 37°C and measures the absorbance at 290 nm (A_5).
8. The decrease of absorbance is the cause of the oxidization of ascorbate by ascorbate oxidase, and it can be estimated through the linear regression equation from the A_{260} of the ascorbate standards.
9. The whole activity of ascorbate oxidase is average of SS and Si.
10. One unit of ascorbate oxidase was defined as the activity that consumed 1 μ mol AsA min^{-1} mg^{-1} protein.

Principles:

Ascorbate oxidase and ascorbate peroxidase were assayed from the decrease in absorbance at 290 nm as ascorbate was oxidized. We used 290 nm in place of 265 nm because the absorbance of our assay mixture was too high at the absorption maximum of ascorbate in Hitachi spectrophotometer.

Notes:

1. There are soluble and ionically bound ascorbate oxidase discovered in plants. Soluble ascorbate oxidase locates in symplast, and ionically bound ascorbate oxidase locates in apoplast.
2. The crude protein is abundant in ascorbate peroxidase but deficient in hydrogen peroxide. Thus the decreasing ascorbate is the cause of the oxidization of ascorbate by ascorbate oxidase.

Reference:

Mukherjee, S.P. and Choudhuri, M.A., (1981). Effect of water stress on some oxidative enzymes and senescence in Vigna seedlings. plant physiology **52:**37-42.

6.19 Ascorbate peroxidase activity analysis

Preparation:

Equipments: spectrophotometer, centrifuge.

Materials: 1.5/2 mL microcentrifuge tubes.

Reagents:

1. Substrate Buffer: 0.4 mL 0.75 mM EDTA plus 1 mL 1.5 mM ascorbate and 1 mL 150 mM potassium phosphate buffer (pH 7.0)

Procedures:

1. Mix 100 μL crude protein with 2.4 mL substrate Buffer and measure the absorbance at 290 nm immediately (A_0).
2. Add 250 μL 0.03% H_2O_2 to the above mixture and mix well.
3. Incubate the mixture for 2 min at 25°C and measure the absorbance at 290 nm immediately (A_2).
4. The decrease of absorbance is the cause of the oxidization of ascorbate by ascorbate peroxidase, and it can be estimated through the linear regression equation from the A_{260} of the ascorbate standards.
5. One unit of ascorbate peroxidase was defined as the activity that consumed 1μ mol AsA min^{-1} mg^{-1} protein.

Principles:

Ascorbate oxidase and ascorbate peroxidase were assayed from the decrease in absorbance at 290 nm as ascorbate was oxidized. We used 290 nm in place of 265 nm because the absorbance of our assay mixture was too high at the absorption maximum of ascorbate in Hitachi spectrophotometer.

Notes:

1. Although the ascorbate oxidase present in the crude protein would interfere the result of the decrease of ascorbate by ascorbate peroxidase, the ascorbate in symplast is oxidized mainly by ascorbate peroxidase.

Reference:

Mukherjee, S.P. and Choudhuri, M.A., (1981). Effect of water stress on some oxidative enzymes and senescence in Vigna seedlings. plant physiology **52:**37-42.

6.20 Monodehydroascorbate reductase activity assay

Preparation:

Equipments: spectrophotometer, centrifuge, oven (37°C).

Materials: 1.5/2 mL microcentrifuge tubes.

Reagents:

1. Substrate Buffer: 2 mM ascorbate in 0.1 M in Tris-HCl (pH 7.2)
2. NADH Buffer: 0.2 mM NADH in 0.1 M in Tris-HCl (pH 7.2)
3. Commercial ascorbate oxidase.
4. Fresh ascorbate (1nM~1mM)

Procedures:

1. Mix 500 μL substrate buffer and 1 μL ascorbate oxidase and measure the absorbance at 290 nm immediately (A_0).
2. Incubate the mixture for 2 min at 25°C and measure the absorbance at 290 nm immediately (A_2).
3. The decrease of absorbance is the cause of the oxidization of ascorbate by ascorbate oxidase, and it is the oxidized form ascorbate, which is the substrate for MDHAR, could be estimated through the linear regression equation from the A_{260} of the ascorbate standards.
4. Add 100 μL NADH Buffer to the above mixture and measure the absorbance at 340 nm immediately (A_{S0})
5. Add 100 μL crude protein to the mixture and incubate at 37°C for 2 min and measure the absorbance at 340 nm (A_{S2})
6. The decrease of absorbance is the cause of the dehydrogenation of NADH to NAD^+ when reducing the oxidized ascorbate by MDHAR.
7. The NADH amount dehydrogenated by MDHAR could be estimated through

the linear regression equation from the A_{340} of the NADH standards.

8. One unit of MDHAR was defined as the activity that oxidized 1nmol NADH min^{-1} mg^{-1} protein.

Principles:

An absorbance spectrum of NAD^+ is at A_{260}, and that of NADH is at A_{260} and A_{340}. NADH is the requirement for reducing monodehydroascorbate to ascorbate by MDHAR. Therefore the value of the absorbance at 340 nm decreases because that NADH would release the hydrogen ion and became NAD^+.

Notes:

The choice of absorbance after adding NADH is dependent on the measure the oxidization of NADH (decreasing of A_{340}) or reduction of oxidized ascorbate (increasing of A_{290}). Unfortunately, the value of A_{290} is cause of the action of ascorbate oxidase and MDHAR and provides the incorrect result.

Reference:

Eltayeb, A.E., Kawano, N., Badawi, G.H., Kaminaka, H., Sanekata, T., Shibahara, T., Inanaga, S., and Tanaka, K. (2007). Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta, **225:**1255-1264.

6.21 Superoxidase dismutase (SOD) activity measurement

Preparation:

Equipments: spectrophotometer, centrifuge, oven (37°C).

Materials: 1.5/2 mL microcentrifuge tubes.

Reagents:

1. Tea-Dea buffer: 100 mM triethanolamine and 100 mM diethanolamine (pH 7.4).
2. Substrate buffer: 80 μL 7.5 mM NADH + 25 μL 0.1 M EDTA (pH 7.0) + 25 μL 50 mM manganese (II) chloride ($MnCl_2$) (pH 7.0).
3. 2-mercaptoethanol.
4. Fresh ascorbate (1 nM~1 mM)

Procedures:

1. Mix 150 μL substrate buffer with 0.8 mL Tea-Dea buffer and measure the

absorbance at 340 nm immediately (A_0).

2. Add 1 mL 2-mercaptoethanol and mix well.
3. Incubate the mixture for 10 min at 37°C and measure the absorbance at 340 nm (A_{10}).
1. The decrease of absorbance at 340 nm is the cause of NADH oxidation by 2-mercaptoethanol in the presence of EDTA and Mn^{2+}.
2. Prepare another 150 µL substrate buffer mix with 0.8 mL Tea-Dea buffer and measure the absorbance at 340 nm immediately (A_{S0}).
3. Add 200 µL crude protein and mix well.
4. Add 1mL 2-mercaptoethanol and mix well.
5. Incubate the mixture for 10 min at 37°C and measure the absorbance at 340 nm (A_{S10}).
6. The A_{S0} should be equal to A_0, and A_{S10} should be more than A_{10}.
7. The inhibition of 2-mercaptoethanol-induced oxidation of NADH by SOD could be estimated through the linear regression equation from the A_{340} of the NADH standards.
8. One unit of SOD was defined as the activity that inhibition of the 50% oxidation NADH min^{-1} mg^{-1} protein.

Principle:

The principle of this method is based on the oxidation of NADH mediated by superoxide radical, which is reactant for SOD. Coenzyme oxidation occurs in the presence of suitable concentrations of EDTA. Mr^{2+} and mercaptoethanol through a free-radical chain of reactions involving thiol oxidation and univalent O_2 reduction. The addition of SOD to the reaction mixture causes a proportionate inhibition, of the rate of NADH oxidation, thus confirming the involvement of superoxide in the process and providing the basis for SOD activity determination.

Reference:

Paoletti, F., Aldinucci, D., Mocali, A., Caparrini, A. (1986). A sensitive spectrophotometric method for the determination of superoxide activity in tissue extracts, Anal. Biochem. **154:** 536-541.

6.22 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Preparation:

Equipments: (Hoefer system) Gel caster, electrode chamber (tank), power source, vortex, centrifuge.
Materials: 1.5/2 mL microcentrifuge tubes.
Reagents:

1. 40% polyacrylamide solution.
2. Lower Tris: 0.1% SDS in 1.5 M Tris (pH 8.8).
3. Upper Tris: 0.1% SDS in 0.5 M Tris (pH 6.8).
4. TEMED
5. 10% Ammonium persulfate (APS) (fresh): 0.02 g APS in 200 µL ddH2O.
6. 1X Chamber buffer: 3 g Tris, 18.8 g glycine and 5 mL 20% SDS in ddH2O up to 1 L.
7. 6X SDS-PAGE sample buffer (1 mL): 600 µL 100% glycerol, 300 µL upper Tris buffer, 50 µL 20% SDS, 50 µL 2-mercaptoethnaol and less than 1 g bromophenol blue.
8. Staining buffer: 1 g Coomassie brilliant blue G-250, 182 mL methanol, 36 mL acetate, 182 mL H_2O.

Procedures:

1. Prepare acrylamide gels
   (1) Clean the gel caster.
   (2) Prepare the separating gel (bottom) by mixing below reagents in order: 3.5 mL ddH_20→ 2 mL Lower Tris→ 2.5 mL 40% acrylamide→8 µL TEMED→ 50 µL 10% APS.
   (3) Load 7 mL above mixture to gel caster and then load 75% ethanol.
   (4) There would be a delaminating line between the ethanol (upper-layer) and solidified separating gel (bottom).
   (5) Pour out the upper ethanol.
   (6) Prepare the stacking gel (upper-layer) by below reagents in order: 3.5 mL ddH_20→ 1.5 mL Upper Tris→ 0.7 mL 40% acrylamide→8 µL TEMED→ 50 µL 10% APS.
   (7) Load 3 mL above mixture to gel caster that has separating gel.
   (8) Insert the suitable comb to the stocking gel.
   (9) Fill the space vacating from the solidification of stacking gel.
2. Electrophoresis
   (1) Setup the prepared acrylamide gel in the electrode chamber (tank).
   (2) Load 200 mL 1X chamber buffer to make sure no gap between the gel and tank.
   (3) Submerge the gel in the tank.

(4) Electrophoresis in 100 mV for 2 h or until the bromophenol blue lane arrives at the bottom.
(5) Unload the gel from the glass plate carefully.

3. Staining
(1) Incubate the gel in the optimal staining buffer for 15 min in the dark.
(2) Pour out the staining buffer.
(3) Destain by wash with H_2O several times.
(4) Photograph the gel.

Notes:

The Coomassie dyes (R-250 and G-250) bind to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waals attractions. R means reddish and G means greenish. Coomassie R-250, which has the more sensitivity than G250, can detect as little as 0.1 μg of protein. Though less sensitive, the gel stained by Coomassie G-250 displayed less background interference and reactive duration. The gel staining with G250 could be removed by water in place of destaining buffer present in acetate. Moreover, the R250 is not suitable for measuring the protein amount by colorimetry because of inaccuracy on higher concentration.

6.23 PME activity in native acidic gel electrophoresis

Preparation:

Equipments: centrifuge, oven (37°C), PAGE electrophoresis set. mortars and pistils.

Materials: 1.5/2 mL microcentrifuge tubes, liquid nitrogen,

Reagents:

1. Extraction buffer (pH4.5): 0.1 M citrate, 0.2 M sodium phosphate dibasic (Na_2HPO_4), 1 M sodium chloride.
2. Sample buffer (15 mL): 3 mL 50% glycerol, 360 μL 1 M potassium hydroxide (KOH), 129 μL 100% acetate and 11.5 mL ddH_2O.
3. Running buffer (500 mL): 483.7 mL ddH_2O, 12 mL 1M KOH, 4.3 mL 100% acetate
4. Acid buffer (500 mL;pH 6.3): 0.1 M citrate in 0.2 M sodium phosphate dibasic
5. Substrate buffer: 0.5% pectin in acid buffer.

Procedures:

1. Homogenize tissue (1 g) with liquid nitrogen in pre-chilled mortar and pestle

2. Add 1mL extraction buffer and mix well.
3. Transfer the slurry to the new microcentrifuge tubes.
4. Delaminate by centrifugating with 8000 rpm for 15 min at 4°C.
5. Transfer the supernatant to the new microcentrifuge tubes and centrifugate again to remove the residue.
6. The supernatant is crude protein and quantify the protein concentration.
7. Mixing the 10 μg crude proteins with equal volume sample buffer and 1 μL methyl green dye.
8. Prepare the acidic continuous native PAGE (20 mL; pH 4.3) by mixing the below chemicals in order:
   13.748 mL ddH_2O→5 mL 40% polyacrylamide→480 μL 1 M KOH→172 μL 100% acetate→100 μL TEMED→500 μL 10% APS
9. Setup the electrode chamber (tank) and electrophoresis in running buffer.
10. Electrophoresis with 100 V for 2 hr at 4°C and polarity reversed
11. Equilibrate the gel in acid buffer for 5 min.
12. Incubate the gel in the substrate buffer at 37°C for 90 min.
13. Rinse the gel with ddH_2O.
14. Stain with 0.02% ruthenium red (w/v) in ddH_2O for 15 seconds.
15. Destain with ddH_2O until the existence of the reddish band.
16. Photograph the gel

Notes:

1. Electrophoresis carries out with polarity reversed
2. Excess total protein loading would display saturated pattern and reduce the resolution.
3. Stain the gel with ruthenium red should be less than 1 min to prevent the background interference.

Reference:

Bosch, M., Cheung, A.Y., Hepler, P.K. (2005). Pectin methylesterase, a regulator of pollen tube growth. Plant Physiol. **138:** 1334-1346.

6.24 RNA extraction

Preparation:

Equipments: mortar and pistil, centrifuge, water bath (65°C), refrigerator (4,-80°C),

30 mL centrifuge tubes.

Materials: liquid nitrogen, 5 mL tip.

Reagents:

1. Extraction buffer (500 mL): 10 g polyvinylpyrrolidone (PVP, average mol wt 40000) + 10 g hexadecyltrimethylammonium bromide (CTAB) + 4.653 g EDTA + 58.44 g NaCl + 0.25 g spermidine.
2. 10X MOPS (200 mL): 8.372 g MOPS + 0.8203 g sodium acetate + 0.7448 g EDTA.
3. Denature buffer: 600 μL formamide + 210 μL formaldehyde + 10X MOPS buffer + 7 μL ethidium bromide (EtBr; 10 mg mL^{-1}).
4. RNase free ddH_2O: 1 mL diethypyrocarbonate (DEPC) in 1 L water and sterilize in autoclave with 2 atm for 40 min.

Procedures:

1. Grind 1 g sample in the mortar treated with liquid nitrogen.
2. Add 10 mL extraction buffer with fresh 200 μL β-mercaptoethanol into the mortar.
3. After thawing the mixture, transfer the slurry to the centrifugation tube.
4. Incubate the mixture at 65°C in water bath for 10 min.
5. Delaminate by centrifugating with 13000rpm at 25°C for 15min.
6. Transferring the supernatant into a new centrifugation tube.
7. Add equal volume chloroform/isoamyl alcohol (24:1) and gently mix it for 1 min.
8. Repeat step 5 to step 7.
9. Delaminate by centrifugating with 13000 rpm at 25°C for 15 min.
10. Transferring the supernatant into a new centrifugation tube.
11. Add one fourth fold volume 10 M lithium chloride (LiCl)
12. Incubate the mixture at 4°C overnight.
13. Collect the RNA by centrifugating with 13000 rpm at 4°C for 15 min.
14. Wash the pellet by 75% ethanol two times.
15. Remove the excess alcohol in the pellet by vacuum dryer
16. Resuspend the pellet with optimal volume RNase free ddH_2O.
17. Check the quality of RNA by electrophoresis.
18. The remaining RNA samples were immediately steeped in liquid nitrogen and keep in -80°C for long time storage.
19. RNA electrophoresis (e.g. 25mL volume):
    (1) 0.25 g agar + 25 mL RNase free ddH_2O
    (2) Melt the agar by microwave oven.

(3) Until the mixture cool down to 40~50°C and add 2.5 mL 10X 3-(N-morpholino) propanesulfonic acid, 4-morpholinepropanesulfonic acid (MOPS) and 0.75 mL 37% formaldehyde.
(4) Solidify the agar in the suitable tank.
(5) Electrophoresis in 50 mV 1 hr.
(6) Photograph the gel under UV exposure.

Notes:

1. There are three methods for RNA precipitation :
   (1) LiCl : For precipitation of RNA which molecular weight is longer than 200 bps. The optimal precipitation condition is at 4°C.
   Supernatant : 10 M LiCl = 4:1(v/v)
   (2) 100% ethanol : The optimal precipitation condition is at -20°C.
   Supernatant : 100% EtOH : 10 M NH_4OAc = 1:2.5:0.25 (v/v/v)
   (3) 100% isopropanol : The optimal precipitation condition is at -20°C.
   Supernatant : Isopropanol : 10 M NH_4OAc = 1:1:0.25
2. All material including mortars, pistils, reagents, etc should be remove the RNase by rinsing with 1‰ DEPC and then sterilize in autoclave with 2 atm for 40 min.
3. The chemicals for extraction buffer should be mixed in order and sterilize in autoclave with 2 atm for 40 min after stirring with 0.5 mL DEPC overnight.

6.25 Rapid amplification of cDNA end; RACE

Preparation:

GeneRacer™ Advanced RACE Kit, Invitrogen.

Procedures:

A. Dephosphorylation

(1) Add the below reagents in a 1.5 mL microcentrifuge tube in order:

RNA(5 μg)	7	μL
10X CIP Buffer	1	μL
RNaseOut™ (40U μL^{-1})	1	μL
CIP (10U μL^{-1})	1	μL

(2) Incubate the above mixture in heating plate at 50°C for 1 hr.
(3) Keep the mixture in the ice for 2 min.
(4) Add 100 μL RNase-free ddH_2O and 100 μL phenol and chloroform and vortex vigorously for 30 seconds.

(5) To delaminate by centrifugating with 13000 rpm for 5 min at room temperature.
(6) Transfer aqueous (top) phase to a new microcentrifuge tube (~100 μL).
(7) Add 2 μL 10 mg mL^{-1} mussel glycogen, 10 μL 3 M sodium acetate (pH 5.2), and mix well.
(8) Add 220 μL 95% ethanol and vortex briefly.
(9) Freeze on liquid nitrogen for 30 min.
(10) To pellet RNA by centrifugating with 13000 rpm at 4°C for 20 min.
(11) Add 500 μL 70% ethanol, invert several times, and vortex briefly.
(12) To collect the RNA by centrifugating with 13000 rpm at 4°C for 5 min.
(13) Carefully remove the remaining ethanol by pipetting and air-dry the pellet for 30 min at room temperature.
(14) Resuspend the pellet in 7 μL RNase-free ddH_2O

B. Decapping

(1) Add the below reagents in a 1.5 mL microcentrifuge tube in order:

Dephosphorylated RNA	7	μL
10X TAP Buffer	1	μL
RNaseOUT™(40 U μL^{-1})	1	μL
TAP(0.5 U μL^{-1})	1	μL

(2) Incubate the above mixture in heating plate at 37°C for 1 hr.
(3) Keep the mixture in the ice for 2 min.
(4) Add 100 μL RNase-free ddH_2O and 100 μL phenol and chloroform and vortex vigorously for 30 seconds.
(5) To delaminate by centrifugating with 13000 rpm for 5 min at room temperature.
(6) Transfer aqueous (top) phase to a new microcentrifuge tube (~100 μL).
(7) Add 2 μL 10 mg mL^{-1} mussel glycogen, 10 μL 3M sodium acetate (pH 5.2), and mix well.
(8) Add 220 μL 95% ethanol and vortex briefly.
(9) Freeze on liquid nitrogen for 30 min.
(10) To pellet RNA by centrifugating with 13000 rpm at 4°C for 20 min.
(11) Add 500 μL 70% ethanol, invert several times, and vortex briefly.
(12) To collect the RNA by centrifugating with 13000 rpm at 4°C for 5 min.
(13) Carefully remove the remaining ethanol by pipetting and air-dry the pellet for 30 min at room temperature.
(14) Resuspend the pellet in 7 μL RNase-free ddH_2O

C. Ligation Reaction

(1) Add 7 μL of dephosphorylated, decapped RNA to the tube containg the pre-aliquoted, lyophilized GeneRacer^{TM} RNA oligo (0.25 μg).
(2) Pipet up and down several times to mix and resuspend RNA Oligo.
(3) Centrifugate briefly to collect the fluid in the bottom of the tube.
(4) Incubate at 65°C for 5 min to relax the RNA secondary structure.
(5) Place on ice to chill for 2 min and centrifugate briefly.
(6) Add the following reagents to the above tube in order, mix gently by pipetting, and centrifugate briefly.

10X Ligase Buffer	1	μL
10 mM ATP	1	μL
RNaseOUT^{TM} (40 U μL^{-1})	1	μL
T_4 RNA ligase (5 U μL^{-1})	1	μL

(7) Incubate at 37°C for 1 hr.
(8) Keep the mixture in the ice for 2 min.
(9) Add 100 μL RNase-free ddH_2O and 100 μL phenol and chloroform and vortex vigorously for 30 seconds.
(10) To delaminate by centrifugating with 13000 rpm for 5 min at room temperature.
(11) Transfer aqueous (top) phase to a new microcentrifuge tube (~100 μL).
(12) Add 2 μL 10mg mL^{-1} mussel glycogen, 10 μL 3 M sodium acetate (pH 5.2), and mix well.
(13) Add 220 μL 95% ethanol and vortex briefly.
(14) Freeze on liquid nitrogen for 30 min.
(15) To pellet RNA by centrifugating with 13000rpm at 4°C for 20 min.
(16) Add 500 μL 70% ethanol, invert several times, and vortex briefly.
(17) To collect the RNA by centrifugating with 13000 rpm at 4°C for 5 min.
(18) Carefully remove the remaining ethanol by pipetting and air-dry the pellet for 30 min at room temperature.
(19) Resuspend the pellet in 10 μL RNase-free ddH_2O

D.Reverse transcription

(1) Add the below reagents to the 10 μL of ligated RNA from above tube in order:

Primers	1	μL
dNTP Mix	1	μL
RNase-free ddH_2O	1	μL

(2) Incubate the mixture at 65°C for 5 min to remove any RNA structure.
(3) Chill on ice for at least 1 min and centrifugate briefly.

(4) Add the following reagents to the above tube in order:

5X First Strand Buffer	4	μL
0.1 M DTT	1	μL
RNaseOUT^{TM} (40 U μL^{-1})	1	μL
SuperScript^{TM} III RT(200 U μL^{-1})	1	μL

(5) Mix well by pipetting gently up and down.

(6) Centrifugate briefly and incubate at 50°C for 60 min.

(7) Inactivate the RT reaction at 70℃ for 15 min.

(8) Chill on ice for 2 min and centrifugate briefly.

(9) Add 1 μL of RNase H (2U) to the reaction mix.

(10) Incubate at 37°C for 20 min.

(11) Centrifugate briefly and use immediately for amplification (or store at -80°C)

(12) This cDNA (RACE ready cDNA) is used for gene cloning or amplifying cDNA end.

E. Gene cloning

(1) Download the amino acid sequence of interested genes in different species from NCBI website.

(2) Align these amino acid sequences and find out the consensus region.

(3) Design the degenerated forward and reverse primer from consensus region.

(4) Amplify the fragment of interested genes from the RACE ready cDNA library.

(5) Check the PCR result in the electrophoresis and elute the band with optimal length in the agarose gel.

(6) Ligate the interested genes with T-A vector.

(7) Transformate the ligated plasmid into competent cell.

(8) Spray the transformated competent cell in the plate containing ampicillin.

(9) Incubated the plate at 37°C overnight.

(10) Select the correct colony by colony PCR.

(11) Sequencing the amplified fragment in plasmid and design the gene reverse primer for 5'RACE or gene forward primer for 3' RACE.

F. Primary PCR for 5' or 3' RACE

(1) Adding the below reagents to a new 0.2mL microcentrifuge tube for primary 5' RACE in order:

Sterile water	33.5	μL
10X High Fidelity PCR Buffer	5	μL

2.5 mM dNTP Mix	4	μL
10 μM GeneRacerTM 5'or 3' Primer	3	μL
50 mM $MgSO_4$	2	μL
10 μM Gene reverse primer(5') or forward primer (3')	1	μL
RACE ready cDNA	1	μL
Platinum Taq DNA Polymerase High Fidelity (5 U μL^{-1})	0.5	μL

(2) Setup a Touch-down PCR reaction following the below condition:

94°C	2 min	
94°C	30sec	} 5cycles
72°C	2 min	
94°C	30sec	} 5cycles
70°C	2 min	
94°C	30 sec	} 25cycles
65°C	30 sec	
68°C	2 min	
68°C	10 min	
16°C	∞	

(3) Check the PCR product by electrophoresis.

G. Nested PCR for 5' or 3' RACE

(1) Adding the below reagents to a new 0.2 mL microcentrifuge tube for nested 5' RACE in order:

Sterile water	35.5	μL
10X High Fidelity PCR Buffer	5	μL
2.5 mM dNTP Mix	4	μL
10 μM GeneRacerTM 5'or 3' Nested Primer	1	μL
50 mM $MgSO_4$	2	μL
10 μM Gene nested reverse primer(5') or forward primer (3')	1	μL
RT template	1	μL
Platinum Taq DNA Polymerase High Fidelity (5 U μL^{-1})	0.5	μL

(2) Setup a Touch-down PCR reaction following the below condition:

94°C	2 min	
94°C	30 sec	} 5cycles
72°C	2 min	
94°C	30 sec	} 5cycles
70°C	2 min	
94°C	30 sec	} 25cycles
65°C	30 sec	

68°C 2 min
68°C 10 min
16°C ∞

(3) Check the PCR product by electrophoresis.

6.26 RT PCR for gene expression (sequences of primers is present in Table. 2)

1. One-step RT PCR

(1) Add the following reagents to a 0.2 mL microcentrifuge tube in order

RNase Free ddH_2O	24	μL
10 X One Step RNA PCR Buffer	5	μL
25 mM $MgCl_2$	10	μL
10 mM dNTP	5	μL
10 μM Gene specific forward primer	1	μL
10 μM Gene specific reverse primer	1	μL
RNA (1 μg μL^{-1})	1	μL
RNase Inhibitor (40 units mL^{-1})	1	μL
AMV RTase XL (5 units mL^{-1})	1	μL
AMV-Optimized Taq (5 units mL^{-1})	1	μL

(2) Setup a PCR reaction following the below condition:

50°C 45 min
94°C 2 min
94°C 30 sec
50~60°C 30 sec } 12~25cycles
72°C 30 sec
72°C 10 min

16°C ∞

(2) The expression level of gene in different samples was quantificated by the intensity of PCR product which displayed in the electrophoresis.

2. Two-step RT PCR

(1) Add the below reagents to the 10μL of ligated RNA from above tube in order:

RNA (1 μg μL^{-1})	5	μL
dN_6 (2.5 mM)	4	μL
RNase-free ddH2O	29	μL

(2) Incubate the mixture at 72°C for 5 min to remove any RNA structure.

(3) Chill on ice for at least 2 min and centrifugate briefly.

(4) Add the following reagents to the above tube in order:

10 X One Step RNA PCR Buffer	5	μL
25 mM $MgCl_2$	10	μL
10 mM dNTP	5	μL
RNase Inhibitor (40 units mL^{-1})	1	μL
AMV RTase XL (5 units mL^{-1})	1	μL

(5) Incubate the mixture at 50°C for 45 min and stop the reaction at 94°C for 2 min.

(6) Setup a PCR reaction following the below condition for gene expression:

50°C	45 min	
94°C	2 min	
94°C	30 sec	} 12~25cycles
50~60°C	30 sec	
72°C	30 sec	
72°C	10 min	
16°C	∞	

6.27 Construction of functional plasmid for overexpressing the interested genes in *Arabidopsis.*

Preparation:

Equipments: Electrophoresis set, centrifuge (4/-20°C), heating plate (65/50/42°C), refrigerator, oven (80/37°C), PCR machine, electroporation machine and cuvette.

Materials: Ligation kit, Gel elution kit, pCAMBIA 2300 vector, 15 mL centrifuge tube, 1.5 mL microcentrifuge tubes, restriction enzymes,

Reagents:

1. LB medium (1 L): Mix 15g (Luria-Bertani) medium and 25 g agar (for bacteria

culture) and sterilize at 2 atm for 20 min.

2. LB broth (1 L): Mix 15g (Luria-Bertani) medium and sterilize at 2 atm for 20 min.
3. Solution 1: 50 mM glucose, 10 mM EDTA in 25 mM Tris-HCl (pH 8.0)
4. Solution 2 (Fresh): 880 μL ddH_2O, 100 μL 10% SDS and 20 μL 10N sodium hydroxide. Keep at room temperature.
5. Solution 3: 60 mL 5 M potassium acetate + 11.5 mL glacial acetic acid and 28.5 mL H_2O.
6. RNase: 0.1 g RNase A solves in 10 mL H_2O, and then boils it for 10 min. Filter sterilize the solution by passing it through 0.22 micron filter and storage at -20°C.
7. PCI: phenol/chloroform/isoamylalcohol (25:24:1).
8. CI: chloroform/isoamylalcohol (24:1)
9. X-gel: 1 g of X-gal in 10mL of dimethyl formamide (DMF).
10. IPTG: isopropyl thiol-D-galactoside, 0.2 g IPTG solves in 10 mL H_2O and filter sterilize the solution by passing it through 0.22 micron filter and store them in -20 ^{0} C.

Procedures:

1. Elute the DNA product form the electrophoresis by following the elution kit protocol.
2. Check and quantify the elution product by electrophoresis.
3. Ligate the DNA fragment with T-A cloning vector (e.g. pGEM-T easy)

DNA fragment	3	μL
2X ligation buffer	5	μL
pGEM-T easy vector	1	μL
T_4 DNA ligase	1	μL

4. Incubate the mixture at 4°C overnight.
5. Transformation by heat shock method following below steps:
   (1) Mix above mixture with 100 μL E.coli (DH5α) competence cell and keep on ice for 30 min.
   (2) Heat the above mixture at 42°C for 90 seconds.
   (3) Keep on ice for 2 min.
   (4) Add 100μL LB broth to above mixture and incubate at 37°C for 30 min.
   (5) Spread the mixture with 40μL 10% X-gel and 7μL 20% IPTG for Blue White screening on the 100 ppm ampicillin LB medium plate.
   (6) Incubate the plate at 37°C overnight.
6. Subculture several single colony in a new plate and operate a colony-PCR to select the colonies transformed successfully.
7. Sequence the plasmid to confirm and analyze the interested gene by

bioinformatics approach.

8. Generation of single colony from the accurate colony by streaking plate method.
9. Extraction the plasmids from the colony by following the below steps:
   (1) Culture the single colony in 5 mL LB broth with 100 ppm ampicillin at 37°C overnight.
   (2) Collect the bacteria by centrifugating with 3000 rpm at room temperature for 10 min.
   (3) Discard the supernatant and resuspend the pellet with 100 μL solution 1.
   (4) Incubate at room temperature for 5 min
   (5) Add the solution 2 and vortex gently until the mixture becomes clear.
   (6) Keep on ice for 5 min.
   (7) Add 150 μL solution 3 and vortex gently until appearance of while slurry.
   (8) Delaminate the above mixture by centrifugating with 13000 rpm at 4°C for 15 min.
   (9) Transfer the 400 μL supernatant to a new 1.5 mL microcentrifuge tube and add 4 μL RNase (10 mg mL^{-1}).
   (10) Incubate the mixture at 50°C for 1 hr.
   (11) Add 400μL PCI and vortex vigorously
   (12) Delaminate the above mixture by centrifugating with 1300 rpm at 4°C for 15 min.
   (13) Transfer 300 μL supernatant to a new microcentrifuge tube.
   (14) Add 300 μL CI and vortex vigorously.
   (15) Delaminate the above mixture by centrifugating with 1300 rpm at 4°C for 5 min.
   (16) Transfer 200 μL supernatant to a new microcentrifuge tube.
   (17) Add 50 μL 10M ammonium acetate (NH_4OAc; pH 6.5) and 500 μL 100% ethanol.
   (18) Keep at -20°C for 2 hr.
   (19) Collect the plasmid by centrifugating with 13000 rpm at 4°C for 20 min.
   (20) Remove the excess salt form the plasmid by washing with 70% ethanol twice.
   (21) Remove the ethanol from the plasmid by vacuating.
   (22) Resuspend the pellet with ddH_2O.
10. Digestion of plasmid with optimal restriction enzymes following the below steps:

Plasmid (50 μg)	85 μL
Restriction enzymes (less than 5% of total volume)	4 μL
10X Digestion buffer	10 μL
100X BSA	0-1 μL

11. Incubate the above mixture at 37°C overnight.

12. Check the digestion product by electrophoresis.
13. Elute the DNA product form the electrophoresis by following the elution kit protocol.
14. Check and quantify the elution product by electrophoresis.
15. Ligate the DNA fragment with pCAMBIA 2300 vector

DNA fragment	6	μL
2X ligation buffer	9	μL
Vector	2	μL
T_4 DNA ligase	1	μL

16. Incubate the mixture at 4°C overnight.
17. Transformation, colony PCR and sequencing to confirm the accuracy of the functional plasmid.
18. Transformation of *Agrobacterium tumefaciens* by electroporation method.
    (1) Keep the electroporation cuvette in the oven (80°C) for 15 min to remove the excess ethanol.
    (2) Capping and keep on ice for 5 min.
    (3) Keep the competence cell on ice for 5 min.
    (4) Add the 2 μL pure plasmid to the competent cell and incubate on ice for 5 min.
    (5) Transfer the above mixture to the electroporation cuvette.
    (6) Charge and trigger the pulse.
    (7) Add 900 μL LB broth and incubate at 28°C for 1 hr.
    (8) Spread 200 μL mixture on the100 ppm kanamycin plate and incubate at28°C for two day.
19. Subculture several single colony in a new plate and operate a colony-PCR to select the colonies transformed successfully.

Notes:

1. The pH of the phenol as shipped is 6.6 ± 0.2. After addition of the Tris alkaline Buffer, the pH is confirmed to be 7.9 ± 0.2, and it is suitable for deproteinization of nucleic acids.
2. The rotational speed when collection of the bacteria by centrifugating should not higher than 5000 rpm to prevent the damage of bacteria.
3. X-gal is light sensitive and hence store in a glass or polypropylene containers wrapped with aluminum foil. Stock solution need to be store in -20°C
4. The plasmid for electroporation is extracted from Viogene Mini Plus™ Plasmid DNA Extraction System.

6.28 Preparation of E.coli (DH5α) competence cell

Preparation:

Equipments: Oven (37°C), Shaker (19°C), centrifuge, spectrophotometer, refrigerator (-80°C)

Materials: LB broth, LB medium plate without any antibiotics, 50mL centrifuge tube, Flask, liquid nitrogen. plastic cuvette

Reagents:

1. Transformation buffer (1L): 3.47 g 4-piperazinediethanesulfonic acid disodium salt (PIPES), 2.20 g calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$) and 18.65 g potassium chloride (KCl) in 800 mL H_2O, and then adjust the pH value to 6.7 by potassium hydroxide or hydrogen chloride. Add 10.88 g manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) and filter sterilize the solution by passing it through 0.22 μm filter and storage at 4°C.
2. DMSO (Dimethyl sulfoxide)
3.

Procedures:

1. Subculture DH5α cell in the LB medium plate without any antibiotics and incubate at 37°C overnight.
2. Culture a single colony with 15 mL LB broth in 50 mL centrifuge tube at 37°C overnight.
3. Pour above medium to a 250 mL LB broth in a 1L flask.
4. Incubate at 19°C overnight.
5. Until the absorbance in 600 nm of this medium ranges in 0.5-0.6, and keep the medium on the ice for 10 min.
6. Collect the bacteria by centrifugating with 3000 rpm at 4°C for 15 min.
7. Resuspend the pellet by 40 mL pre-chilled transformation buffer on the ice.
8. Collect the bacteria by centrifugating with 3000 rpm at 4°C for 15 min.
9. Resuspend the pellet by 10 mL pre-chilled transformation and 0.75 mL DMSO gently on the ice.
10. Transfer each 100 μL mixture to a 1.5 mL microcentrifuge tube and immerse in liquid nitrogen immediately.
11. Storage the competent cell at -80℃

6.29 Preparation of *A. tumefaciens* (GV3101) competence cell

Preparation:

Equipments: Oven (28°C), Shaker (28°C), centrifuge, spectrophotometer, refrigerator (-80°C)

Materials: LB broth, LB medium plate with 20 μg mL^{-1} rifampicine, 50 mL centrifuge tube, Flask, liquid nitrogen, plastic cuvette

Reagents:

1. 10% glycerol: 10 μL 100% glycerol in 90 mL H_2O and filter sterilize the solution by passing it through 0.22 μm filter and storage at 4°C.
2.

Procedures:

1. Subculture GV3101 cell in the LB medium plate with 20 μg mL^{-1} rifampicine and incubate at 28°C for 2 day.
2. Culture a single colony with 15 mL LB broth in 50 mL centrifuge tube at 28°C overnight.
3. Pour above medium to a 250 mL LB broth in a 1000 mL flask.
4. Incubate at 28°C until the absorbance in 600 nm of this medium ranges in 0.5-0.6, and keep the medium on ice for 10 min.
5. Collect the bacteria by centrifugating with 3000 rpm at 4°C for 15 min.
6. Resuspend the pellet by 20 mL pre-chilled 10% glycerol on the ice.
7. Collect the bacteria by centrifugating with 3000 rpm at 4°C for 15 min.
8. Resuspend the pellet by 10mL pre-chilled 10% glycerol gently on the ice.
9. Transfer each 50μL mixture to a 1.5mL microcentrifuge tube and immerse in liquid nitrogen immediately.
10. Storage the competent cell at -80°C

6.30 Agrobacterium infiltration (Host: Arabidopsis thaliana Col.)

Preparation:

Equipment: Oven (28°C), centrifuge.

Materials: Arabidopsis with inflorescence, 50/250 mL centrifuge tubes, triangle flask,

Reagents:

1. infiltration broth (1L; pH 5.7): 2.2 g MS with vitamin, 50g sucrose, 0.5g MES, 0.1mL Silweet L-77.
2. 100 ppm kanamycin

Procedure:

1. Subculture the colony containing the plasmid in the kanamycin-selected plate and incubate it at 28°C for 2days.
2. Select a single colony form the plate and culture it with 40 mL LB broth in 50 mL centrifuge tube (Agro is anaerobic) overnight.
3. Transfer the bacteria solution to 500 mL LB and culture it at 28°C overnight.
4. While the absorbance at 600 nm is higher than 0.8, collect the bacteria by centrifugating with 5000 rpm at 4°C for 10 min.
5. Resuspend the pellet with 250 mL infiltration broth.
6. Dip the optimal plants in the 250 mL infiltration broth for 45 seconds.
7. Repeat step. 6.
8. Seal the plant with the plastic wrap at the dark overnight.
9. Remove the plastic wrap.
10. Until the whole plant product the fully mature siliques, collect and sieve the seeds in the 1.5 mL microcentrifuge tubes.
11. Remove the water on the surface of the seeds by keep in the humidity-controlled oven.

Note:

1. Remove the growing siliques from the *Arabidopsis* inflorescence before the dipping could decrease the population of wild-type seeds in the later collection of seeds.
2. 250 mL infiltration broth is enough for dipping 30 plants.
3. Infiltration broth is not necessary to sterilize.
4. Adjust the pH value of infiltration buffer by 1 N potassium hydroxide.

6.31 Selection of the transgenic *Arabidopsis* overexpressing interested gene

Preparation:

Equipments: Laminar flow, centrifuge, culture room, PCR machine.

Materials: kanamycin plate, *Arabidopsis* seeds, sterilized H_2O, liquid nitrogen.

Reagents:

1. sterilized buffer (1mL): 0.5 mL H_2O, 0.5 mL bleach with 6% sodium hypochlorite (NaClO) and 10 μL Tween 20.
2. ethanol
3. 1X Tris-EDTA buffer (pH 8.0): 1 mM EDTA in 10 mM Tris-HCl (pH 8.0)
4.

Procedure:

A. Preparation of germination (GM) medium plate (1L) :

(1) Mix the below chemicals in order:
4.4 g MS medium with vitamin→ 10 g sucrose→ 0.5 g MES.

(2) Adjust the pH value of the medium to 5.7 by 1N potassium hydroxide.

(3) Sterilize the medium with optimal weight of phytoagar.

(4) Cool down the medium to 40~50°C and then add cefatriaxone and kanamycin to the final concentration 200 ppm and 75 ppm particularly.

(5) Pour 20mL medium for each petri-dish which size is 9x2 cm.

B. Planting the Arabidopsis seeds

(1) Collect about 300 seeds of transgenic plant in 1.5 mL microcentrifuge tube.

(2) Add 1mL 70% ethanol and vortex 15 second.

(3) Remove the ethanol and add the 1mL sterilized buffer.

(4) Vortex vigorously for 10 min.

(5) Add the ddH_2O to replace of the excess sterilized buffer several times.

(6) Spread the seeds in the kanamycin plate evenly.

(7) Keep the plates in the dark at 4°C for 2 days.
(8) Transfer the plates to the culture room (22°C; 16/8 long-day photoperiod).
(9) Pick up the plants, which grow successfully in the kanamycin plates, and planting them in the soil.

C. Leaf PCR (to amplify the interested genes from plants)

(1) Homogenize one current leaf in 1.5mL microcentrifuge tube with liquid nitrogen.
(2) Add 20 μL 0.5N sodium hydroxide and vortex vigorously.
(3) Incubate at room temperature for 20 min.
(4) Increase the efficiency of the basic digestion by evacuation twice for 1 min or until the production of the bubble.
(5) Boil the mixture for 30 second.
(6) Add 40 μL Tris (pH 8.0) and 160 μL 1X Tris-EDTA buffer (pH 8.0) and vortex vigorously.
(7) Take 1 μL mixture for PCR reaction.

7. References:

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

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell **92:** 773-784.

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55:** 373-399.

Attolico, A.D., and De Tullio, M.C. (2006). Increased ascorbate content delays flowering in long-day grown *Arabidopsis thaliana* (L.) Heynh. Plant Physiol. Biochem. **44:** 462-466.

Aziz, A., Heyraud, A., and Lambert, B. (2004). Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *Botrytis cinerea*. Planta **218:** 767-774.

Badiani, M., Paolacci, A.R., Miglietta, F., Kimball, B.A., Pinter, P.J., Garcia, R.L.,

Hunsaker, D.J., Lamorte, R.L., and Wall, G.W. (1996). Seasonal variations of antioxidants in wheat (*Triticum aestivum*) leaves grown under field conditions. Funct. Plant Biol. **23:** 687-698.

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

Barth, C., Moeder, W., Klessig, D.F., and Conklin, P.L. (2004). The timing of senescence and response to pathogens is altered in the ascorbate-deficient *Arabidopsis* mutant vitamin c-1. Plant Physiol. **134:** 1784-1792.

Bergey, D.R., Orozco-Cardenas, M., de Moura, D.S., and Ryan, C.A. (1999). A wound- and systemin-inducible polygalacturonase in tomato leaves. Proc. Natl. Acad. Sci. U S A **96:** 1756-1760.

Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U., and Tomsett, A.B. (1998). An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat. Biotechnol. **16:** 177-180.

Colville, L., and Smirnoff, N. (2008). Antioxidant status, peroxidase activity, and PR protein transcript levels in ascorbate-deficient *Arabidopsis thaliana* vtc mutants. J. Exp. Bot. **59:** 3857-3868.

Conklin, P.L., and Barth, C. (2004). Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ. **27:** 959-970.

Davey, M.W., Gilot, C., Persiau, G., Ostergaard, J., Han, Y., Bauw, G.C., and Van Montagu, M.C. (1999). Ascorbate biosynthesis in *Arabidopsis* cell suspension culture. Plant Physiol. **121:** 535-543.

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

Debolt, S., Melino, V., and Ford, C.M. (2007). Ascorbate as a biosynthetic precursor in plants. Ann. Bot. (Lond) **99:** 3-8.

Downie, A., Miyazaki, S., Bohnert, H., John, P., Coleman, J., Parry, M., and Haslam, R. (2004). Expression profiling of the response of *Arabidopsis thaliana* to methanol stimulation. Phytochemistry **65:** 2305-2316.

Fall, R., and Benson, A.A. (1996). Leaf methanol - the simplest natural product from plants. Trends in Plant Sci. **1:** 296-301.

Finkelstein, R.R. (2006). Studies of abscisic acid perception finally flower. Plant Cell **18:** 786-791.

Foyer, C.H., and Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell **17:** 1866-1875.

Frenkel, C., Peters, J.S., Tieman, D.M., Tiznado, M.E., and Handa, A.K. (1998). Pectin methylesterase regulates methanol and ethanol accumulation in ripening tomato (*Lycopersicon esculentum*) fruit. J. Biol. Chem. **273:** 4293-4295.

Fukui, Y., and Doskey, P.V. (1998). Air-surface exchange of nonmethane organic compounds at a grassland site: Seasonal variations and stressed emissions. J. Geophys. Res. **103:** 13153–113168.

Galbally, I.E., and Kirstine, W. (2002). The production of methanol by flowering plants and the global cycle of methanol. J. Atmos. Chem. **43:** 195-229.

Gout, E., Aubert, S., Bligny, R., Rebeille, F., Nonomura, A.R., Benson, A.A., and Douce, R. (2000). Metabolism of methanol in plant cells. carbon-13 nuclear magnetic resonance studies. Plant Physiol. **123:** 287-296.

Hancock, R.D., Walker, P.G., Pont, S.D.A., Marquis, N., Vivera, S., Gordon, S.L., Brennan, R.M., and Viola, R. (2007). L-Ascorbic acid accumulation in fruit of *Ribes nigrum* occurs by in situ biosynthesis via the l-galactose pathway. Funct. Plant Biol. **34:** 1080-1091.

Hanson, A.D., and Roje, S. (2001). One-carbon metabolism in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52:** 119-137.

Hirai, N., Kojima, Y., Shinozaki, M., Koshimizu, K., Murofushi, N., and Takimoto, A. (1995). Accumulation of ascorbic acid in the cotyledons of morning glory (*Pharbitis nil*) seedlings during the induction of flowering by low-temperature treatment and the effect of prior exposure to high-intensity light. Plant Cell Physiol. **36:** 1265-1271.

Kanwischer, M., Porfirova, S., Bergmuller, E., and Dormann, P. (2005). Alterations in tocopherol cyclase activity in transgenic and mutant plants of *Arabidopsis* affect tocopherol content, tocopherol composition, and oxidative stress. Plant Physiol. **137:** 713-723.

Kotchoni, S.O., Larrimore, K.E., Mukherjee, M., Kempinski, C.F., and Barth, C. (2009). Alterations in the endogenous ascorbic acid content affect flowering time in Arabidopsis. Plant Physiol. **149:** 803-815.

Lokhande, S.D., Ogawa, K., Tanaka, A., and Hara, T. (2003). Effect of temperature on ascorbate peroxidase activity and flowering of *Arabidopsis thaliana* ecotypes under different light conditions. J. Plant Physiol. **160:** 57-64.

Lorence, A., Chevone, B.I., Mendes, P., and Nessler, C.L. (2004). Myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant

Physiol. **134:** 1200-1205.

Micheli, F. (2001). Pectin methylesterases: cell wall enzymes with important roles in plant physiology. Trends Plant Sci. **6:** 414-419.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. Trends Plant Sci. **9:** 490-498.

Noctor, G. (2006). Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ. **29:** 409-425.

Nonomura, A.M., and Benson, A.A. (1992). The path of carbon in photosynthesis: improved crop yields with methanol. Proc. Natl. Acad. Sci. USA **89:** 9794-9798.

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

Pavet, V., Olmos, E., Kiddle, G., Mowla, S., Kumar, S., Antoniw, J., Alvarez, M.E., and Foyer, C.H. (2005). Ascorbic acid deficiency activates cell death and disease resistance responses in *Arabidopsis*. Plant Physiol. **139:** 1291-1303.

Pelloux, J., Rusterucci, C., and Mellerowicz, E.J. (2007). New insights into pectin methylesterase structure and function. Trends Plant Sci. **12:** 267-277.

Penuelas, J., Filella, I., Stefanescu, C., and Llusia, J. (2005). Caterpillars of *Euphydryas aurinia* (Lepidoptera: Nymphalidae) feeding on *Succisa pratensis* leaves induce large foliar emissions of methanol. New Phytol. **167:** 851-857.

Pignocchi, C., Kiddle, G., Hernandez, I., Foster, S.J., Asensi, A., Taybi, T., Barnes, J., and Foyer, C.H. (2006). Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. Plant Physiol. **141:** 423-435.

Pnueli, L., Liang, H., Rozenberg, M., and Mittler, R. (2003). Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (*Apx1*)-deficient Arabidopsis plants. Plant J. **34:** 187-203.

Ramírez, I., Dorta, F., Espinoza, V., Jiménez, E., Mercado, A., and Peña-Cortés, H. (2006). Effects of foliar and root applications of methanol on the growth of *Arabidopsis*, tobacco, and tomato plants. J. Plant Growth Regul. **25:** 30-44.

Ridley, B.L., O'Neill, M.A., and Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry **57:** 929-967.

Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F., Sakurai, N., Suzuki, H., Hirai, M.Y., Noji, M., Saito, K., Masuda, T., Takamiya, K.,

Shibata, D., and Ohta, H. (2005). Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in *Arabidopsis*. Plant J. **44:** 653-668.

Shen, C.-H., Ramanarayan, K., and Yeh, K.-W. (2009). Decreased L-ascorbate content mediating bolting is mainly regulated by the galacturonate pathway in *Oncidium*. Plant Cell Physiol. **50:** 935-46.

Shen, C.H., and Yeh, K.W. (2009). Hydrogen peroxide mediates the expression of ascorbate-related genes in response to methanol stimulation in *Oncidium*. J. Plant Physiol. (in press)

Smirnoff, N. (2003). Vitamin C booster. Nat. biotechnol. **21:** 134-136.

Smith, A.G., Croft, M.T., Moulin, M., and Webb, M.E. (2007). Plants need their vitamins too. Curr. Opin. Plant Biol. **10:** 266-275.

Tan, J., Wang, H.L., and Yeh, K.W. (2005). Analysis of organ-specific, expressed genes in *Oncidium* orchid by subtractive expressed sequence tags library. Biotechnol. Lett. **27:** 1517-1528.

Veljovic-Jovanovic, S.D., Pignocchi, C., Noctor, G., and Foyer, C.H. (2001). Low ascorbic acid in the vtc-1 mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. Plant Physiol. **127:** 426-435.

von Dahl, C.C., Havecker, M., Schlogl, R., and Baldwin, I.T. (2006). Caterpillar-elicited methanol emission: a new signal in plant-herbivore interactions? Plant J. **46:** 948-960.

Wang, C.Y., Chiou, C.Y., Wang, H.L., Krishnamurthy, R., Venkatagiri, S., Tan, J., and Yeh, K.W. (2008). Carbohydrate mobilization and gene regulatory profile in the pseudobulb of *Oncidium* orchid during the flowering process. Planta **227:** 1063-1077.

Wang, H.L., Chung, J.D., and Yeh, K.W. (2003). Changes of carbohydrate and free amino acid pools in current pseudobulb of *Oncidium* Gower ramsey during inflorescence development. J. Agri. Assoc. China **4:** 476-488.

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

Wolucka, B.A., and Van Montagu, M. (2003). 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.

Wolucka, B.A., Goossens, A., and Inze, D. (2005). Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. J. Exp. Bot. **56:** 2527-2538.

Yamamoto, A., Bhuiyan, M.N., Waditee, R., Tanaka, Y., Esaka, M., Oba, K.,

Jagendorf, A.T., and Takabe, T. (2005). Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and Arabidopsis plants. J. Exp. Bot. **56: 1785-1796.**

Tables:

Table 1. H_2O_2 amount and pectin concentration of PLBs incubated with various treatments

Treatments	H_2O_2 (μM g^{-1} FW)	Pectin (mg g^{-1} FW)
CK (½ MS)		
0 h	28.29±2.83	31.46±0.64
6 h	28.57±1.98	32.20±0.93
12 h	28.92±1.73	30.85±1.13
24 h	26.50±1.24	31.65±1.67
30 h	28.68±2.32	30.45±1.01
50 mM L-Gal		
0 h	28.12±2.44	31.21±1.03
6 h	28.39±2.58	30.70±0.55
12 h	28.35±1.33	29.98±0.51
24 h	30.00±1.42	30.13±0.57
30 h	28.38±2.32	30.88±0.58
50 mM D-GalUA		
0 h	26.86±2.81	30.09±0.56

6 h	28.75±1.85	30.07±0.56
12 h	28.85±2.15	30.56±0.93
24 h	27.47±2.72	31.43±1.16
30 h	27.73±1.89	30.95±0.16
50 mM MeOH		
0 h	27.75±1.72	30.92±0.58
6 h	39.14±1.28	26.42±0.96
12 h	33.14±1.52	25.65±0.58
24 h	31.43±1.30	26.02±1.45
30 h	30.74±1.61	27.57±1.00
50 mM D-GalUA + 50 mM MeOH		
0 h	29.03±2.09	30.86±0.16
6 h	40.11±1.28	26.84±0.99
12 h	34.61±1.05	25.48±1.14
24 h	32.46±2.89	26.94±1.58
30 h	28.82±0.98	27.35±0.43

PLBs = protocorm-like bodies; FW = fresh weight. Mean values ± S.E. were obtained from three independent experiments.

Table 2. The sequence of primers for RT-PCR

PME-F	5-GCTCAAGCTTTGTTCTATGGT-3
PME-R	5-AAAGAAAAAACAAGATAAAATATAGC-3
PG-F	5-ACGGCGGTGGCGGCAGAGGA-3
PG-R	5-ACACTGCCCCTGCCCTCTATAGTGCC-3
GalUAR-F	5-TCCCTGCTTTACAGAAGTCCCT-3
GalUAR-R	5-CCTGGTTTACAAATGGAGGCA-3
GMP-F	5-TTCGAGCGGCTGCCCGTCCA-3
GMP-R	5-GGCTGCCCGATGTCCATCCA-3
GalDH-F	5-TACTCGGAAATTGCCTCCATG-3

GalDH-R	5-CCACACGATCCAAAACATATCTG-3
GalLDH-F	5-TCAAAGAGCACGGGCTTACG-3
GalLDH-R	5-AGGGGAAACCTCCATTGTTCC-3
APX-F	5-TGGCACTCGGCTGGGACTTACGATGT-3
APX-R	5-GTGGTCGGAACCTTTGGTAGCATCAGG-3
MDHAR-F	5-AGCA-GACGATGGATCGCTATCGCCGAA-3
MDHAR-R	5-CGAGTTGAGGCGAGTAGAGCACGTTGA-3

Figures:

A

B

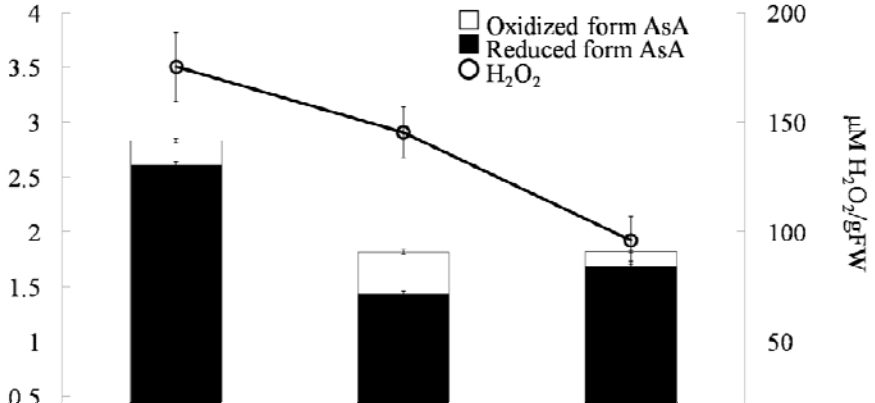


Fig. 1. Ascorbic acid content and H_2O_2 level in different developmental stages of *Oncidium* pseudobulb

A. Diagram of *Oncidium* plant, and AsA content varied with H_2O_2 level in different developmental stage. The inflorescent bud (i) and axillary bud (a) are concomitantly formed at each side of pseudobulb (p) base during the vegetative stage (V). When the inflorescence emerges at bolting period (B), the plant switches into the reproductive stage (R), and the inflorescence stalk develops. L denotes the leaf numbering from top to base.

B. Transverse section of pseudobulb stained with 3,3'-diaminobenzidine for H_2O_2, and $AgNO_3$ for AsA. Spots indicate H_2O_2 and AsA compounds accumulating in tissues. FW=fresh weight

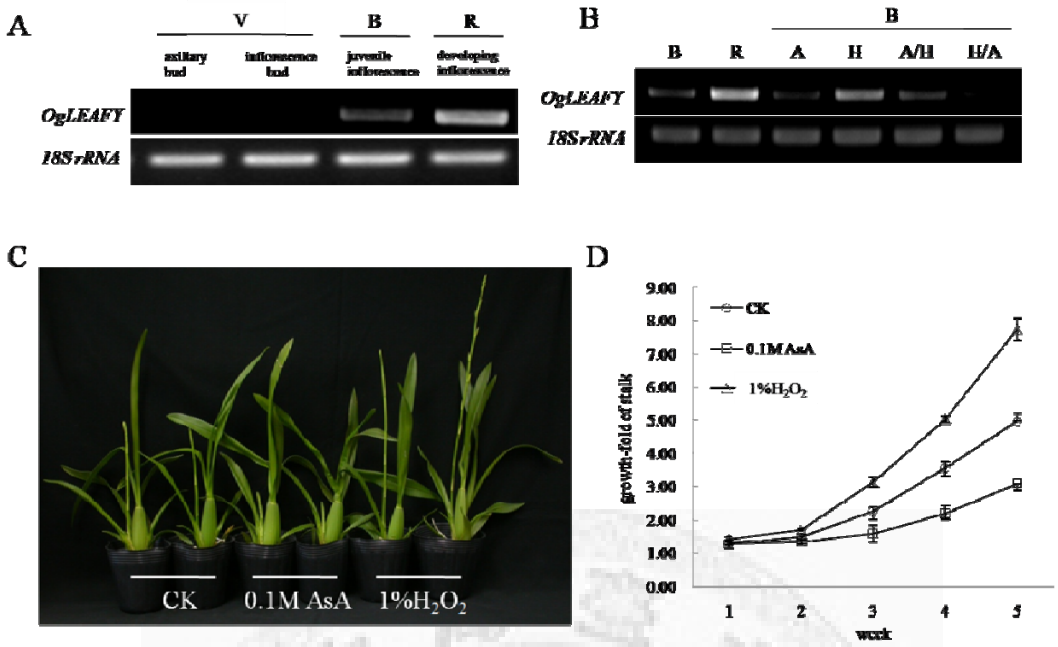


Fig. 2. The expression pattern of *OgLEAFY* at different developmental stages and under artificial treatments of chemicals

A. RT-PCR analysis of *OgLEAFY* expression at the axillary and inflorescence bud of the vegetative stage (V), juvenile inflorescence of the bolting period (B), and developing inflorescence of the reproductive stage (R).

B. RT-PCR analysis of *OgLEAFY* expression under artificial spray of AsA and H_2O_2 at B. A: 0.1 M AsA was sprayed on juvenile inflorescent buds for 4 h; H: 1% H_2O_2 was sprayed on juvenile inflorescent buds for 4 h; A/H: 0.1 M AsA was sprayed and followed by 1% H_2O_2 sprayed for 4 h; H/A: 1% H_2O_2 spray and followed by 0.1 M AsA sprayed for 4 h.

C, D. Bolting time of *Oncidium* affected by artificial spray of AsA and H_2O_2. Bolting time was delayed by 0.1 M ascorbic acid but hastened by 1% H_2O_2 spray

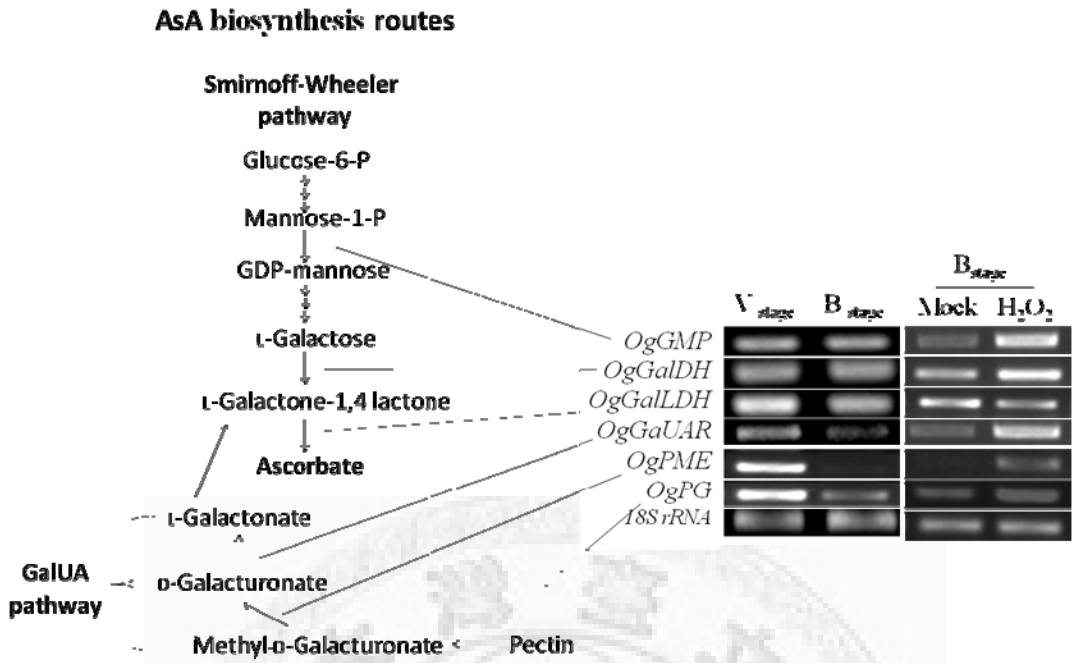


Fig. 3. Expression pattern of AsA biosynthetic genes during V and B in *Oncidium*. Expression level of AsA-related genes at V and B (left panel), and gene expression under 1 % H_2O_2 induction and mock (by H_2O) (right panel).

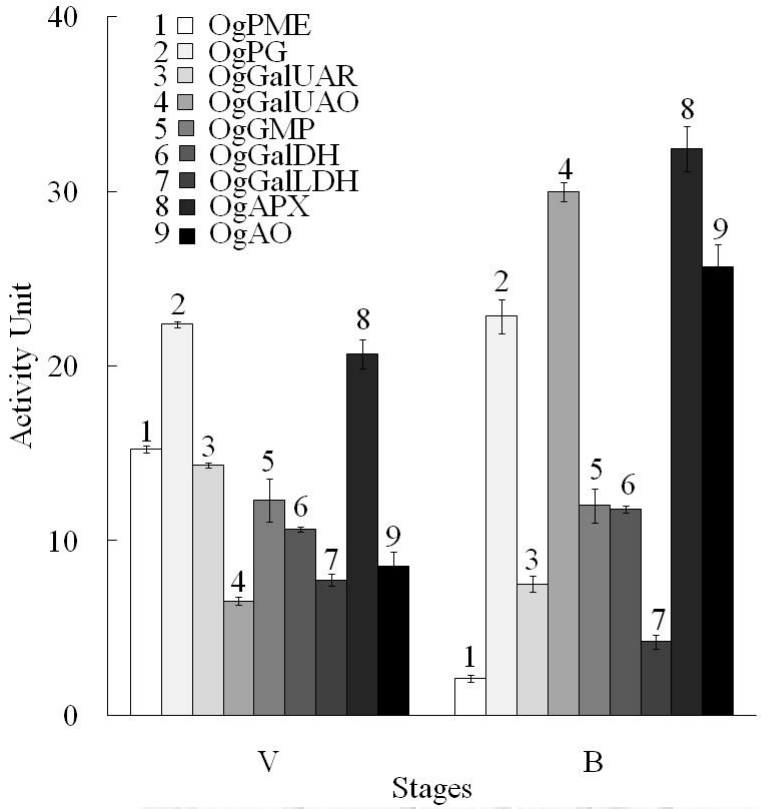


Fig. 4. Enzymatic activities of AsA biosynthetic genes during V and B in *Oncidium*. Enzymatic activity assay during V and B. Ascorbate peroxidase (OgAPX) and ascorbate oxidase (OgAO) belong to AsA catabolic enzymes. Vertical bars represent standard deviation of the mean (n=5).

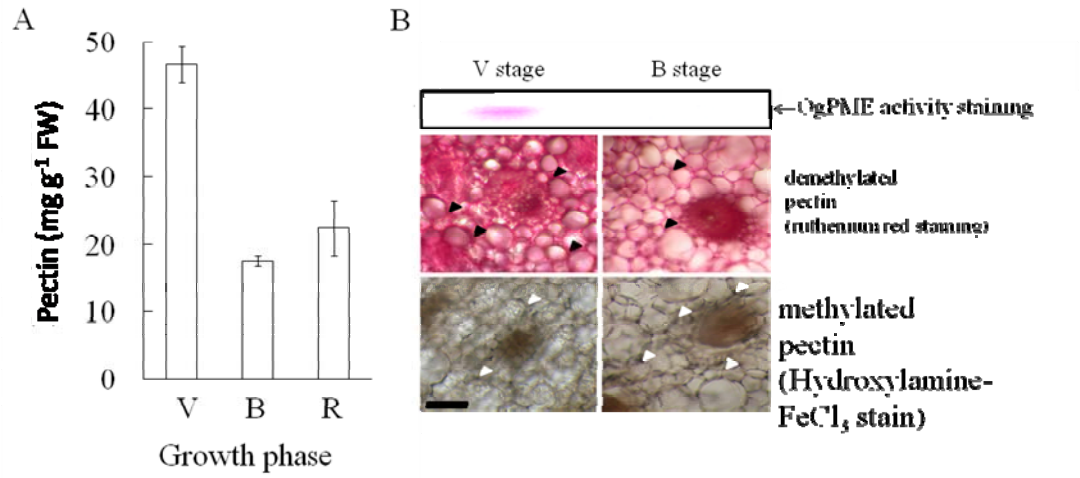


Fig. 5. Pectin content and composition in pseudobulb varied with pectin methylesterase activity.

A. Variation of pectin content in growth stage of V, B and R. Vertical bars represent standard deviation of the mean value (n=5). FW=fresh weight

B. PME activity staining (upper) and histogram staining of transverse sections of pseudobulbs. Demethylated pectin was examined by ruthenium-red staining (black triangle) and methylated pectin by hydroxylamine-$FeCl_3$ staining (white triangle). The black scale represents 50 μm.

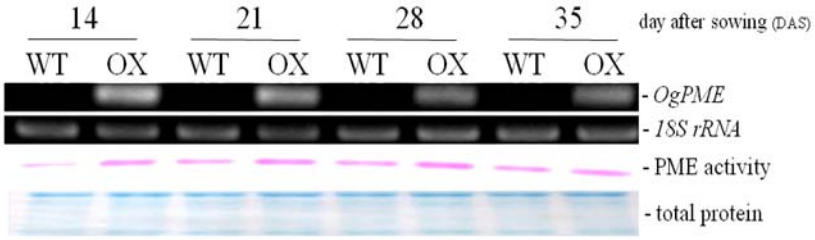


Fig. 6. Overexpression of pectin methylesterase in *Arabidopsis.*
OgPME overexpressed in transgenic *Arabidopsis* (T2) was detected by RT-PCR and activity staining at different growth durations. WT: wild type; OX: transgenic *Arabidopsis*.

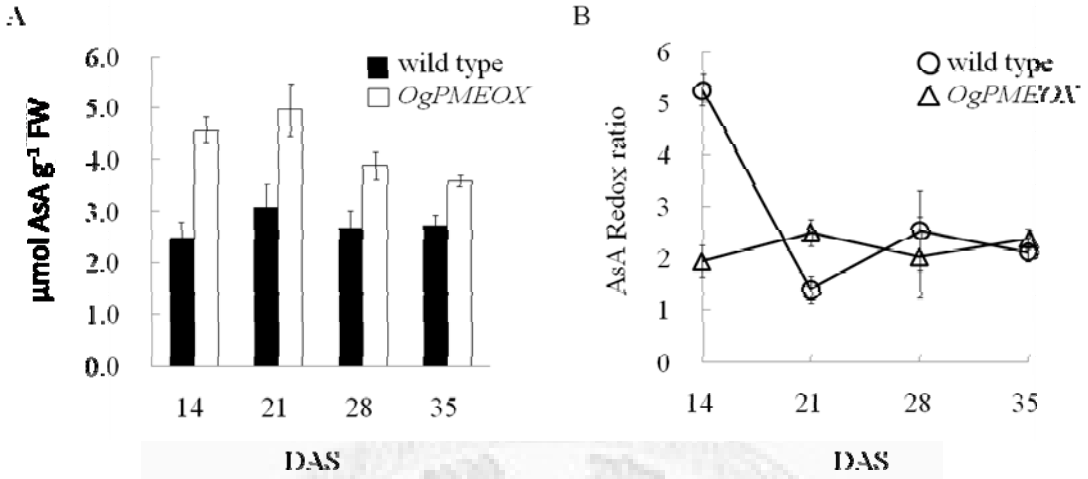


Fig. 7. AsA content and AsA redox state in transgenic *Arabidopsis* overexpressing *OgPME*

A. AsA content in *OgPMEOX* and the wild type measured at different growth durations in transgenic lines. Vertical bars represent standard deviation (n_{wt}= 30, $n_{OgPMEOX}$= 36).

B. AsA redox state measured at different growth durations from transgenic lines and wild type. DAS: day after sowing.

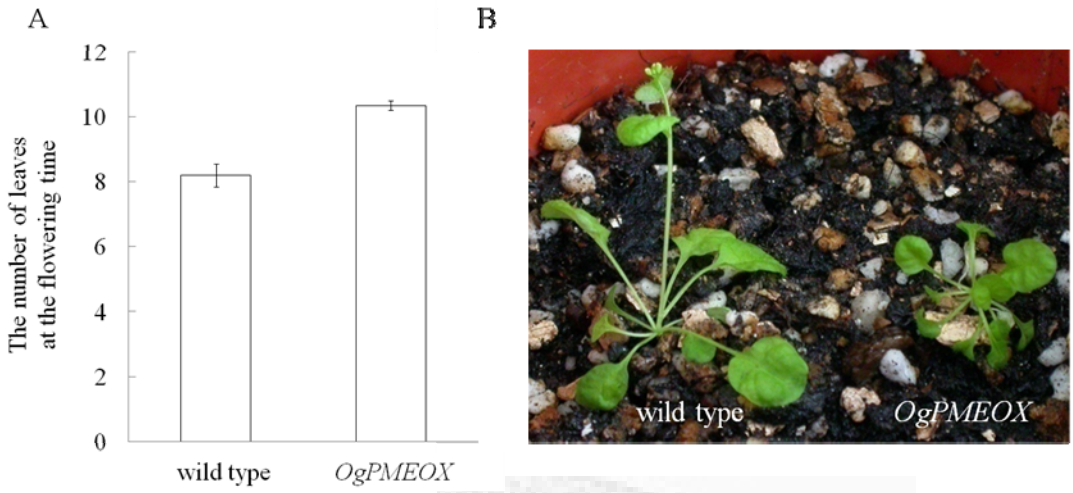


Fig. 8. Delayed flowering phenotype of transgenic *Arabidopsis* overexpressing *OgPME*

A. Number of rosette leaves at flowering time in *OgPMEOX* (10±0.14 leaves) and wild-type plants (8±0.34 leaves). (n_{wt}= 30, $n_{OgPMEOX}$= 36).

B. Phenotype of *OgPMEOX* plant and wild type grown under 23±2°C and long-day photoperiod (16h light and 8h dark).

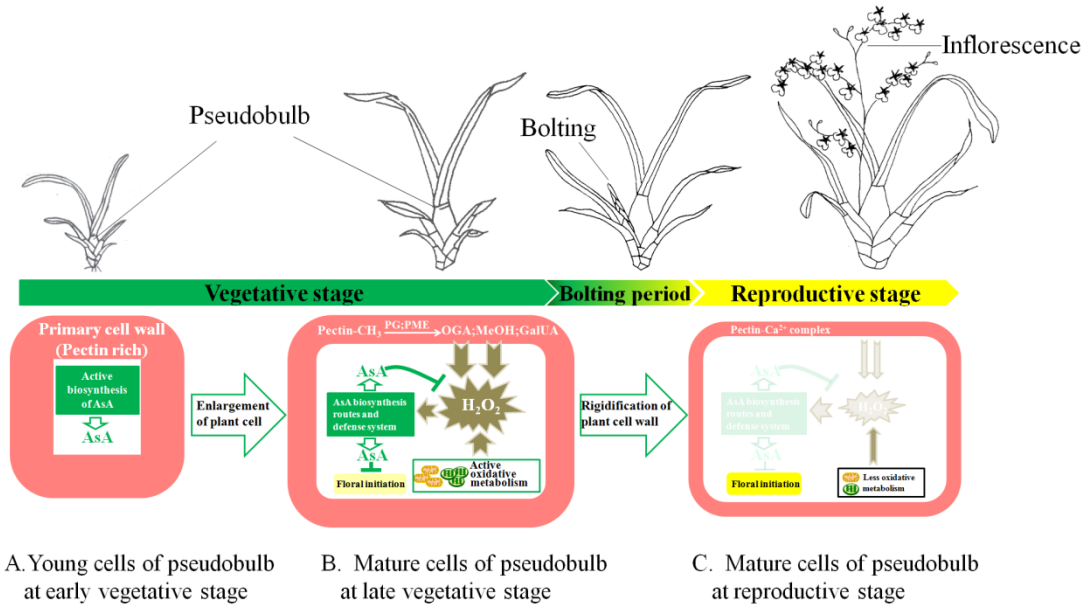


Fig. 9. Schematic representation of ascorbate homeostasis in pseudobulb cell of *Oncidium* orchid in three developmental stages.

A. At early vegetative growth stage, young pseudobulb cells are sink for nutrition storage transported from photosynthetic leaves. Copious pectin and ascorbate are actively biosynthesized in pseudobulb cells.

B. At late vegetative growth stage, pectin is being demethylated by pectin methylesterase (PME) and polygalacturonase (PG) in cell wall enlargement process. Methanol, OGA and GalUA are generated from pectin modification in cell wall. Ascorbate level is not only induced by ROS network, such as methanol oxidation and NADPH oxidase, but also functions to eliminate the oxidative toxicity during the whole vegetative stage. The elevated ascorbate level consequently represses the floral initiation.

C. At bolting period, methanol and OGA are largely decreased due to the outcome of pectin demethylation and less pectin content in cell wall. The effect of ROS-induced ascorbate level is mitigated. The decreasing ascorbate level thus triggers the expression of floral genes for phase transition.

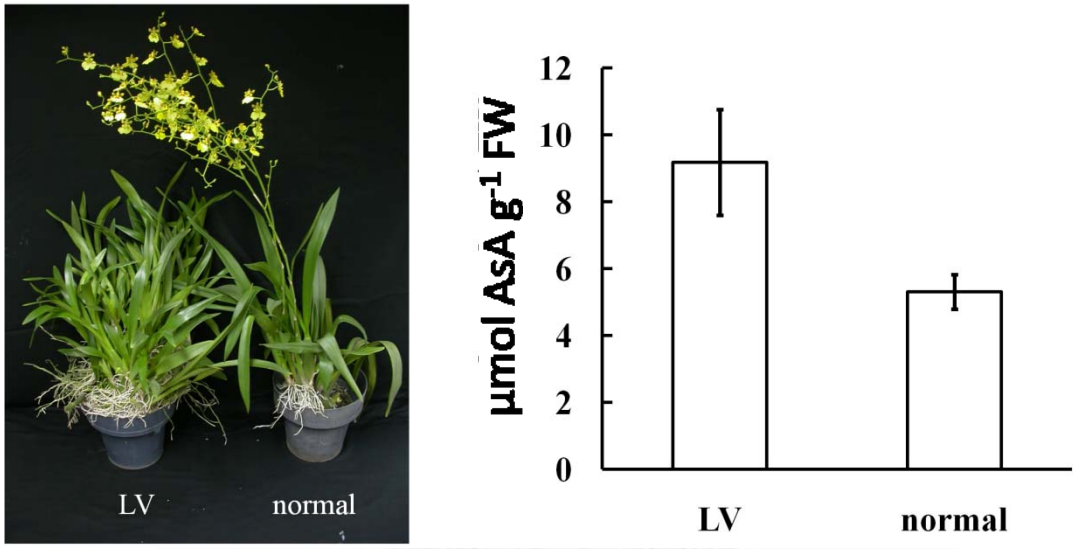


Fig. 10. An *Oncidium* cultivar with long vegetative phase (LV) is caused by the higher endogenous ascorbate concentration.

A. An *Oncidium* cultivar variety (left, LV) is unable to bolt and abolishes the phase transition.

B. LV cultivar contains higher endogenous ascorbate concentration (9~10 μmol g^{-1} FW) two fold to the normal *Oncidium* plants (3~4 μmol g^{-1} FW)

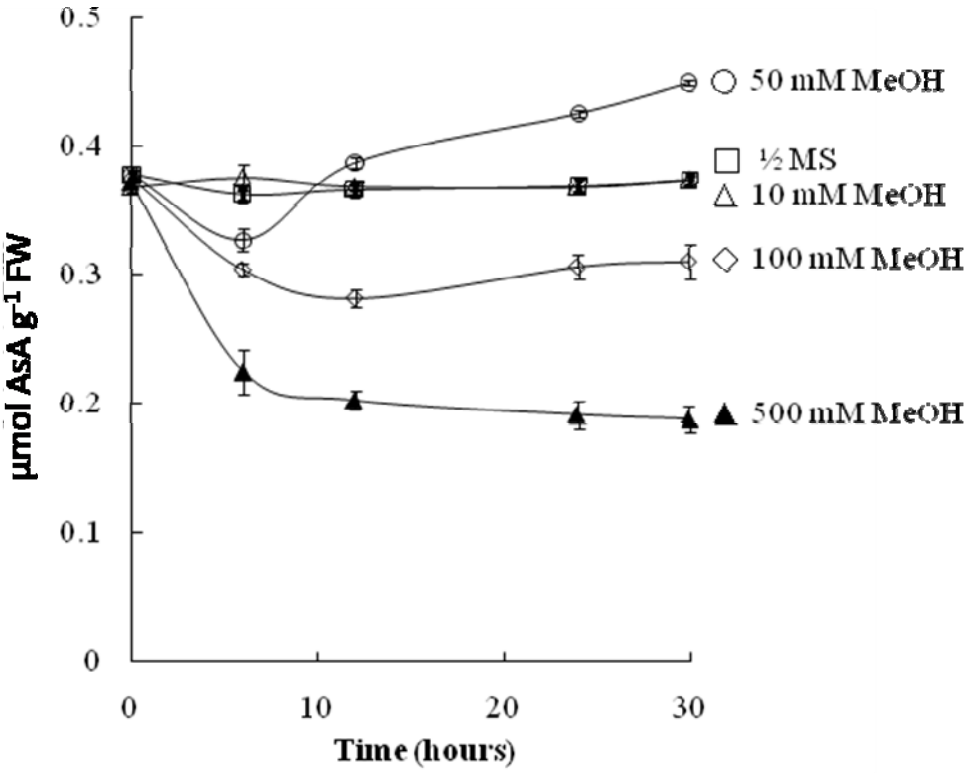


Fig. 11. Effect of methanol (MeOH) doses on ascorbate (AsA) level in *Oncidium* protocorm-like body (PLB) cultures. *Oncidium* PLB cultures were incubated with 500 mM (▲), 100 mM (◇), 50 mM (○), and 10 mM (□) MeOH and ½ MS medium as a control (△) for 30 h. Vertical bars represent standard deviation of the mean obtained from three independent experiments.

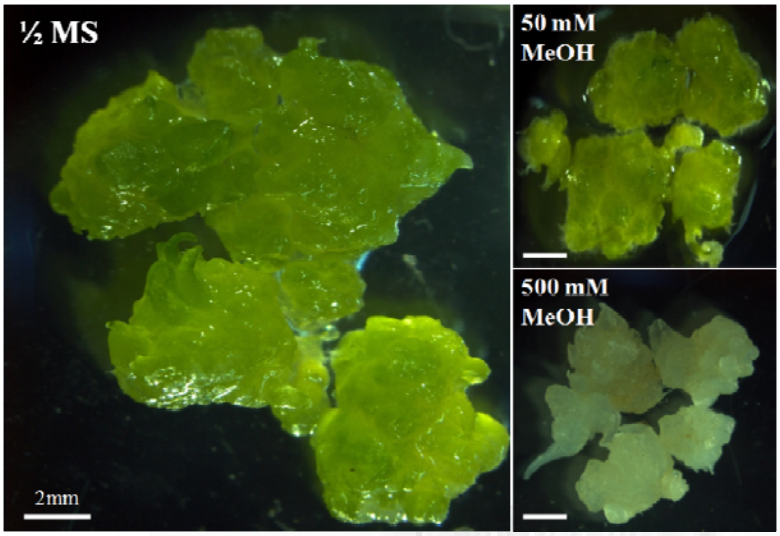


Fig. 12. The effect of MeOH on the PLB culture is associated with MeOH dosage, with 500 mM MeOH being lethal to *Oncidium* PLB culture, and 50 mM has no effects. All *Oncidium* PLB cultures were photographed after 12 h treatment.

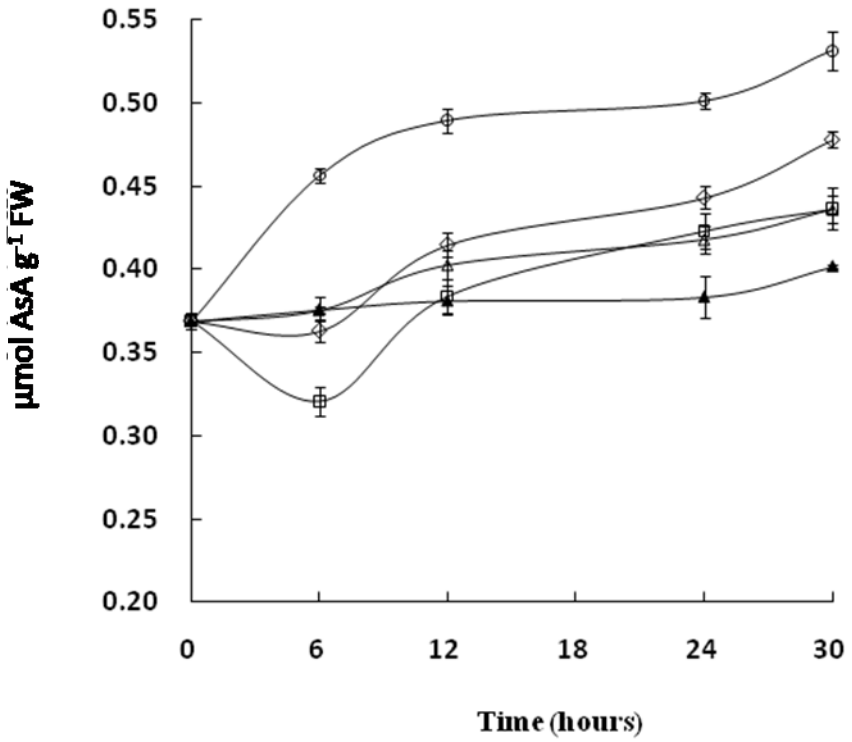


Fig. 13. The AsA level in *Oncidium* PLB cultures incubated with various compounds for 30 h. Vertical bars represent standard deviations of the means obtained from three independent experiments. 50 mM MeOH (□), 50 mM D-galacturonate (D-GalUA) (◇), 50 mM MeOH and 50 mM D-GalUA (△), 50 mM L-galactose (L-Gal) (○) and ½ MS (▲).

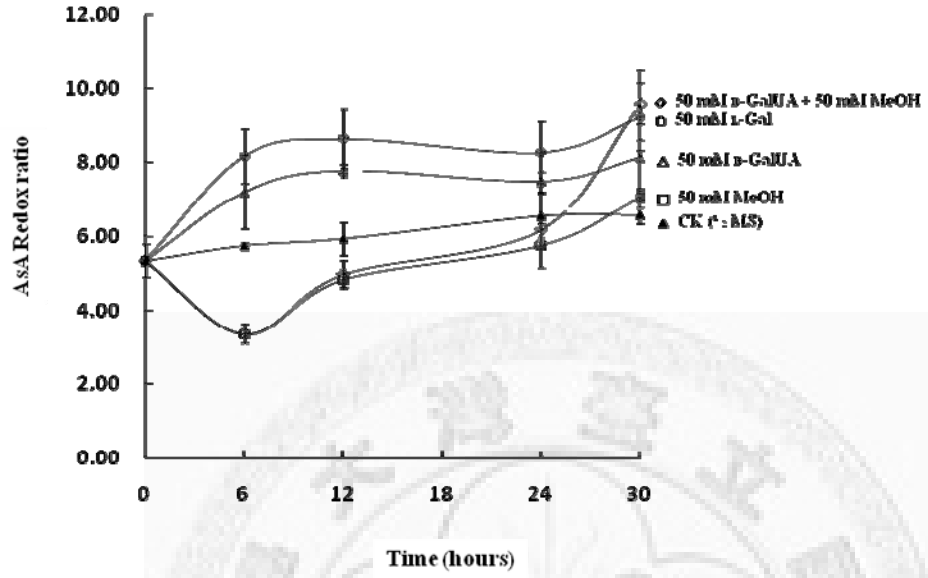


Fig. 14. The redox state in *Oncidium* PLB cultures incubated with various compounds for 30 h. Vertical bars represent standard deviations of the means obtained from three independent experiments. 50 mM MeOH (□), 50 mM D-galacturonate (D-GalUA) (◇), 50 mM MeOH and 50 mM D-GalUA (△), 50 mM L-galactose (L-Gal) (○) and ½ MS (▲).

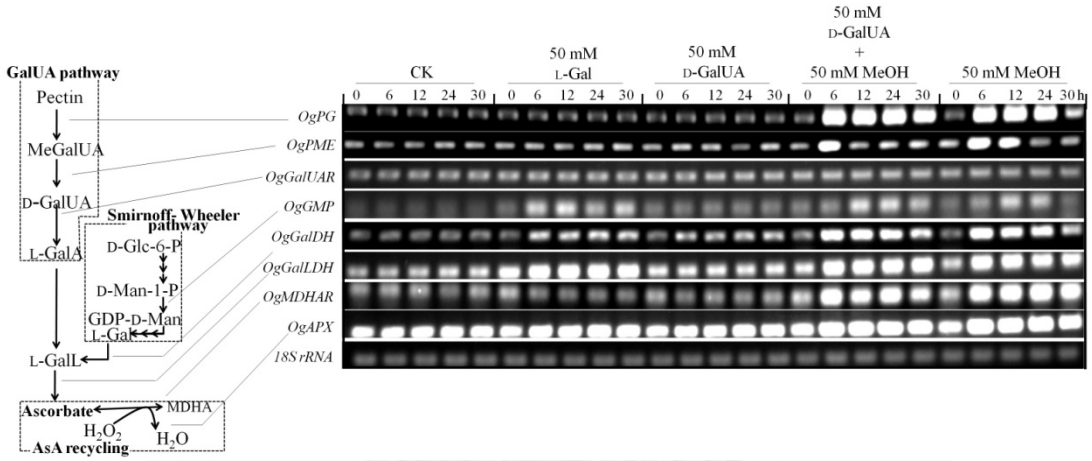


Fig. 15. Expression of AsA-related genes on treatment of *Oncidium* PLB cultures with various compounds. The relative amount of transcripts of *OgPME, OgPG* and *OgGalUAR* in the GalUA pathway; *OgGMP*, *OgGalDH* and *OgGalLDH* in the Smirnoff-Wheeler pathway and *OgAPX* and *OgMDHAR* in the defense system were determined by RT-PCR.

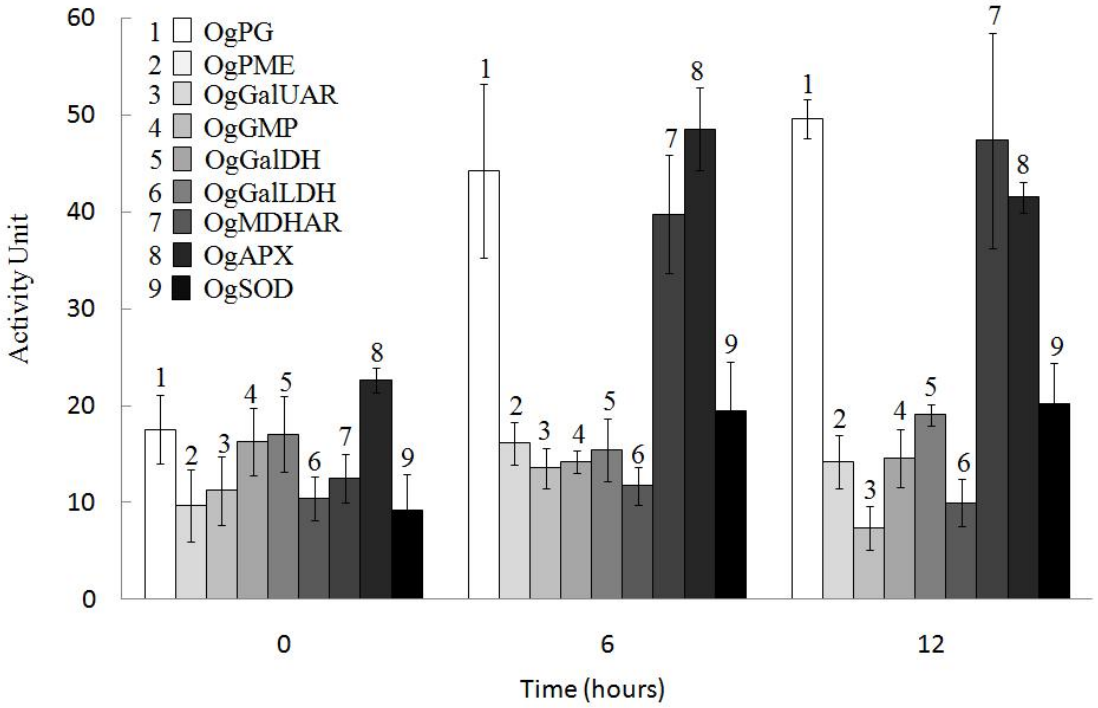


Fig. 16. Activity assays of AsA-related enzymes under 50 mM MeOH treatments. Activity units of AsA-related enzymes were defined in Materials and Methods, and vertical bars represent standard deviations of the means obtained from three independent experiments.

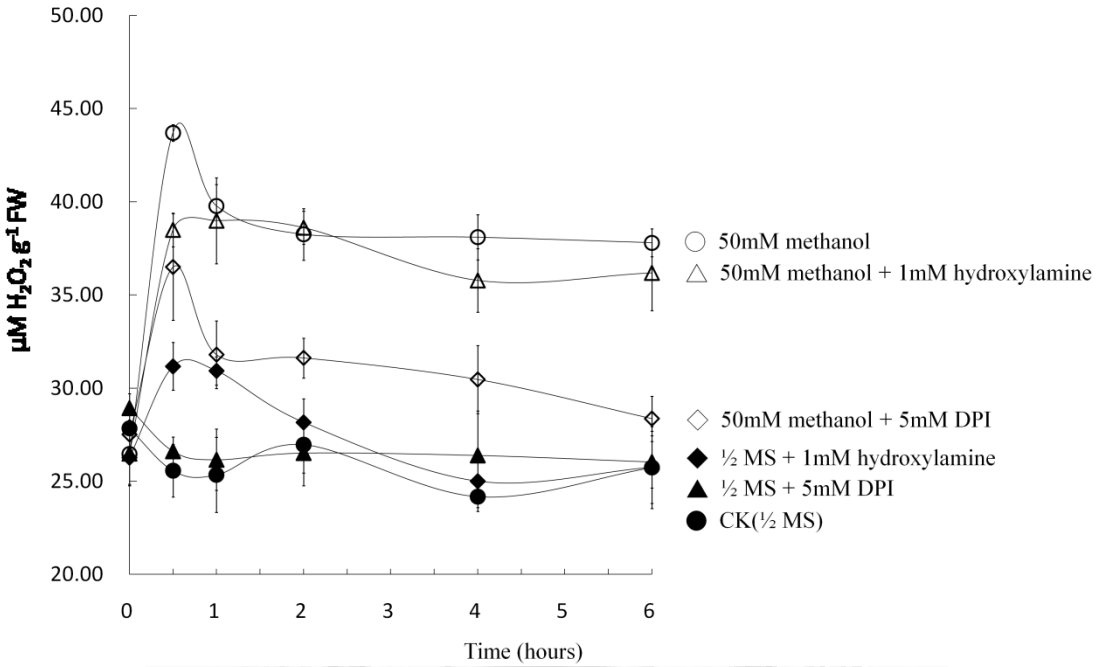


Fig. 17. The effects of hydroxylamine (inhibitor of alcohol oxidase) and diphenyleneiodonium chloride (DPI; inhibitor of NADPH oxidase) on H_2O_2 production in *Oncidium* PLB cultures for 6 h. Vertical bars represent standard deviations of the means obtained from three independent experiments.

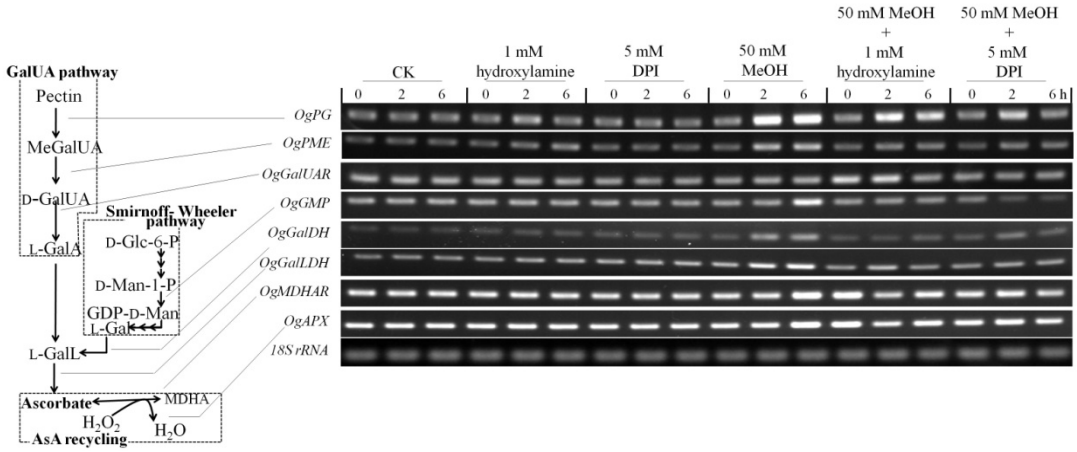


Fig. 18. Expression of AsA-related genes on treatment with H_2O_2-producing inhibitors. Total RNA was isolated from PLBs incubated with hydroxylamine or DPI. The relative amount of transcripts for *OgPME, OgPG* and *OgGalUAR* in the GalUA pathway; *OgGMP*, *OgGalDH* and *OgGalLDH* in the Smirnoff-Wheeler pathway; and *OgAPX* and *OgMDHAR* in the defense system were determined by RT-PCR**.**

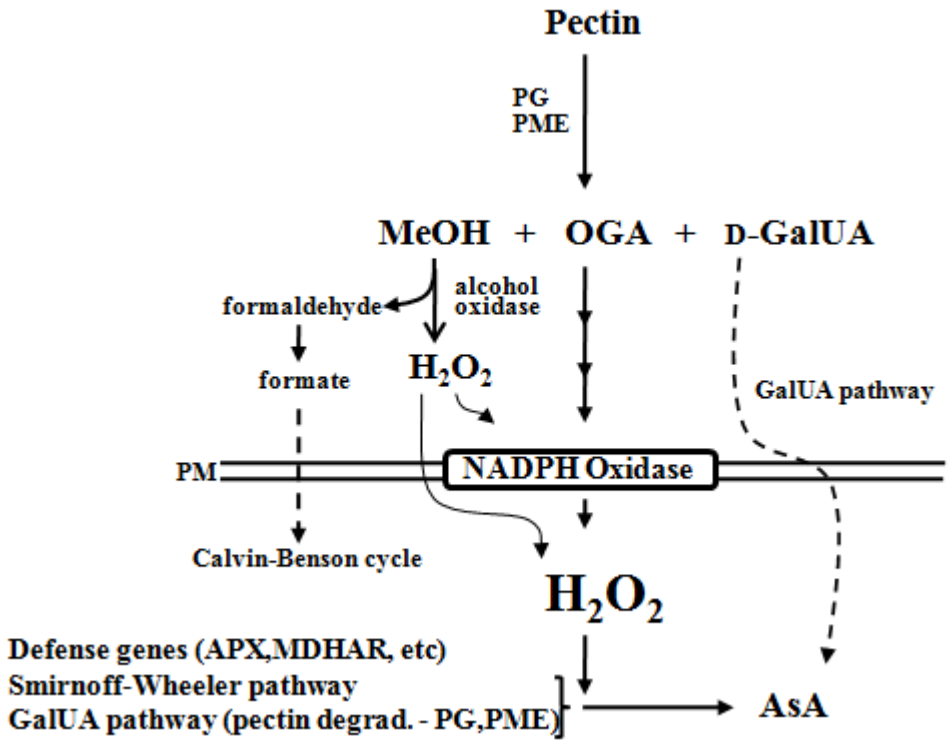
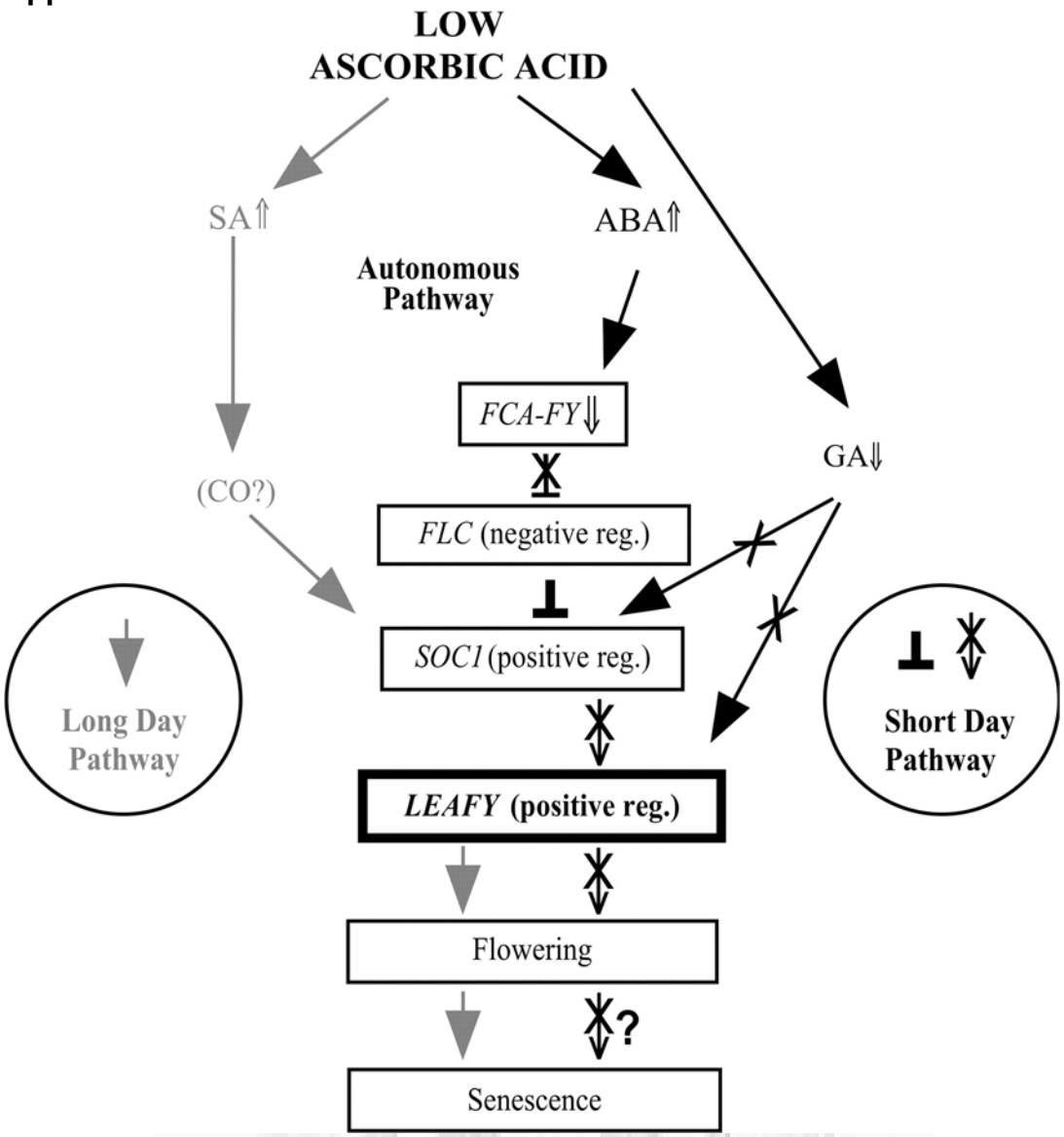
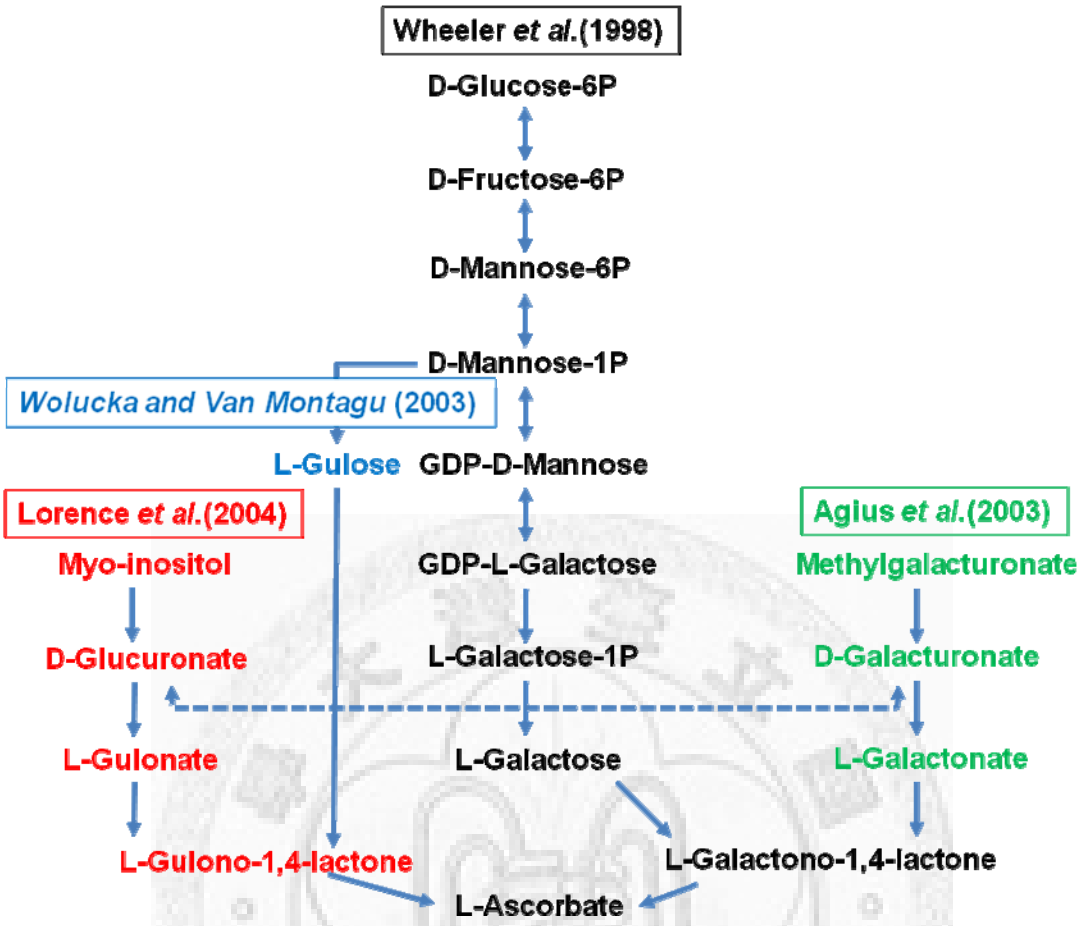


Fig. 19. The proposed model of the H_2O_2-signaling network under MeOH stimulation in *Oncidium* PLB cultures. Methanol is produced along with oligogalacturonic acid (OGA) and D-GalUA during the degradation of pectin in the plant cell wall. Methanol is preferentially oxidized (detoxified) by alcohol oxidase to H_2O_2 and formaldehyde. Subsequently, H_2O_2 activates NADPH oxidase to create more H_2O_2, which acts as secondary messenger to induce the expression of AsA-related biosynthetic genes. In addition, OGA also enhances H_2O_2 production. D-GalUA might be a precursor for AsA synthesis in the GalUA pathway. The products of pectin degradation involved in H_2O_2 signal transduction could function in elevating AsA levels in cells. A high AsA level could scavenge reactive oxygen species and protect the cell from stresses. APX= ascorbate peroxidase; MDHAR= monodehydroascorbate reductase.

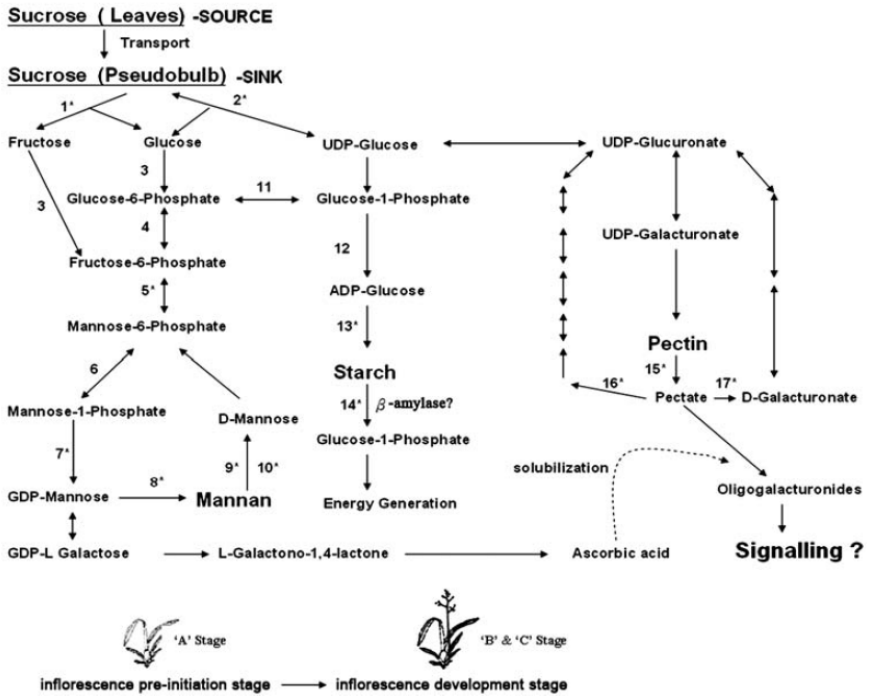
Appendixes:



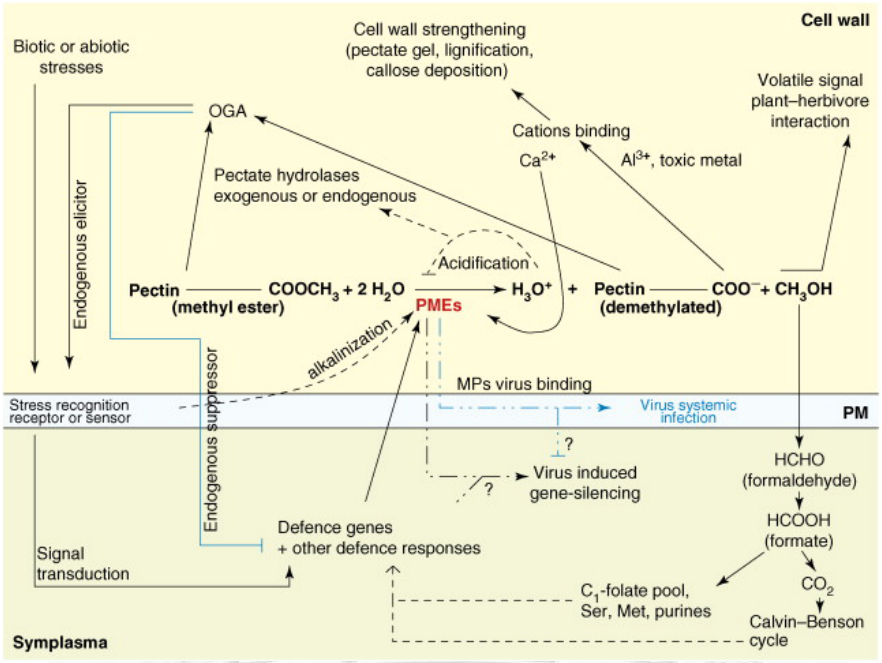
Appendix 1. A simplified diagram illustrating the hypothetical effects of low levels of ascorbic acid in regulating flowering time and senescence via three of the major genetic pathways of flowering in Arabidopsis. (Reproduced from Barth et al., 2006)



Appendix 2. Four potential branch pathways operating in plants, the Smirnoff-Wheeler (mannose) pathway, galacturonate pathway, the gulose pathway, and the *myo*-inositol pathway. (Adapted from Agius et al., 2003)



Appendix 3. Carbohydrate metabolic pathway in the pseudobulb of *Oncidium* during the flowering process. (Reproduced from Wang et al., 2008)



Appendix 4. The production and functional roles of methanol in plant cell. (Reproduced from Pelloux et al., 2007)

Abbreviation:

A_{525}	Absorbance at 525nm
ADP	Adenosine diphosphate phosphate
ADP	Adenosine diphosphate phosphate
AIR	Alcohol-insoluble residue
AO	Ascorbate oxidase
APS	Ammonium persulfate
APX	Ascorbate peroxidase
AsA	Ascorbate
B	Bolting period
BSA	Bovine Serum Albumin
CaMV	Cauliflower mosaic virus
Col.	Columbia
CTAB	Hexadecyltrimethylammonium bromide
DAB	3,3-Diaminobenzidene
DAS	Days after sowing
ddH_2O	Distilled and deionized water
DEPC	Diethypyrocarbonate
DHA	Dehydroascorbate
DMSO	Dimethylsulfoxide
DPI	Diphenyleneiodonium chloride
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
FCR	Folin-Gocalteu phenol reagent
FW	Fresh weight
G6PDH	Glucose-6-phosphate dehydrogenase
GalDH	L-galactose dehydrogenase
GalL	L-galactono-1,4-lactone
GalLDH	GalL dehydrogenase
GalUA	D-galacturonate
GalUAR	galacturonate reductase
GMP	GDP-D-mannose pyrophosphorylase
GMP	Guanosine 5-monophosphate
GTP	Guanosine 5-triphosphate
H_2O_2	Hydrogen peroxide

	Monodehydroascorbate reductase
HA	Hydroxylamine
MDHAR	Monodehydroascorbate reductase
MeGalUA	Methyl-galacturonate
MeOH	Methanol
MOPS	3-(N-morpholino)propanesulfonic acid
MOPS	3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
OGA	Oligogalacturonic acid
P/C/I	Phenol/chloroform/isoamyl alcohol
PG	Polygalacturonase
PLB	Protocorm-like body
PME	Pectin methylesterase
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
PVPP	Polyvinylpolypyrrolidone
R	Reproductive stage
RACE	Rapid amplification of cDNA ends
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SOD	Superoxidase dismutase
TCA	Trichloroacetic acid
Tea-Dea	Triethanolamine-diethanolamine
V	Vegetative stage