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Effects of Benzyladenine and Abscisic Acid on the Disassembly Process of Photosystems in an *Arabidopsis* Delayed-Senescence Mutant, *ore9*

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Using wild-type (WT) leaves and those from an *ore9* delayed-senescence *Arabidopsis* mutant, we investigated the delaying and accelerating effects of benzyladenine (BA) and abscisic acid (ABA), respectively, on the degradation process of the photosynthetic apparatus during dark-induced senescence (DIS). In the mutant, delays were seen for both the breakdown of chlorophyll (Chl) and the decrease in photochemical efficiency of photosystem II (Fv/Fm). Moreover, each step was prolonged in the disassembly process of the Chl-protein complexes. In the presence of BA, Chl degradation was retarded to a similar extent for both the mutant and the WT, but the decrease in Fv/Fm was not. However, in the presence of ABA, the two processes were accelerated in both genotypes. Therefore, although the *ore9* mutation causes this functional delayed-senescence, it may not be related to the non-functional delay that happens afterwards. In contrast, BA seems to affect both processes.

Keywords: abscisic acid, *Arabidopsis*, benzyladenine, dark-induced senescence, photosynthesis, stay-green mutant

Leaf senescence, an important stage in the plant life cycle, follows a characteristic metabolic pathway that elicits a change in structure before cell death (Nooden, 1988). It has been assumed that this process is internally programmed because its appearance is so specific and orderly in terms of its occurrence (Ronning et al., 1991; Thomas et al., 1992).

The progression of leaf senescence is commonly and easily monitored by measuring the loss of photosynthetic pigments, particularly chlorophyll (Chl) (Thimann, 1980; Gepstein, 1988). Changes in the CO_2-fixation rate or other indicators of total photosynthesis often roughly parallel this decline in Chl (Thimann, 1980; Gepstein, 1988; Bachmann et al., 1994; Wingler et al., 1998). However, the existence of 'stay-green' mutants of soybean (Kahanak et al., 1978) and meadow fescue grass (Thomas and Stoddart, 1975) raises questions about the suitability of Chl loss serving as an index of senescence. Therefore, other parameters for cellular metabolic processes are being examined. For example, the maximum photosynthetic activity of photosystem II (PSII), Fv/Fm, has been shown to be an efficient measure of the functional aspect of the photosynthetic apparatus in a 'non-functional stay-green' mutant of *Arabidopsis* (Oh et al., 2000).

Mutants with delayed-senescence phenotypes are essential tools for studying the regulatory mechanisms of leaf senescence (Thomas and Smart, 1993; Bachmann et al., 1994; Nooden and Guiamet, 1996; Nam, 1997; Oh et al., 1997). For example, the well-known stay-green grass mutant, *Bf993*, of *Festuca pratensis* possesses a lesion at the ring-opening step in the Chl degradation pathway. The *Sid* gene of *Bf993* regulates degradation of the thylakoids during senescence (Thomas, 1977; Harwood et al., 1982; Hilditch et al., 1989; Vincentini et al., 1995; Matile et al., 1996). A cytoplasmic gene, *cytG*, in soybeans also causes a stay-green phenotype, rendering the Chl b of senescing leaves more than Chl a (Guiamet et al., 1991). Consistent with the differential retention of those two pigment types, stability of the light-harvesting complex II (LHCII) is greatly increased. In fact, the role of *cytG* contrasts with that of *Bf993* in stabilizing only the LHC rather than the porphyrin-binding pro-

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Abbreviations: ABA, abscisic acid; BA, benzyladenine; Chl, chlorophyll; DIS, dark-induced senescence; LHCII, light-harvesting complex II; WT, wild-type

teins throughout the membrane.

Some *Arabidopsis* mutants also manifest delayed leaf senescence (Oh et al., 2000; Woo et al., 2001), including *ore1*, *ore3*, and *ore9*, which are differently regulated in the senescence process by their corresponding recessive nuclear gene (Oh et al., 1996, 1997). The *ore9* mutant delays the onset of declines in Chl content and Fv/Fm during natural- and hormone-induced senescence. Its protein, ORE9, contains an F-box motif, which is a component of the ubiquitin E3 ligase complex, thereby extending the longevity of leaves (Woo et al., 2001). In contrast to this functional stay-green mutant, the *ore10* and *ore11* genes are characterized by their induction of non-functional stay-green properties (Oh et al., 2000).

Although leaf senescence occurs in an age-dependent manner, its initiation and progression can be modulated by a variety of external factors, e.g., temperature, mineral deficiency, drought, or pathogen infection, as well as by internal factors, such as plant growth regulators (Thomas and Stoddart, 1980; Smart, 1994). Cytokinin is a phytohormone known to delay senescence symptoms (Richmond and Lang, 1957; Leshem, 1984; Singh et al., 1992; Badenoch-Jones et al., 1996). In contrast, a wide range of species is sensitive to senescence-promoters, such as ABA and methyl jasmonate (Smart, 1994). Ethylene is also a potent senescence-promoter in both the flowers and leaves of many dicots (Hall, 1977; Oh et al., 1997).

Here, we examined how ABA and BA might regulate degradation of the photosynthetic apparatus during dark-induced senescence (DIS) in *ore9*, an *Arabidopsis* functional delayed-senescence mutant. Using several *in vivo* and *in vitro* techniques for monitoring intracellular changes, we divided the entire DIS process into distinguishable steps so that we could determine how those phytohormones affected the initiation and duration of each specific stage of DIS.

MATERIALS AND METHODS

Plant Materials

Seeds of the *Arabidopsis ore9* mutant and a wild-type (WT) were sown in a 1:1:1 mixture of vermiculite:peat moss:perlite and reared in a growth chamber with a 16-h photoperiod and a day/night temperature cycle of 22°C/18°C.

For our phytohormone treatments, leaves were excised at about 21 d-old seedlings just prior to bolting. The tissues were floated on a 3 mM MES buffer solution (pH 5.8), with or without the addition of 5 μM BA or 100 μM ABA (Sigma-Aldrich, USA). All treatments were performed at 22°C under continuous darkness.

Measurements of Chl Content and Photochemical Efficiency of PSII

Leaves were ground in 2 ml of 80% acetone with a glass homogenizer. Their Chl content was calculated on a tissue fresh-weight basis, according to the method of Arnon (1949). Chl fluorescence was measured with a PAM-2000 fluorometer (Walz, Germany) after dark-adaptation for 10 min at room temperature (RT). Minimal fluorescence (Fo), with all open PSII reaction centers, was obtained by measuring the amount of modulated light sufficiently low enough (<0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. Maximal fluorescence (Fm), with all closed PSII reaction centers, was determined by applying a 0.8-s saturating pulse at 3200 μmol m^{-2} s^{-1} to dark-adapted leaves. Variable fluorescence (Fv) was equal to Fm minus Fo; hence, the photochemical efficiency of PSII was defined as Fv/Fm (Kitajima and Butler, 1975).

Preparation of Thylakoid Membranes

Thylakoid membranes were prepared as described by Aro et al. (1993), with some modifications. Detached leaves were ground with a glass homogenizer in ice-cold grinding buffer [50 mM Hepes (pH 7.6), 0.3 M sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$]. The homogenates were filtered through two layers of Miracloth (Calbiochem, USA) and micro-centrifuged (Micro 17R; Hanil, Korea) at 20,000g for 7 min. The pellet was then washed twice and re-suspended in a buffer of 50 mM Hepes (pH 7.6), 0.1 M sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$. The suspension was kept on ice in the dark after Chl concentrations were measured.

Native Green Gel Analysis of Chl-Protein Complexes

Native Chl-protein complexes were separated as described by Allen and Staehelin (1991). The resolving gel contained 25 mM Tris-HCl (pH 8.3), 50 mM glycine, and 10% glycerol, while the stacking gel consisted of 25 mM Tris-HCl (pH 6.3), 50 mM glycine, and 10% glycerol. The electrode buffer contained 25

mM Tris, 192 mM glycine (pH 8.3), and 0.1% SDS. An 8% polyacrylamide gel was used, with a 100:1 ratio of acrylamide to bisacrylamide. Polymerization was initiated at RT by the addition of 0.1% ammonium persulfate and 0.05% TEMED (*NNN'N'*-tetramethylethylenediamine).

After the isolated thylakoid membrane suspension was centrifuged, the pellet was washed twice in 2 mM Tris-maleate (pH 7.0) and re-suspended in a solubilization buffer [0.45% octyl glucoside, 0.45% decyl maltoside, 0.1% lithium dodecyl sulfate, 10% glycerol, and 2 mM Tris-maleate (pH 7.0)] to yield a 20:1 ratio (w/w) of total nonionic detergents to Chl. Samples were kept on ice for 30 min, and insoluble materials were removed by centrifugation. The Chl concentration of the remaining suspension was measured, and an aliquot of the solubilized thylakoid membranes containing 13.5 μg of Chl per lane was loaded on a 1.5-mm-thick gel. After the samples were electrophoresed at 4°C and a constant 6 mA, the green gel was photographed under visible light.

RESULTS

Changes in Chl Content and Photochemical Efficiency of PSII, Fv/Fm, during Dark-Induced Senescence (DIS)

When detached leaves of the wild-type (WT) *Arabidopsis* were incubated in MES buffer (pH 5.6) under continuous darkness for 5d, most turned completely yellow. In contrast, those of the *ore9* mutant reached a similar state at about Day 10 after the onset of DIS, as quantified by Chl content (Fig. 1A, 2A). To compare the changes in Chl concentration with those associated with functioning of the photosynthetic apparatus, we simultaneously monitored Fv/Fm, the photochemical efficiency of PSII (Fig. 1B, 2B).

In the WT, Chl content began to decline at 2 d after the onset of DIS; by Day 3, the level was down to approximately 50% of the original, and only 10% by Day 5. However, Fv/Fm did not decrease until Day 3. Even at 4 d, Fv/Fm values were maintained at 85% of the starting level. However, photosynthetic efficiency steeply decreased to about 0.2 by Day 5. In contrast, these decreases in Chl content and Fv/Fm were deferred in the delayed-senescence mutant, only beginning to show significant changes at Days 3 and 6, respectively (Fig. 2).

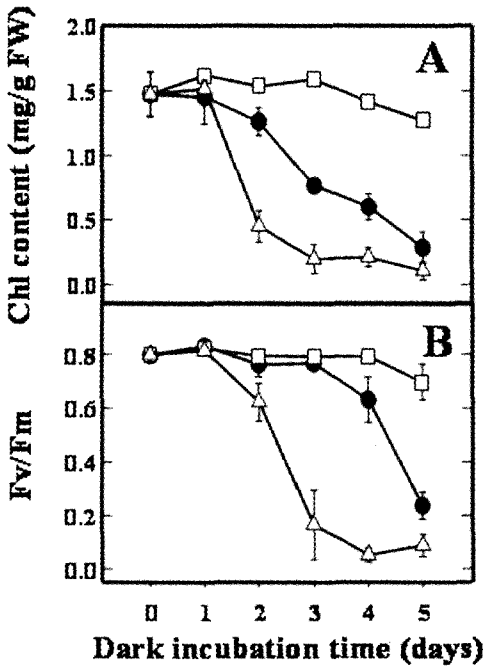

Figure 1. Effects of BA and ABA on changes in Chl content **(A)** and Fv/Fm **(B)** during dark-induced senescence (DIS) in *Arabidopsis* wild-type leaves. Fresh weight (FW) was determined before start of DIS. Fv/Fm during DIS was measured without dark incubation; Fv/Fm at Time 0 was measured after 10-min dark-adaptation period. ●, MES buffer (pH 5.6); □, 5 μM BA; Δ, 100 μM ABA.

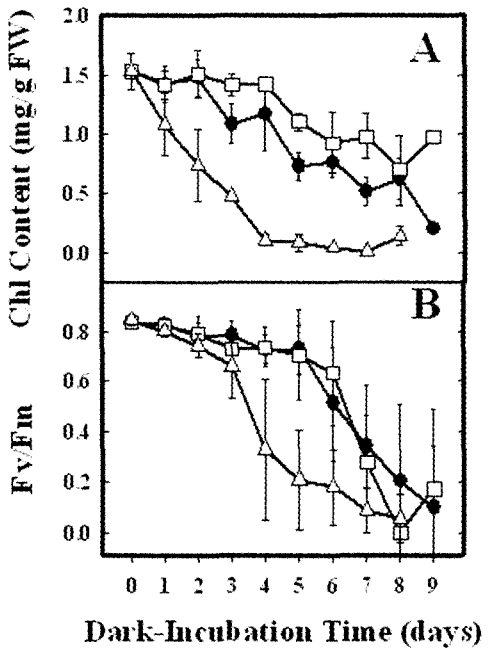

Figure 2. Effects of BA and ABA on changes in Chl content **(A)** and Fv/Fm **(B)** during dark-induced senescence in *Arabidopsis* senescence-delayed mutant *ore9*. Experimental conditions are same as described for Figure 1. ●, MES buffer (pH 5.6); □, 5 μM BA; Δ, 100 μM ABA.

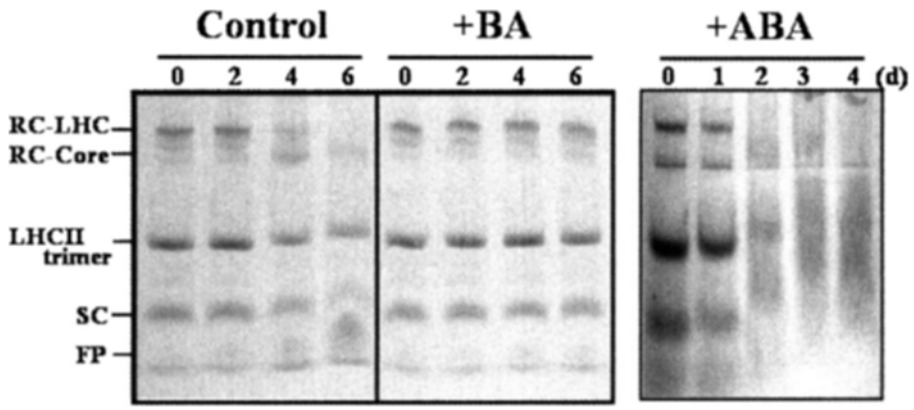

Figure 3. Fractionation of thylakoid pigment-protein complexes in wild-type leaves of *Arabidopsis* during dark-induced senescence. In each lane, 30 μl of solubilized thylakoids (13.5 μg of Chl) was loaded. Band names are marked according to Allen and Staehelin (1991). 'RC-LHC' represents a number of large PSI and PSII complexes with attached antennas; 'RC-Core', some partial PSI and PSII complexes that were largely stripped of antennas; 'LHCII trimer', light-harvesting complex II trimer; 'SC', small complex; 'FP', free pigment. Leaves were incubated either in MES buffer (pH 5.6) (Control) or in presence of 5 μM BA (+BA) or 100 μM ABA (+ABA).

Effects of BA and ABA on Changes in Chl Content and Fv/Fm during DIS

BA and ABA are senescence-retarding and senescence-promoting agents, respectively. As expected, both Chl degradation and decreased Fv/Fm were retarded when WT leaves were treated with 5 mM BA (Fig. 1). In fact, at 5 d after DIS, both parameters were at nearly the same levels as those measured from the non-senesced control leaves. In contrast, ABA accelerated the decline in both Chl and Fv/Fm, beginning at Day 2. Interestingly, BA did not retard the decrease in Fv/Fm in the *ore9* mutant, but did delay Chl degradation (Fig. 2). However, this mutant did show an ABA-induced acceleration of decreases in Chl content and Fv/Fm, with values of the latter starting to drop significantly after Day 3 (Fig. 2B).

Changes in the Composition of Chl-Protein Complexes during DIS, and Effects of BA and ABA

To determine whether changes in PSII functioning are correlated with alterations in the structure of the photosynthetic apparatus, we investigated fluctuations in the Chl-protein complexes during DIS (Fig. 3, 4). After solubilization in a detergent system consisting of octyl glucoside, decyl maltoside, and lithium dodecyl sulfate, those complexes were resolved and identified on a green gel. Their degradation pattern in WT leaves (Fig. 3) was similar to what we had reported previously (Oh and Lee, 1996). Briefly, during DIS, the LHCII-less core complexes that disassembled from the RC-LHC region were accumulated in the RC-Core region. In contrast, the LHCII trimers released from the RC-LHC and/or the free LHCII trimers decreased without accumulation, decomposing into free Chl and polypeptides. Therefore, this degradation phenomenon seemed to be sequential, being more noticeable at Day 4, before the leaves then turned completely yellow after 5 to 6 d of DIS. The steps in degradation in the *ore9* mutant proceeded in a similar fashion, although each was markedly delayed compared with the WT (Fig. 4). The release of LHCII from the RC-LHC that was initiated at Day 4 in the WT was not observed until Day 6 in the mutant. Similarly, the RC-LHC disappeared almost completely at Day 10 in the mutant, compared with its occurrence at Day 6 in the WT.

In the presence of BA, almost no alterations in the Chl-protein complexes were observed during DIS of the WT for 6 d, except for some minor changes in RC-LHC and RC-Core (Fig. 3). Interestingly, this stabilizing effect of BA on the Chl-protein complexes in those regions was not present in the *ore9* leaves (Fig. 3). Instead, only LHCII remained as a very stable form until 10 d of DIS (Fig. 4). In contrast, ABA treatment helped to initiate degradation in the Chl-protein complexes at Day 1 for the WT leaves (Fig. 3), but not until Day 4 for the mutant tissues (Fig. 4).

DISCUSSION

Degradation of Chl and Loss of PSII Activity during DIS

The kinetics of Chl degradation in WT leaves during

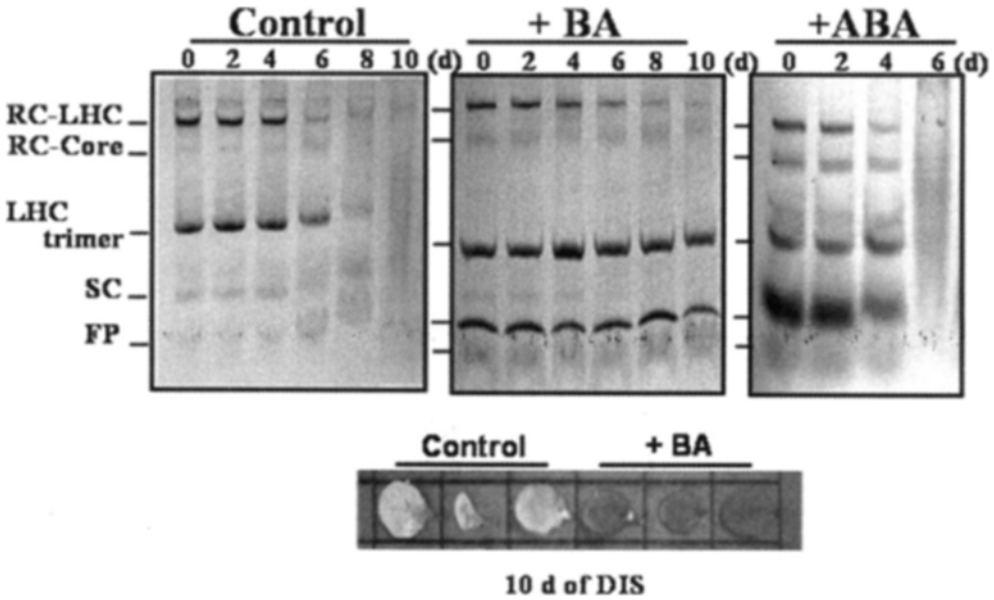

Figure 4. Fractionation of thylakoid pigment-protein complexes in *ore9* mutant leaves of *Arabidopsis* during dark-induced senescence. Details are same as described for Figure 3. Leaves were incubated either in MES buffer (pH 5.6) (Control) or in presence of 5 μM BA (+BA) or 100 μM ABA (+ABA). At bottom, pictures are of 10 d-senesced leaves in absence (Control) or presence of BA (+BA).

DIS differed from that of the loss in PSII function (Fig. 1), and the two processes seemed not to be directly correlated. In fact, although about 50% loss of Chl content was observed at Day 3 in the WT leaves, Fv/Fm values remained unchanged. Similar observations were made with the *ore9* mutant, albeit with a 2-d delay. Likewise, the intensity of the RC-LHC band on the green gel showed the same trend, with decreases being observed at Day 4 in the WT, and at Day 6 in the *ore9* leaves. This decline in Chl content without an accompanying shift in Fv/Fm has also been reported for young spinach plants grown in Mg- and S-deficient media (Dannehl et al., 1995). In our experiments, the rapid degradation of the photosynthetic apparatus without any loss of PSII activity resulted in greater D1 protein turnover because that rate of turnover was high enough to prevent any inhibition of PSII. However, because D1 protein turnover was not active under darkness, our data cannot account for this and further clarification will be needed.

Sequential Disassembly Process of Chl-Protein Complexes during DIS, and the Effects of BA

Previously, we reported that the disassembly of Chl-protein complexes proceeds sequentially (Oh and Lee, 1996). The PSII reaction center complexes are degraded earlier than the LHCII, whereas the PSI antenna complexes are degraded earlier than the PSI reaction center complexes, indicating that the loss of PSI antennas is regulated by an entirely different mechanism from that of PSII (Oh and Lee, 1996; Yamazaki et al., 2000; Guiamet et al., 2002). The RC-LHC region is divided into the RC-Core proteins and the LHCII trimers, in which the former are degraded during DIS, while the latter are first converted to LHCII monomers and then further dissociated into pigments and LHCII polypeptides (Oh and Lee, 1996).

In the *ore9* mutant, the intensity of the RC-LHC band started to decrease at Day 6 of DIS (Fig. 4). Simultaneously, the Fv/Fm values declined about 25% compared with the control (Fig. 2). This decrease in photosynthetic activities was due to the increase in Fo, which was pronounced after 6 d of DIS in the *ore9* mutant (data not shown). In the presence of BA, a relatively large amount of Chl still remained even after Fv/Fm had significantly decreased. This delay in Chl degradation in the BA-treated *ore9* leaves may have been due to the greater stability of LHCII during DIS (Fig. 4). We have previously reported that LHCII in *ore10*, a non-functional stay-green mutant, is stable during DIS, a response attributed to some defect in LHCII-associated proteolysis (Oh et al., 2000, 2003). In those *ore10* leaves, all the Chl-protein complexes except LHCII are degraded at the late stages of DIS.

Moreover, the excitation energy coupling between LHCII and PSII core complexes is blocked, resulting in a higher value for Fo (Oh et al., 2000). Interestingly, the delayed senescence shown in this current study in BA-treated *ore9* leaves was also non-functional.

Possible Modes of Action for BA and ABA in Relation to the Role of the ORE9 Product

Here, we have demonstrated that delayed senescence can be achieved by regulating the degradation of Chl and/or proteins in the Chl-protein complexes. Various phytohormones control leaf senescence, with each functioning at a different site during that process. Both the degradation of Chl and the decrease in Fv/Fm during DIS were effectively protected by BA in the WT, but only the former process could be delayed in the presence of BA in the *ore9* mutant. As we have suggested previously (Oh et al., 2004), the proteolysis of Chl-protein complexes occurs in two steps: initial degradation to render the photosynthetic apparatus non-functional and a secondary furthering of degradation. In the case of the non-functional stay-green mutant *ore10*, that second step seems to be blocked, and the remaining partially degraded Chl-protein complexes tend to form aggregates that protect their further degradation. However, this significant accumulation of aggregates was not observed in our current BA-treated *ore9* mutant leaves (data not shown). Therefore, the non-functional delayed senescence observed in our mutant leaves treated with BA is related to the stability of LHCII, but differs from that measured in *ore10* leaves.

Woo et al. (2001) have reported that the *ore9* mutation blocks ubiquitin-mediated proteolysis. ORE9 is a protein that contains an F-box motif, which, as a component of the ubiquitin E3 ligase complex, extends the longevity of leaves. Although the *ore9* mutation causes functional delayed senescence, it may not be related to the non-functional delay that happens afterward. BA seemed to be associated with both processes, but its action does not influence the effect of the ORE10 mutation. Our BA treatment of *ore10* leaves may have delayed this functional senescence process (data not shown).

ABA stimulated senescence in both WT and *ore9* leaves, with the mutant rate of senescence being roughly similar to that measured in the ABA-untreated WT leaves. When BA was co-treated with ABA during DIS of WT leaves, Chl degradation slowed down compared with leaves treated with ABA alone (data not shown). The modes of regulation for these phytohormones may not be simple. Some senescence-associated genes (SAGs) that are transcriptionally up-regulated in an age-dependent manner in senescing leaves are also regulated by environmental factors, including stress and nutrient supply (Gan and Amasino, 1997; Nam, 1997; Yoshida, 2001). Moreover, factors such as aging, darkness, ethylene, ABA, or methyl jasmonate seem to have different modes for regulating some SAGs (Park et al., 1998). For example, SAG12 encoding a cysteine protease is repressed when treated with cytokinin (Noh and Amasino, 1999). Therefore, the mechanisms of phytohormone regulation, with regard to the role of ORE9, may be complicated, and are probably superimposed with each other on the developmental age-dependent senescence process, as has been suggested by Nam (1997).

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