学位論文

Ethylene Inhibits Abscisic Acid - Induced Stomatal Closure in *Arabidopsis thaliana*

シロイヌナズナのアブシジン酸による気孔閉鎖に対する

エチレンの阻害作用

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東京大学大学院　新領域創成科学研究科　先端生命科学専攻

田中　洋子

Ethylene Inhibits Abscisic Acid - Induced Stomatal Closure in *Arabidopsis thaliana*

TANAKA Yoko

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Department of Integrated Bioscience,

Graduate School of Frontier Science,

The University of Tokyo

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Contents

Abbreviations

ABA : abscisic acid

ACC : 1- aminocyclopropane-1-carboxylic acid

ACS : ACC synthase

AVG : aminoethoxyvinyl glycine

BA 6- benzyl adenine

ein3-1 : *ethylene insensitive3-1*

eto1-1 : *ethylene over-producer1-1*

etr1-1 : *ethylene resistant1-1*

GCP guard cell protoplast

GFP : green fluorescent protein

1-MCP : 1- methylcyclopropene

MES : 2- morpholinoethanesulfonic acid, monohydrate

NAA : 1- naphtaleneacetic acid

PCR : polymerase chain reaction

WT : wild type

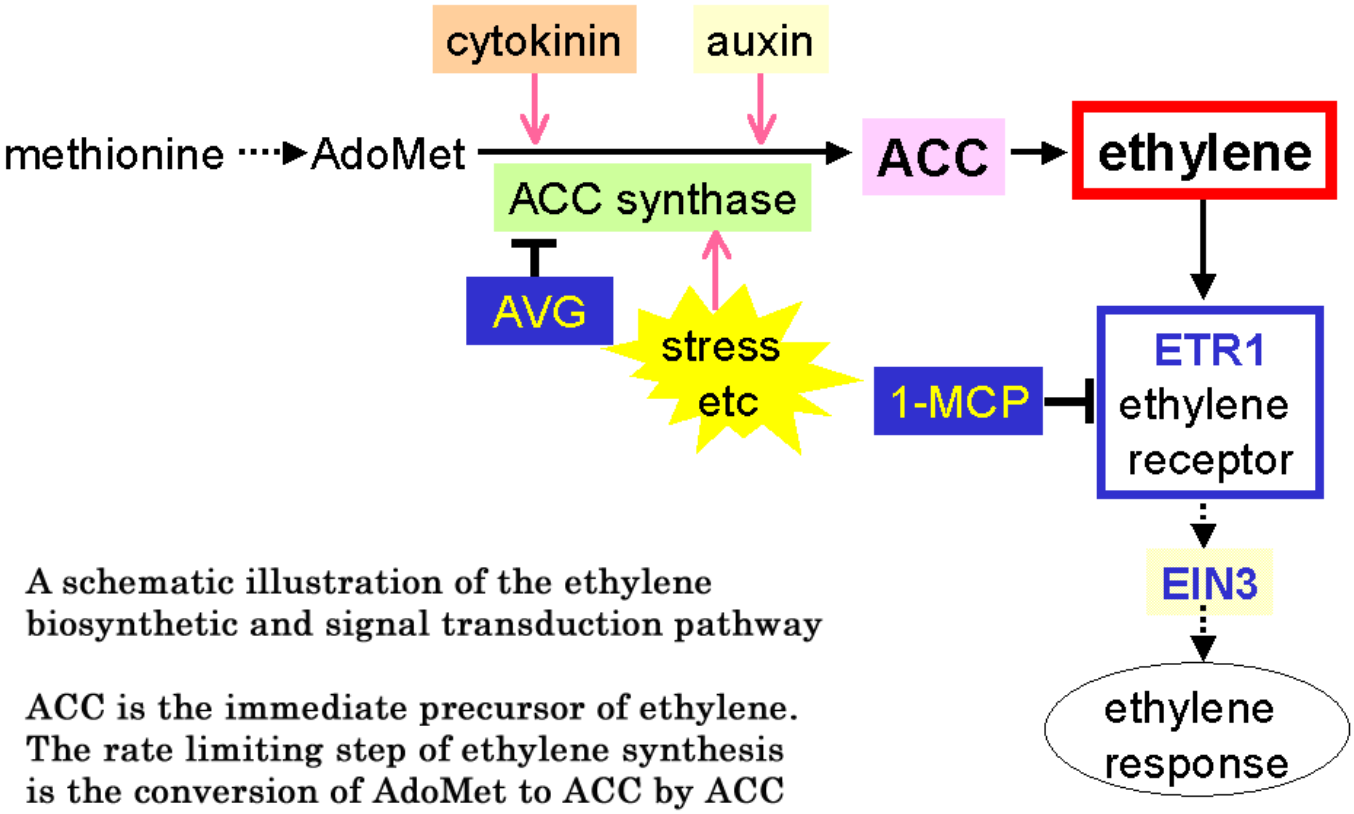
General Introduction

Stomata are pores located in the epidermis of the aerial parts of plants. These pores open and close by the shape change of the pairs of surrounding specialized cells, termed “guard cells”, Plants regulate the aperture of the stomatal pore in response to water stress and photosynthetic capacity. Since drought is a major environmental factor that influences plant growth, a lot of studies have been performed about the regulation of the stomatal opening and closing.

Stomata close in response to environmental signals such as light, carbon dioxide, and humidity. During this process, a phytohormone abscsic acid (ABA) plays a major role. Other than stomata closing, this phytohormone is involved in bud and seed dormancy, growth regulation, leaf senescence and abscission and a variety of plant stress responses. A rapid increase in ABA concentration triggers ion efflux from the guard cells resulted in a decrease of the osmotic pressure. This decrease then causes water efflux and the guard cell volume decreases. The volume change in the guard cells is the major motive power for the stomatal opening and closing.

To adapt the environmental stress, it is well known that gaseous phytohormone ethylene is also involved. However, the effect of ethylene on the ABA-induced stomatal closure has not yet been clarified. Therefore, in Chapter I, I investigated the interaction between ABA and ethylene during stomatal closing. In this study, I used *Arabidopsis thaliana* for an experimental material because various mutant plants are available in this species including ethylene signaling. In Chapter II, I next investigated the effect of cytokinin and auxin against ABA-induced stomatal closure. They are major phytohormones and widely accepted to increase stomatal aperture and inhibit the effect of ABA, Since they are also known to increase ethylene biosynthesis, I tried to explain the above established theory from a

point of view of ethylene synthesis. In Chapter III, I further investigated the cross talk point in which ethylene inhibit the effect of ABA from view points of guard cell volume, vacuolar volume in the guard cells and vacuolar structural changes.



A schematic illustration of the ethylene biosynthetic and signal transduction pathway

ACC is the immediate precursor of ethylene. The rate limiting step of ethylene synthesis is the conversion of AdoMet to ACC by ACC synthase under most conditions. The ethylene signal is perceived by a family of receptors including ETR1, which is inactivated in the presence of ethylene. Downstream components in the ethylene pathway include several positive regulators including EIN3. The synthesis of ACC is enhanced by stress, cytokinin and auxin through the activation of ACC synthase. The activity of ACC synthase is inhibited by AVG, while 1-MCP inhibits ethylene receptors.

Chapter I

Ethylene inhibits ABA – induced stomatal closure in *Arabidopsis thaliana*

Summary

In order to examine the crosstalk between the abscisic acid (ABA) and ethylene signal transduction pathways, signaling events during ABA-induced stomatal closure were examined in *Arabidopsis* wild type (WT) plants, in an ethylene-overproducing mutant (*eto1-1*), and in two ethylene-insensitive mutants (*etr1-1* and *ein3-1*). Using isolated epidermal peels, stomata of WT plants were found to close within a few minutes in response to ABA, whereas stomata of the *eto1-1* mutant showed a similar but less sensitive ABA response. In addition, ABA-induced stomatal closure could be inhibited by application of ethylene or the ethylene precursor, ACC. In contrast, stomata of the *etr1-1* and *ein3-1* mutants were able to close in response to concomitant ABA and ACC application, although to a lesser extent than in WT plants. Moreover, expression of the ABA-induced gene, *RAB18,* was reduced following ACC application. These results indicate that ethylene delays stomatal closure by inhibiting the ABA signaling pathway. The same inhibitive effects of ethylene on stomatal closure were observed in ABA-irrigated plants and the plants in drought condition. Furthermore, upon drought stress, the rate of transpiration was greater in *eto1-1* and WT plants exposed to ethylene than in untreated WT control plants, indicating that the inhibitive effects of ethylene on ABA-induced stomatal closure was also observed *in planta*.

Introduction

Guard cells are highly specialized epidermal cells that are located in pairs on the aerial organs of plants. Each pair of guard cells forms a pore or "stoma" that closes and opens in response to osmotic shrinking and swelling of the guard cells, respectively. Stomata play a major role in controlling gaseous exchange, especially of photosynthetic carbon dioxide uptake, and in water release by transpiration in response to changes in the surrounding environment. The regulation of stomatal conductance is thus extremely important for the survival of plants. Abscisic acid (ABA), synthesized in response to drought stress, is known to induce stomatal closure and to reduce transpirational water loss (Schroeder et al., 2001). ABA also regulates other plant growth and developmental processes, such as embryo maturation, seed dormancy and adaptation to environmental stresses (Leung and Giraudat, 1998). Among the various phytohormones, ABA appears to play the most important role in the control of stomatal responses (Dodd, 2003). However, as stomata respond to various external conditions that may alter the plants' phytohormone balance, stomatal movement may in fact be controlled by the interactions between multiple phytohormones. Indeed, numerous recent studies indicate a cooperative effect between ABA and other phytohormones in various physiological events, and that these effects are dependent on the species, conditions, periods and sites in which the hormones function. Such examples include the antagonism between gibberellins and ABA in cereal aleurone layers (Lovegrove and Hooley, 2000); the effects of methyl jasmonate on plant transpiration (Lee et al., 1996; Wang, 1999), through the regulation of stomatal closure (Raghavendra and Reddy, 1987) in cooperation with ABA and mediated by production of reactive oxygen species (Suhita et al., 2004); as well as the interaction between ethylene and ABA.

Ethylene regulates numerous plant processes, including seed germination, root-hair initiation, leaf and flower senescence and abscission, fruit ripening, nodulation, and plant responses to a wide variety of stresses (Bleecker and Kende, 2000). Although its role in stomatal closure has been suggested (Giulivo 1986), its effect in this process seems rather contradictory. In the early stages of *Arabidopsis* seedling development, ethylene appears to act as a negative regulator of ABA action while in *Arabidopsis* roots it has a positive synergistic effect on ABA action by modulating the overall carbon status (Ghassemian et al., 2000). Under water stress conditions, the increased endogenous ABA levels limit ethylene production and so maintain the growth ratio between shoots and roots (Sharp, 2002).

Despite these advances in my understanding of both ABA and ethylene, studies on the mechanisms by which ethylene interacts with ABA in guard cells are still in their infancy. Over the past decade, genetic screens that were based on ethylene's triple-response phenotype have been extensively conducted on *Arabidopsis*, and more than a dozen unique mutants have been identified. Ethylene is synthesized from methionine via *S*-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). The conversion from AdoMet to ACC, catalyzed by ACC synthase (ACS), is generally the rate limiting step of ethylene biosynthesis. In the *ethylene over-producer1-1* (*eto1-1*) mutant, ethylene is overproduced due to a reduction of ETO1 activity, which promotes ACS5 degradation by a proteasome-dependent pathway (Wang et al., 2004). The ethylene perception and signal transduction pathways have also been investigated using *Arabidopsis* mutants. Genetically dominant mutations in *ETHYLENE RESISTANT1* (*ETR1*) result in ethylene insensitivity (Bleecker and Kende, 2000), whereas mutations at the *ETHYLENE INSENSITIVE3* (*EIN3*) locus cause reduced ethylene sensitivity (Roman et al., 1995).

To further investigate the interactions between ABA and ethylene, I have in this chapter compared the stomatal responses of three *Arabidopsis* mutants, *eto1-1* (*ethylene overproducer1-1*), *etr1-1* (*ethylene resistant1-1*) and *ein3-1* (*ethylene insensitive3-1*) to those of wild type (WT) plants. In addition to these phenotypic observations, the differential expression of the ABA-induced gene, *RAB18,* which accumulates only in guard cells (Nylander et al., 2001), was investigated in these plant.

Materials and Methods

Plant materials and culture conditions

Arabidopsis thaliana seeds of Col-0, *eto1-1* (stock# CS3072), *etr1-1* (CS237) and *ein3-1* (CS8052) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). All mutants used were of the Col-0 background. Seeds were germinated and grown on vermiculite that was irrigated daily with mineral nutrients as described by Naito et al. (1994) in growth chambers at 23.5°C, a relative humidity of 60%, and under a photosynthetic photon flux density (PPFD) of 50 μmol photons m^{-2} s^{-1} in 12-h light / 12-h dark cycles.

Measurement of stomatal aperture

The abaxial epidermis was peeled from rosette leaves of 5- to 6-week-old plants 3 h after the beginning of the light period. Epidermal peels were floated, peeled-side down, on opening buffer (10 mM KCl, 10 mM $CaCl_2$ and 10 mM MES, 0.01% Tween 20, pH 6.5) and incubated under light conditions for 2 h to open the stomata. Subsequently, the epidermal peels with preopened stomata were transferred to the

same buffer supplemented with 10 μM ABA (Sigma-Aldrich, MO, USA), either with or without bubbling 100 μL L^{-1} ethylene gas (GL sciences, Tokyo, Japan) or alternatively with the addition of 10 μM ACC. For dark conditions, preopened epidermal peels were incubated in the dark for 2 h. Stomatal apertures were measured from the pore widths that were observed by light microscopy (Olympus BX51), using a fitted camera (Olympus DP70 digital camera unit), and measured with a digital ruler in Adobe Photoshop 6 (Adobe systems, CA, USA).

Treatment with 1-methylcyclopropene (1-MCP) and aminoethoxyvinyl glycine (AVG)

Treatment of samples with 1-MCP was conducted by evaporating 5.6 mg 1-MCP, dissolved in 85 μL distilled water, in a closed chamber (5.8 L) for 12 h. The final concentration of 1-MCP in the gas phase was expected to be about 500 pL L^{-1} (Tamaoki et al., 2003). For treatment with aminoethoxyvinyl glycine (AVG), 100 μM AVG was added to the opening buffer during the experiment.

Expression Analyses

For analysing ABA responses of epidermal tissues, rosette leaves from 5- to 6-week old-plants were homogenized in opening buffer for 2 min and the epidermal fragments then collected on a 20 μm (pore size) nylon mesh as described in Allen et al. (1999). These fragments were then incubated in the opening buffer without Tween 20 for 2 h, and then treated with 10 μM ABA or with 10 μM ABA and 100 μM ACC for another 2 h. The epidermal fragments were again collected on a 20 μm nylon mesh and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg of frozen tissue using the Qiagen Plant RNeasy Kit (Qiagen, CA, USA), according to the manufacturer's specifications. For reverse transcription PCR (RT-PCR) analysis, 5 μg of total RNA was reverse transcribed with M-MLV Reverse

Transcriptase (Promega, WI, USA) and the resulting cDNAs then used for the subsequent PCR steps.

Real-time quantitative PCR was conducted in a Smart Cycler II System (Cepheid, CA, USA) and using SYBR Premix Taq (Takara Bio, Shiga, Japan) according to the manufacture's specifications. As an internal standard for cDNA amounts, a 143-bp fragment of *actin-7* cDNA was amplified with PCR primers 5' -GGAAATTGTCCGTGACATAAAGGAG- 3' (upstream primer) and 5' –CTCTCAGCTCCGATGGTTATGACTT- 3' (downstream primer). A 226-bp fragment of the *RAB18* cDNA was amplified with PCR primers 5' –ACGAGTACGGAAATCCGATG- 3' (upstream primer) and 5' –ACCACCACTTTCCTTGTGGA- 3' (downstream primer).

Treatment with ABA and drought stress in planta

Treatment with ABA *in planta* was carried out by irrigation of 100 μM ABA dissolved in distilled water for 30 min. For drought stress, the aerial parts of 5-week-plants were detached from the roots. Total RNA was extracted from rosette leaves of treated plants, before or 30 min after the treatment and real-time quantitative PCR was conducted as described above.

Stomatal imaging of in vivo leaves

Stomatal shapes were observed by Suzuki's Universal Micro-Printing (SUMP) method using SUMP liquid and SUMP plate (SUMP laboratory, Tokyo, Japan). Here, the abaxial side of the leaves was pressed onto 10 uL of SUMP liquid placed on a cover glass until the liquid became solid. The copied SUMP resin images were then observed by light microscopy (Olympus BX51, Olympus, Tokyo, Japan).

Ethylene gas treatment and water loss measurements

Five-week-old WT plants were exposed to a dose of 100 μL L^{-1} ethylene gas (GL sciences, Tokyo, Japan) for 3 h in a transparent plastic, air-tight container. An appropriate amount of 100% ethylene was injected into the chamber so as to bring the gas composition to 100 μL L^{-1}, and the presence of ethylene gas was verified by observing the triple response of etiolated *Arabidopsis* seedlings (Guzmán and Ecker, 1990). Conditions within the container were maintained at 23.5°C, with a PPFD of 50 μmol photons m^{-2} s^{-1} under continuous light. Control plants were kept in the same conditions described above but without ethylene gas. After the treatments, the aerial parts of the plants were detached from the roots and their fresh weights measured.

Results

Ethylene inhibits ABA - induced stomatal closure

To first examine the effects of ABA on stomata, I employed an *in vitro* system using isolated epidermal peels in which I could measure stomatal apertures. After light illumination of WT plants (Fig. I-1A, C and E), the stomatal apertures reached approximately 1.47 μm (Fig. I-1G). Time course observations demonstrated that within 5 min of start of ABA application, the stomata almost completely closed (Fig. I-1D), with stomatal apertures decreasing to approximately 0.64 μm and being maintained in this condition for at least 30 min (Fig. I-1G). Using the same system, I examined the effects of ethylene on ABA-induced stomatal closure. When gaseous ethylene was applied together with ABA to the isolated epidermal peels, the stomata started to close within 5 min after the treatment. However, stomatal closure was

incomplete (Fig. I-1F, G), with stomatal apertures reaching about 0.83 μm and being maintained at this level until 30 min (Fig. I-1E, G). To confirm the efficacy of exogenous ethylene treatment, I applied the ethylene precursor, ACC, instead of ethylene. Following treatment with 10 μM ACC, ABA-induced stomatal closure was inhibited again, with the stomata remaining in a half-opened state. Similar pattern of changes in stomatal aperture was observed in the *eto1-1* plants (Fig. I-1B, G), a mutant that overproduces ethylene without treatment with either ethylene or ACC. These observations indicate that both endogenous and exogenous ethylene have an inhibitive effect on ABA-induced stomatal closure.

Ethylene signaling impairs ABA regulation of stomatal closure

To examine the effects of ethylene on ABA-induced stomatal closure in further detail, I measured the stomatal apertures in the following three experimental systems: using ethylene biosynthesis and/or signaling mutants; treatment with ethylene reception inhibitors; and treatment with an ACS inhibitor.

First, I compared the stomatal responses of WT plants with those of the three ethylene mutants, *eto1-1, ein3-1* and *etr1-1*. The stomata in WT plants closed in response to either dark or ABA (Fig. I-2A). In contrast, the ABA-induced stomatal closure of *eto1-1* plants was suppressed, although stomatal closure in response to darkness occurred to the same extent as in WT plants (Fig. I-2A). As with WT plants, stomatal apertures of the *etr1-1* and *ein3-1* mutants decreased to about 0.60 μm after either dark or ABA treatment (Fig. I-2A). However, the ABA-induced stomatal closure could not be restored by ACC treatment in these mutants (Fig. I-2A).

I next examined the effects of a competitive inhibitor of ethylene receptor(s), 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997), on stomatal responses. After an overnight treatment with 1-MCP, stomata of both WT and *eto1-1* closed

after ABA treatment and the closure was not restored even in the presence of ACC (Fig. I-2B). These results suggest that ethylene signaling is necessary for the inhibition of ABA-induced stomatal closure.

I subsequently investigated the effects of ethylene in WT and *eto1-1* plants treated with aminoethoxyvinyl glycine (AVG), an inhibitor of ACS (Yoshii and Imaseki, 1982). In WT plants, ABA-induced stomatal closure did not differ between AVG treated- and non-treated-plants (compare Fig. I-2A and Fig. I-2C). In contrast, AVG-treated *eto1-1* plants showed an ABA-induced stomatal closure response similar to that of the WT plants (Fig. I-2C). Such a stomatal response was not the result of the toxic side effects of AVG treatment, since AVG together with exogenous ACC inhibited ABA-induced stomatal closure in both WT and *eto1-1* plants (Fig. I-2C). These results suggest that the inhibition of ABA-induced stomatal closure observed in the *eto1-1* plants resulted from the over-production of ethylene.

Differential expression of an ABA-induced gene in guard cells

To monitor the inhibition of ABA-signaling by ethylene in guard cells tissues at the molecular level, the expression of an ABA-induced gene, *RAB18,* was examined by quantitative real-time PCR. Following treatment with ABA, *RAB18* expression was enhanced in both WT and *eto1-1*, but the level of *RAB18* expression in *eto1-1* plants was significantly lower than that in WT plants ($P<0.01$). Simultaneous treatment with ACC and ABA resulted in decreased *RAB18* transcript levels in both WT ($0.01 \leq P<0.05$) and *eto1-1* plants (Fig. I-3). These results suggest that induction of *RAB18* expression by ABA is inhibited by exogenous ACC application.

Ethylene inhibits stomatal closure upon ABA treatment and drought stress *in*

planta

The above results indicated that ethylene repressed the ABA-induced closure of stomata *in vitro*, but provided no evidence *in planta*. I therefore carried out an examination of whether ethylene affects stomatal closure even *in planta* or not. I irrigated ABA to WT, *eto1-1* and WT plants previously exposed to gaseous ethylene. The level of *RAB18* expression was elevated in leaves of WT plants by ABA treatment to roots (Fig. I-4A) and stomata in WT plants closed rapidly after ABA treatment (Fig. I-4B). In contrast, stomata of *eto1-1* plants and ethylene gas-exposed plants closed slowly and not completely even 30 min after ABA treatment (Fig. I-4B).

Stomata are known to close in response to drought so as to limit water loss by transpiration. During this process, ABA is synthesized and plays a role in closing stomata. To study the effect of ethylene on stomatal closure under drought stress, I subjected the plants, WT, *eto1-1* and WT priorly exposed to gaseous ethylene, to drought stress by detaching the above-ground portion of the seedlings from their roots. After the detachment, the level of *RAB18* expression in the leaves was elevated in WT plants (Fig. I-5A). The stomata of WT plants rapidly closed within 5 min and after that the stomatal aperture was not so changed (Fig. I-5B, D and E). Although there is no clear difference in appearance between these plants after detachment (Fig. I-5-C), the stomata of *eto1-1* plants and WT plants exposed to ethylene closed slowly after detachment (Fig. I-5B, F to K).

To gain further insight into the observed delay of stomatal closure, the changes in fresh weight under drought stress were measured in the *eto1-1* plants and WT plants exposed to ethylene. Results demonstrated that *eto1-1* plants and WT plants exposed to ethylene showed an accelerated decrease of the fresh weight in comparison to non-treated WT plants (Fig. I-6A). The rate of the fresh weight decrease in *eto1-1* and ethylene-treated plants was higher than in non-treated

plants 20 min after detachment (Fig. I-6B). This result will reflect the fact that the *eto1-1* plants and WT plants exposed to ethylene fail to limit their transpirational water loss by the delay of stomatal closure in an early stage of drought condition.

Discussion

The prime objective of this current study was to elucidate the crosstalk between ABA and ethylene signal transduction on stomatal closure using *Arabidopsis* leaf epidermal peels as a model. Towards this goal, I evaluated the inhibition of ABA-induced stomatal closure by ethylene using genetic, molecular, biochemical and physiological approaches.

Ethylene inhibits ABA-induced stomatal closure

Gaseous ethylene or ACC treatment of the epidermal peels inhibited ABA-induced stomatal closure (Fig. I-1). In contrast, inhibition of ABA-induced stomatal closure by ACC treatment was not observed if the ethylene reception or signaling pathway were inhibited (Fig. I-2A, B). Besides, ACC treatment did not induce the dark-closed stomata into the open state (data not shown). Taken together, these results suggest that ethylene physiologically inhibited ABA-induced stomatal closure through the ethylene signaling pathway.

In my experimental system, I found that in response to ABA, stomata of *Arabidopsis* plants closed much faster than other plants. For example in *Vicia faba,* their stomata started to close 8 min after ABA treatment and reached its maximal closure within 20 min (Roelfsema et al, 2004). A rapid response to ABA might be necessary for survival strategy of plants like *Arabidopsis*, in which whole plant size

as well as stomatal size are much smaller than the plants like *Vicia faba*. Still, the same inhibitive effect of ethylene against ABA was observed also in stomata of *Vicia faba* (data not shown).

Effect of ethylene on stomatal closure *in planta*

Since the expression of *RAB18* was elevated in leaves in *in planta* analysis by ABA irrigation, I assumed that the amount of ABA was also elevated by this culture conditions. As the same inhibitive effects of ethylene on stomatal closure were observed, it is suggested that the inhibitive effect of ethylene against ABA coincided with functions also *in planta*.

However, in my three timecourse experiments, there were some differences in patterns of stomatal closure upon ethylene gas treatment or in *eto1-1* plants (Fig. I-1G, 4B, 5B). In response to ABA irrigation, the stomata of WT plants exposed to ethylene gas gradually closed by 30 min (Fig. I-4B) while those of peels floated on buffer kept half open (Fig. I-1G). This difference will come from the amount of ethylene exposure on plants. Ethylene gas exposed to the plant tissue in advance of ABA irrigation might diminish gradually (Fig. I-4B) while ethylene gas once dissolved in the buffer might remain at least through the experimental period (Fig. I-1G). This effusion of ethylene gas exposed to the plant tissue will explain the difference between the *eto1-1* and WT plants exposed to ethylene gas since in *eto1-1* plants ethylene should be overproduced continuously. In addition, the gradual closure of the stomata in WT plants exposed to ethylene gas may suppose an idea that the effect of ethylene against ABA is reversible.

On the other hand, in contrast to the ABA-irrigated plants and peels floated on buffer, the stomata of detached leaves of both WT plants treated with gaseous ethylene and *eto1-1* plants finally closed at the same level as control WT plants (Fig.

I-5B), though they exhibited a retardation of stomatal closure compared to the control (Fig. I-5B, D to K). This complete closure in the detached plants by 30 min might be caused by the loss of water in guard cell itself, since the stomata are known to be closed by water loss itself from the guard cells upon dehydrated condition (Asai et al. 1999). Although I did not measure ABA amount in my experiments, an increase of the ABA concentration in detached leaflets within 15 min in Vicia faba (Harris and Outlaw 1991) will support the idea that the stomatal closure after detachment will be caused by ABA response and ethylene may inhibit the ABA signalling.

Still, in those plants in which the delay in stomatal closure was observed, an acceleration of water loss rate appeared to result in their fresh weights decrease faster than WT plants (Fig. I-6A, B). A rapid decrease in fresh weight was also observed in the WT plants treated with ACC, but not in the *etr1-1* and *ein3-1* plants (data not shown). Therefore, the delay in stomatal closure appears to result from the inhibition by ethylene, and the result of my *in vitro* experiments could be extrapolated to the *in planta* observations.

In this chapter, I have shown that ethylene inhibits ABA-induced stomatal responses. What is the physiological role of ethylene inhibition of ABA-mediated stomatal closure? One possibility may be that ethylene ensures the minimum supply of carbon dioxide for photosynthesis by keeping the stomata half opened. It is well known that both ethylene and ABA are affected by drought stress of plants (Leung and Giraudat, 1998) and the rate of photosynthesis rate is almost dependent on the stomatal aperture. Indeed, even though the antagonist relationship between ABA and ethylene under drought has been reported (Spollen et al., 2000), ethylene synthesis is often promoted in response to drought (Xu and Zou, 1993). With regards to the relationship between ethylene and photosynthesis, it has been reported that

the rates of ethylene release, photosynthesis and transpiration increased simultaneously in rice, and were especially promoted during the light period (Saito et al., 1996). Furthermore, in unstressed WT *Arabidopsis* plants, ethylene production levels were found to be controlled by light and by the circadian clock (Thain et al., 2004). Therefore, ethylene may play a role in keeping a minimum level of photosynthesis upon a drought stress for a long period.

Another possible role of ethylene on the inhibition of ABA-mediated stomatal closure, may be related to the involvement of ethylene in regulating leaf senescence, since defects in ethylene synthesis or perception were shown to delay leaf senescence (Davies and Grierson, 1989; Picton et al., 1993; Grbic and Bleecker, 1995; John et al., 1995). Drought can also induce senescence, and is known to promote increased ethylene production in plants (McMichael et al., 1972; Apelbaum and Yang, 1981). A recent report demonstrated that the loss of *ACS* expression in transgenic maize plants resulted in a delay in drought-induced leaf senescence that was associated with increased stomatal conductance (Young et al., 2004), indicating that ethylene promotes drought-induced leaf senescence.

In the present study, I showed a physiological effect of ethylene on ABA-induced stomatal closure. Among various ABA signal transduction mechanisms, rapid ABA-induced Ca^{2+} influx and S-type anion channel activation are required for *RAB18* expression (Schroeder et al., 2001). Although I did not measure anion channel activities in this study, the repression of the ABA-induced *RAB18* expression by ACC treatment (Fig. I-3) suggest that ethylene signaling might impair the Ca^{2+} influx or the S-type anion channels activation. At least, ethylene seems not to interfere the early ABA-signaling pathway since the stomata started to close by ABA application even in the presence of ethylene, but some later stage of ABA-signaling since the stomata were kept half-opened by ethylene treatment. It

would be excellent to find out the cross point of interaction between ABA- and ethylene- signaling pathway. However, to clarify this point needs further study.

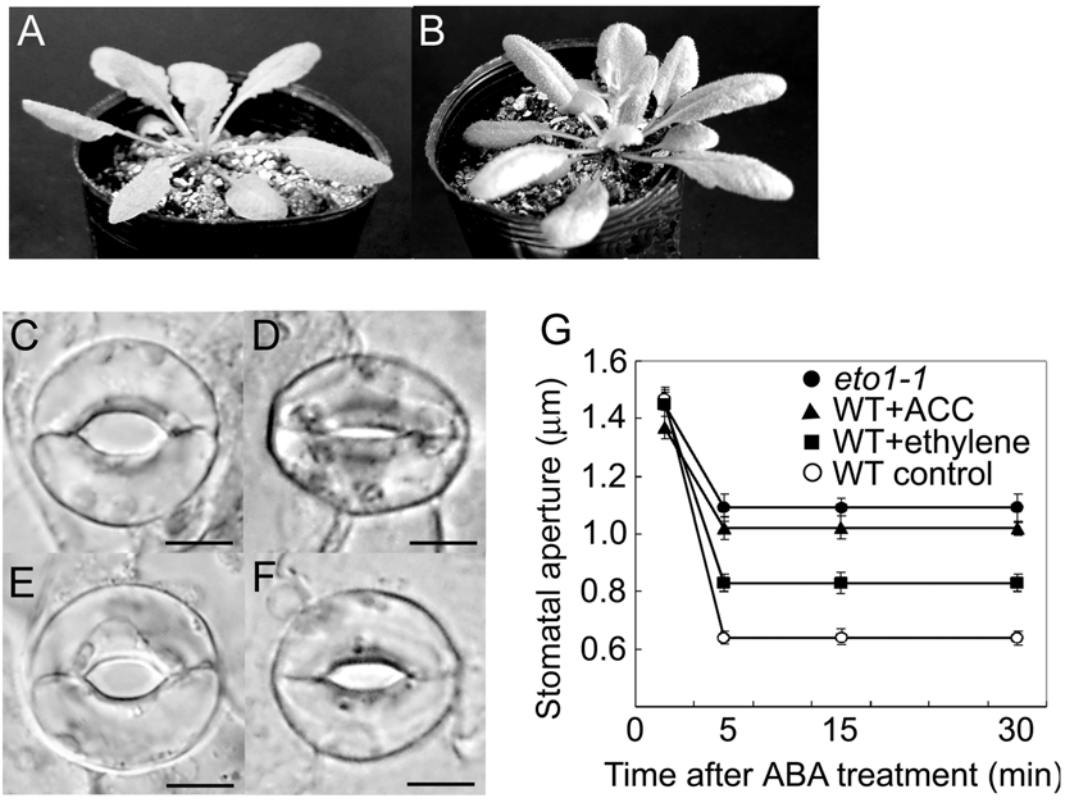


Figure I-1.
Ethylene treatment impairs stomatal closure induced by ABA treatment.

Overall phenotype of (A) WT and (B) *eto1-1* plant.

WT stomata preopened in light conditions (C) without and (E) with ethylene gas.
WT stomata after incubation with 10 μM ABA for 30 min (D) without and (F) with ethylene.

(G) Time course changes in stomatal apertures (width μm) after ABA treatment of
WT (open circles), WT exposed to ethylene gas (closed squares),
WT treated with ACC (closed triangles), and *eto1-1* plants (closed circles).
Bars represent means ± SEs.

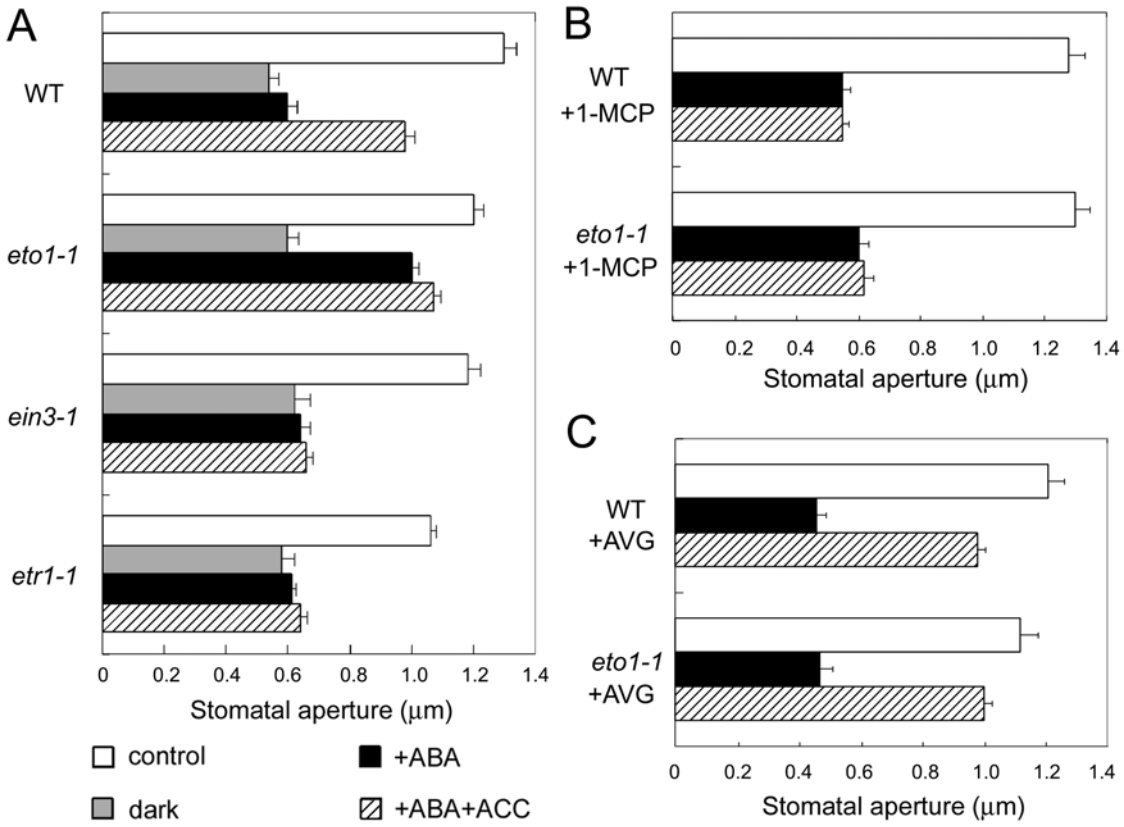


Figure I-2.
ACC treatment impairs stomatal closure induced by ABA treatment only when plants can transmit the ethylene signal.

Stomatal apertures of
(A) WT, *eto1-1*, *ein3-1* and *etr1-1* plants,
(B) 1-MCP-treated WT and *eto1-1* plants,
(C) AVG-treated WT and *eto1-1* plants, in the preopened condition (open columns),
after incubation in darkness for 2 h (gray columns),
after incubation with 10 μM ABA for 2 h in the light (closed columns),
and after incubation with 10 μM ABA and 10 μM ACC in the light (striped columns).
The data is representative of three independent experiments, showing the means of 100 stomata. Bars represent means ± SEs.

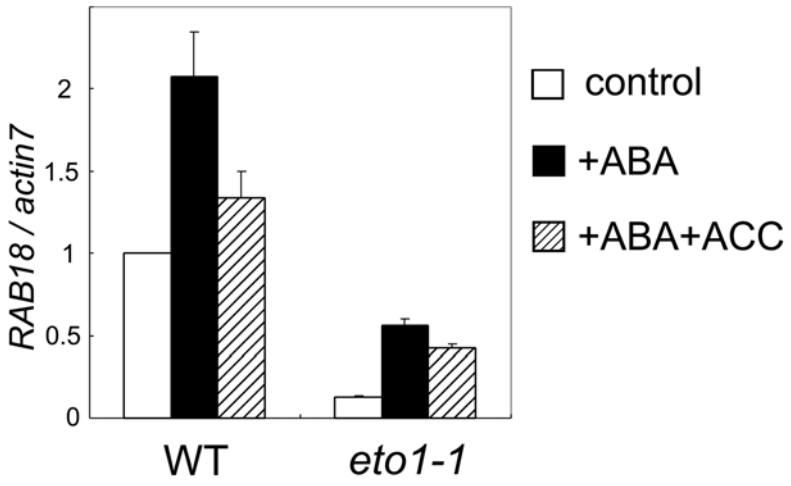


Figure I-3.
ABA responses of epidermal tissues of WT and *eto1-1* plants.

Expression patterns of an ABA-induced gene, RAB18, were compared between the WT and *eto1-1*. mRNA was extracted from epidermal tissues before treatment (open columns), and after treatment with 10 μM ABA (closed columns) or 10 μM ABA plus 100 μM ACC (striped columns) in opening buffer without Tween 20 for 2 h. Gene expression levels were measured by real-time quantitative PCR and the relative amounts of transcripts then calculated and normalized with actin7 mRNA. Bars represent the means ± SEs (n = 6-9). The data were analyzed by ANOVA. Difference between WT+ABA and WT+ABA+ACC was significant ($0.01 < P < 0.05$), and between WT+ABA and *eto1-1*+ABA was significant ($P < 0.01$).

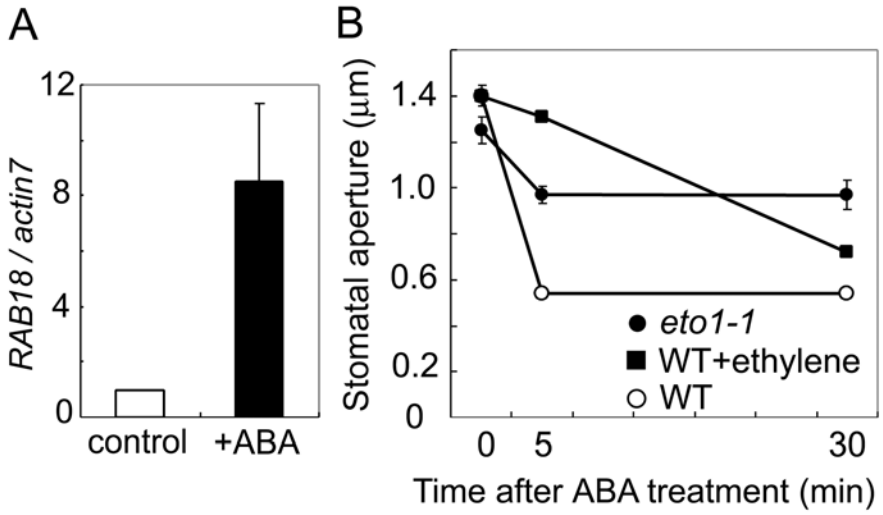


Figure I-4.
Ethylene impairs stomatal closure induced by ABA treatment in planta

(A) Expression patterns of an ABA-induced gene, *RAB18*, were compared between before and after ABA treatment. mRNA was extracted from leaves of WT plants befo treatment (open columns), and after irrigation with 100 μM ABA (closed columns) for 30 min. Gene expression levels were measured by real-time quantitative PCR and th relative amounts of transcripts then calculated and normalized with *actin7* mRNA. Bars represent the means ± SEs (n = 4-8).

(B) Time course changes in stomatal apertures after ABA irrigation of WT (open circl WT exposed to ethylene gas (closed squares), and *eto1-1* plants (closed circles).

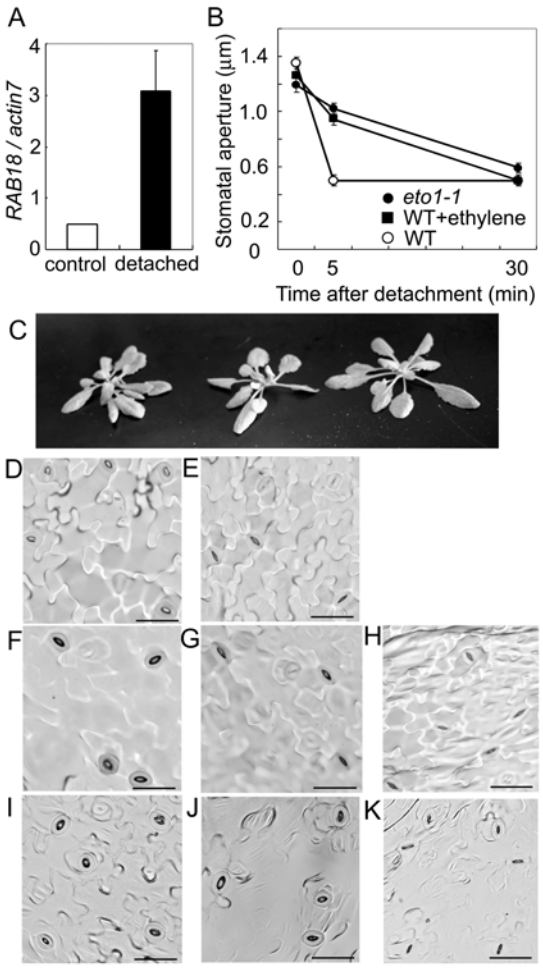


Figure I-5.
Delay in stomatal closure of *eto1-1* and WT plants exposed to ethylene compared to WT plants under drought stress.

(A) Expression patterns of an ABA-induced gene, *RAB18*, were compared between before and after detachment. mRNA was extracted from leaves of WT plants before detachment (open columns), and 30 min after detachment (closed columns). Gene expression levels were measured by real-time quantitative PCR and the relative amounts of transcripts then calculated and normalized with *actin7* mRNA.
Bars represent the means ± SEs (n = 4-8).

(B) Time course changes in stomatal apertures after detachment of WT (open circles), WT exposed to ethylene gas (closed squares), and *eto1-1* plants (closed circles).

(C) Overall phenotype of WT, *eto1-1* and WT plant exposed to gaseous ethylene 30 min after detachment (from left to right).

Stomata of (D) WT plants before detachment of leaves, (E) WT plants 5 min after leaf detachment, (F) eto1-1 plants before detachment, (G) *eto1-1* plants 5 min or (H) 30 min after detachment, (I) WT exposed to ethylene gas before detachment, (J) 5 min or (K) 30 min after detachment, as observed by optical microscopy (×400) (bars = 20 μm).

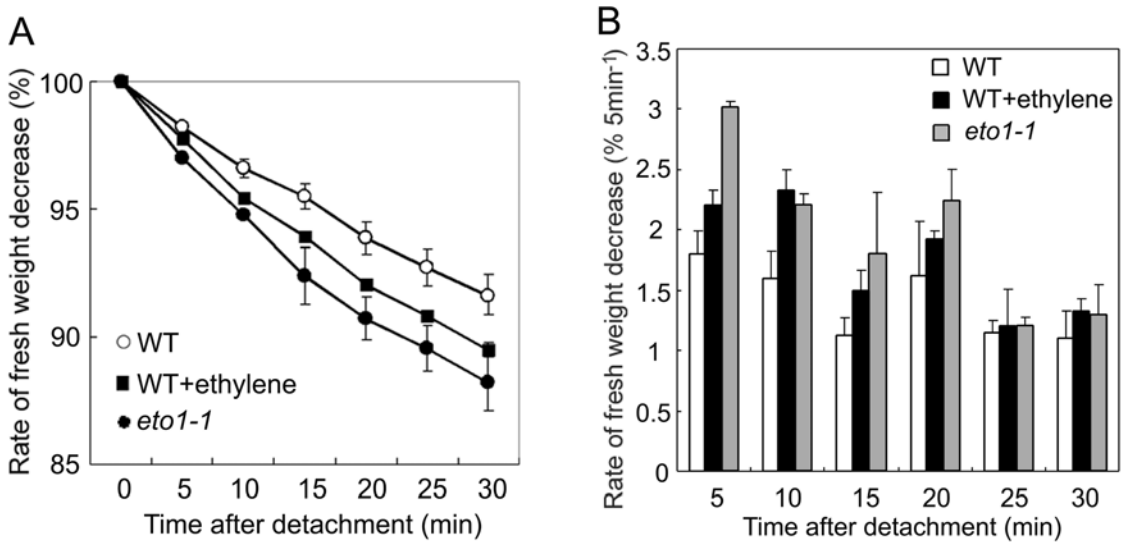


Figure I-6.
Accelerated decrease of the fresh weight in *eto1-1* and WT plants exposed to ethylene gas.

(A) Changes in fresh weight decrease ratio of aerial parts of WT (open circles), WT plants exposed to ethylene gas (closed squares) and *eto1-1* plants (closed circles) after detachment from roots.

(B) The rate of the fresh weight decrease calculated every 5 min after detachment from roots. WT (open columns), WT plants exposed to ethylene gas (gray columns) and *eto1-1* plants (closed columns).
Fresh weights of the aerial parts after detachment were measured, and the ratios of reduced weights and original fresh weights were then calculated. The data is representative of three independent experiments, and the means of 20 individual plants are shown. Error bars represent SEs.

Chapter II

Summary

Cytokinins and auxins are major phytohormones that are involved in plant growth and development. It is widely accepted that during stomatal movement these phytohormones increase stomatal aperture and inhibit the effects of ABA. In Chapter I, I showed an antagonistic effect of ethylene on ABA -induced stomatal closure. As both cytokinins and auxins are known to affect ethylene biosynthesis, I investigated in this Chapter whether these phytohormones inhibit the effect of ABA through ethylene biosynthesis. As with ACC treatment, both the cytokinin, 6-benzyladenine (BA), and the auxin, 1-naphthaleneacetic acid (NAA), inhibited ABA-induced stomatal closure, and these effects were negated when ethylene signaling, perception or biosynthesis were blocked. The above observations suggest that cytokinins and auxins inhibit ABA-induced stomatal closure through the modulation of ethylene biosynthesis.

Introduction

Stomata are located at the end of the transpiration stream within a plant. Regulation of stomatal aperture by guard cells is crucial for minimizing water loss from leaf tissues and maximizing CO_2 exchange for photosynthesis. In response to various environmental stresses, such as drought, cold, air pollutants and high CO_2 concentrations, stomata close in a process that involves the phytohormone, abscisic

acid (ABA) (Schroeder et al, 2001). In Chapter I, I described the antagonistic effect of ethylene on ABA-induced stomatal closure (Tanaka et al, 2005).

Other phytohormones are also involved in the ABA-signaling pathways, and these include cytokinins and auxins that are the major phytohormones regulating plant growth and development. Cytokinin is a classic phytohormone involved in cell division, growth, and organogenesis. However, with respect to the stomata, cytokinin is able to induce stomatal opening and increase the rate of transpiration in leaves independent of light illumination (Livnè and Vaadia, 1965) and also inhibit the effect of ABA (Das et al, 1976, reviewed by Incoll and Jewer, 1987). Auxin, like cytokinin, is involved in a myriad of developmental and environmental processes; embryo patterning, cell division and elongation, vascular differentiation, lateral root initiation, gravitropism and phototropism (Berleth and Sachs, 2001). Concerning stomatal movement, auxin is also able to accelerate stomatal opening and respiration (Pemadasa, 1982) and reduce stomatal closure by darkness, CO_2 and ABA (Snaith and Mansfield, 1982, reviewed by Davies and Mansfield, 1987).

On the other hand, both auxins and cytokinins are known to affect the biosynthesis of ethylene (reviewed by Mattoo and Suttle, 1991). In vegetative tissues, which normally produce quite low amounts of ethylene, auxin markedly induced ethylene biosynthesis (Abeles and Rubinstein, 1964). Pineapple flowering, which was once considered to be a response to auxin, is now understood to be a result of auxin-induced ethylene formation (Burg and Burg, 1966). Ethylene is synthesized from methionine via *S*-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979), and the conversion from AdoMet to ACC, which is catalyzed by ACC synthase (ACS), is generally considered as the rate-limiting step of ethylene biosynthesis. Auxin enhanced ACS gene expression (Nakagawa et al, 1991; Liang et al, 1992), and in

guard cells, the inhibition of ACS activity completely inhibited auxin-induced stomatal opening in darkness (Merritt et al, 2001), supporting the involvement of ethylene production in these processes.

The effects of cytokinins on ethylene biosynthesis have also been the focus of numerous studies. Fuchs and Lieberman (1968) demonstrated the promotion of ethylene biosynthesis by cytokinins in pea seedlings, and this promotion was found to be due to enhanced endogenous ACC production (McKeon et al, 1982). A recent study revealed that cytokinins increase the stability of the ACS protein (Chae et al, 2003). Therefore, the various physiological responses to auxins and cytokinins could be modulated by ethylene biosynthesis.

In this chapter, I examined the effects of cytokinin and auxin on the inhibition of ABA-induced stomatal closure. To investigate whether ethylene biosynthesis and/or the ethylene signaling pathway are involved in this process, I used three different conditions in which the signaling, perception or biosynthesis of ethylene were defective. First, I examined the effect of cytokinin and auxin on stomatal closure in the *Arabidopsis thaliana ein3*-1 (*ethylene insensitive3-1*) mutant that cannot transmit the ethylene signal (Roman et al., 1995). In addition, I inhibited the ethylene receptor(s) using the inhibitor, 1-methylcyclopropene (1-MCP, Sisler and Serek, 1997). Furthermore, I used an inhibitor of ethylene biosynthesis, aminoethoxyvinyl glycine (AVG), to inhibit ACS activity. In addition to these phenotypic observations, the differential expression patterns of an ABA-induced gene, *ABI2,* and an ethylene responsive gene, *SAG13,* were investigated.

Materials and Methods

Plant materials and culture conditions

Arabidopsis thaliana seeds of Col-0, *ein3-1* (CS 8052), and *amp1-1* (CS 8324) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). All mutants used had the Col-0 background. Plant culture conditions were as described in Chapter I.

Measurement of stomatal apertures

Preparation of epidermal tissues was described in Chapter I. For application of phytohormones, the epidermal peels with preopened stomata were transferred into the same buffer supplemented with 10 μM ABA (Sigma-Aldrich, MO, USA), with or without the addition of 10 μM ACC, 10 μM BA, 10 μM NAA, or 10 μM gibberellin acid (GA_3). Measurements of stomatal apertures were also described in Chapter I.

Isolation of guard cell protoplasts (GCPs)

GCPs were isolated from leaves of 4- to 5-week-old *Arabidopsis thaliana* plants by overnight enzymatic digestion according to the method of Pandey et al. (2002). In brief, excised leaves were homogenized in distilled water for 2 min and the epidermal fragments collected on a 20 μm (pore size) nylon mesh. These fragments were then incubated overnight in an enzyme solution containing 0.65 % Onozuka RS cellulase (Yakult Honsha Co, Ltd), 0.35 % Macerozyme R10 (Yakult Honsha Co, Ltd), 0.25 % (w/v) BSA and 0.001 % kanamycin dissolved in 55 % (v/v) of basic solution containing 5 mM MES (pH 5.5), 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 μM KH_2PO_4, 0.5 mM ascorbic acid, 0.55 M sorbitol and 45 % of (v/v) distilled water. Released, isolated and collected GCPs were briefly preincubated in a GCP reaction buffer (0.4 M

mannitol, 10 mM KCl, and 1 mM $CaCl_2$) and incubated for 1 h in the light after phytohormone application.

Treatments with 1-methylcyclopropene (1-MCP) and aminoethoxyvinyl glycine (AVG)

Treatment of samples with 1-MCP and aminoethoxyvinyl glycine (AVG) was as described in Chapter I.

Gene expression Analyses

Total RNA extraction, reverse transcription PCR (RT-PCR) analysis, and real-time quantitative PCR were performed as described in Chapter I. As an internal standard for cDNA amounts, a 143-bp fragment of *actin-7* cDNA was amplified with the PCR primers, 5'-GGAAATTGTCCGTGACATAAAGGAG-3' (upstream primer) and 5'-CTCTCAGCTCCGATGGTTATGACTT-3' (downstream primer). A 172-bp fragment of the *ABI2* cDNA was amplified with the PCR primers, 5'-TTCTATCCTCGCCGCTTCAT-3' (upstream primer) and 5'-ACCACCACTTTCCTTGTGGA-3' (downstream primer). A 180-bp fragment of the *SAG13* cDNA was amplified with PCR primers, 5'-TCGTCAACAATGTGGGAACG-3' (upstream primer) and 5'-CGACTCCAGCAGCAGAGGAT-3' (downstream primer).

Results

Cytokinin and auxin inhibited ABA-induced stomatal closure through ethylene synthesis

To first examine the inhibitory effects of cytokinin and auxin on

ABA-induced stomatal closure, I employed an *in vitro* system using isolated epidermal peels in which I could measure stomatal apertures. After light illumination of WT plants, the stomata opened and their apertures increased to approximately 1.47 μm. ABA treatment closed the stomata almost completely, and stomatal apertures decreased to approximately 0.46 μm (Fig. II-1A). When ACC was applied together with ABA to the isolated epidermal peels, ABA-induced stomatal closure was inhibited, and stomatal apertures reached only 0.90 μm in a half-opened state. When I applied BA as a cytokinin or NAA as an auxin in addition to ABA, stomatal closure was again inhibited similar to that following ACC application (Fig. II-1A). In contrast, GA_3 had no inhibitory effect on the ABA-induced stomatal closure.

Time course observations demonstrated that within 5 min of ABA application, the stomata almost completely closed and remained in this state for at least 30 min (Fig. II-1B). When I applied ABA together with ACC, BA or NAA, the stomata started to close within 5 min. However, stomatal closure was incomplete, with stomatal apertures reaching only 1.00 to 1.15 μm and being maintained at this level for 30 min (Fig. II-1B).

In the previous Chapter, I reported that treatment of epidermal peels with gaseous ethylene or ACC inhibited ABA-induced stomatal closure (Tanaka et al. 2005). To examine whether the effects of BA or NAA on ABA-induced stomatal closure were mediated by ethylene biosynthesis, I examined their effects in an ethylene signaling mutant and in WT plants treated with inhibitors of ethylene receptor(s) and biogenesis. In the *ein3-1* ethylene signaling mutant, which cannot transmit the ethylene signal, ABA induced the closure of stomata whereas ACC application did not inhibit this ABA effect as also demonstrated in Chapter I (Fig II-2A). Furthermore, neither BA nor NAA treatment inhibited ABA-induced

stomatal closure in this mutant (Fig. II-2A). In addition, an overnight treatment with 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene receptor(s), negated the ACC effect on stomatal responses (Chapter I), as also observed for the BA and NAA treatments (Fig. II-2B). Subsequently, I examined the effects of aminoethoxyvinyl glycine (AVG), which inhibits ACC synthase (ACS) activity and results in the inhibition of endogenous ethylene biosynthesis (Yoshii and Imaseki, 1982), on stomatal closure. In the presence of AVG, the inhibitive effects of neither BA nor NAA could be observed, while exogenous ACC application could still inhibit ABA-induced stomatal closure (Fig. II-2C). Furthermore, in a cytokinin over-producing mutant, *amp1-1* (Chaudhury et al., 1993), ABA-induced stomatal closure was inhibited whereas AVG treatment in addition to ABA closed the stomata (Fig. II-3). These results suggest that the inhibitive effects of BA and NAA on ABA-induced stomatal closure resulted from enhanced ethylene production.

Differential expression of an ethylene-responsive gene and an ABA-induced gene in guard cell protoplasts

Arabidopsis guard cells are surrounded by epidermal cells with which they exchange materials, such as water, ions and sugars, during stomatal movement. To examine whether the above responses invoked by BA or NAA application occurred within the guard cells themselves, or whether ethylene was supplied from the adjacent epidermal tissue, I isolated guard cell protoplasts (GCPs) from the epidermal tissue. To monitor the responses of the GCPs to phytohormones at the molecular level, the expression patterns of an ABA-induced gene, *ABI2,* and an ethylene-response gene, *SAG13,* were examined by quantitative real-time PCR. Following ABA treatment, *ABI2* expression was enhanced, whereas simultaneous treatment with ABA and ACC, BA or NAA decreased the *ABI2* transcript levels

(0.01≤P<0.05) (Fig. II-4A). In contrast, the *SAG13* transcript levels were elevated by treatment with ACC and also with BA or NAA (0.01≤P<0.05) (Fig. II-4B). These results suggest that the guard cell responses to phytohormones occurred in the GCPs themselves.

Discussion

In the leaf epidermis of *Arabidopsis thaliana*, cytokinin or auxin treatment inhibits ABA-induced stomatal closure similar to that following ACC treatment (Fig. II-1A, Chapter I). Although the inhibitive effects of these phytohormones were described previously (Dodd, 2003), results from our studies with the *ein3-1* ethylene-insensitive mutant together with those using 1-MCP and AVG, as inhibitors of ethylene receptors and biosynthesis, respectively, demonstrate an involvement of the ethylene signaling pathway in stomatal movement. AVG inhibits the activity of ACS, which catalyses the reaction from AdoMet to ACC (Chi et al, 1991). As this inhibitor treatment completely repressed the effects of BA and NAA on stomatal closure, these phytohormones appear to inhibit ABA-induced stomatal closure by accelerating ethylene biosynthesis, especially upstream of ACS activity. Expression analysis of the *ABI2* and *SAG13* genes in the GCPs suggests that the GCPs themselves were responsive to the phytohormones. Repression of *ABI2,* an ABA-induced gene, by BA or NAA as well as by ACC application, suggests the involvement of these phytohormones in the ABA-signaling pathway. Further observations of the changes in GCP diameter upon phytohormone application are in accordance with the effects of cytokinin or auxin on ethylene production (see Chapter III). Although I did not directly measure the levels of ethylene produced, the

increase in gene expression levels of *SAG13* (Fig. II-4A), of ACS in the guard cells (Tsuchisaka and Theologis, 2004), and of ACS in Arabidopsis leaves following treatment with the auxin, indole-3-acetic acid (IAA, Arteca and Arteca, 1999), suggest an effect on ethylene production. The observation that ACC application compensated for the effect of AVG (Fig. II-2C) is in agreement with the inhibitive effects of AVG on ACC synthesis and indicates that this inhibitor had little side-effect on stomatal closure. As application of kinetin, an alternative cytokinin, or IAA, an alternative auxin, demonstrated the same effects as BA or NAA (data not shown), I conclude that cytokinins and auxins inhibit ABA-induced stomatal closure through ethylene biosynthesis.

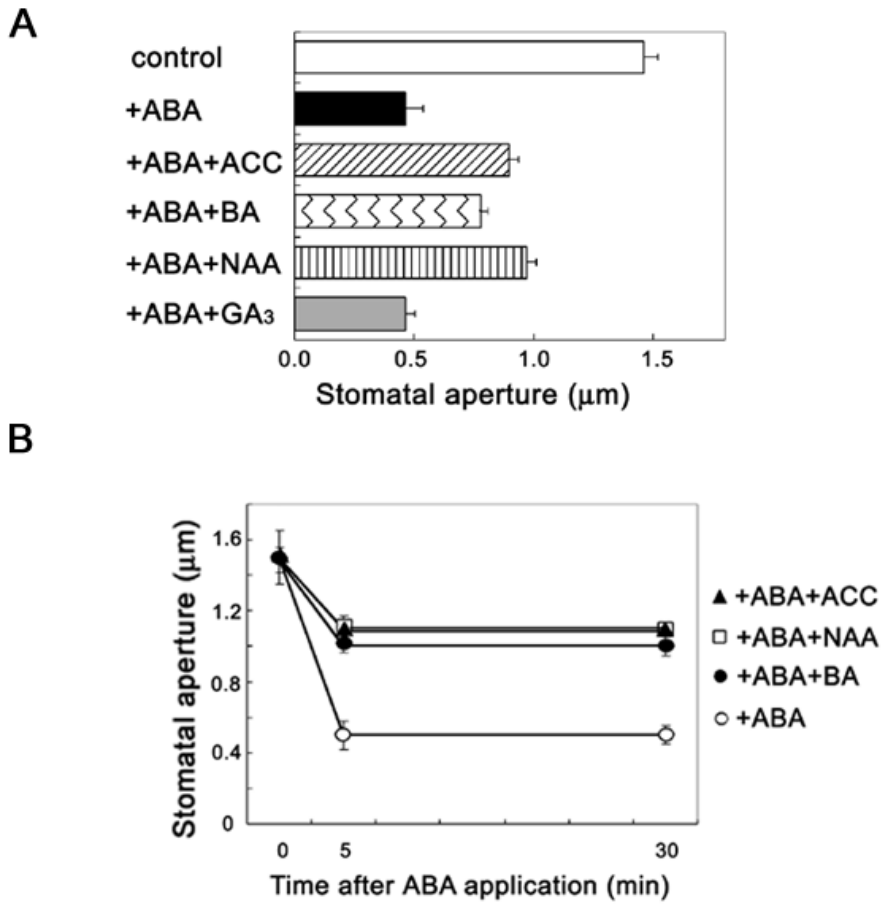


Figure II-1.
ACC, BA, and NAA applications impair stomatal closure induced by ABA.

(A) Stomatal apertures of WT plants preopened by white light illumination (control), after incubation with 10 μM ABA and with 10 μM ACC, 10 μM BA, 10 μM NAA, or 10 μM GA3 in addition to ABA application for 2 h.
The data is representative of three independent experiments with the means of 100 stomata. Bars represent means ± SEs.
(B) Time course changes in stomatal apertures after ABA application (open circles), and after ACC (closed triangles), BA (closed circles) or NAA (open squares) application in addition to the ABA treatment.
Bars represent means ± SEs.

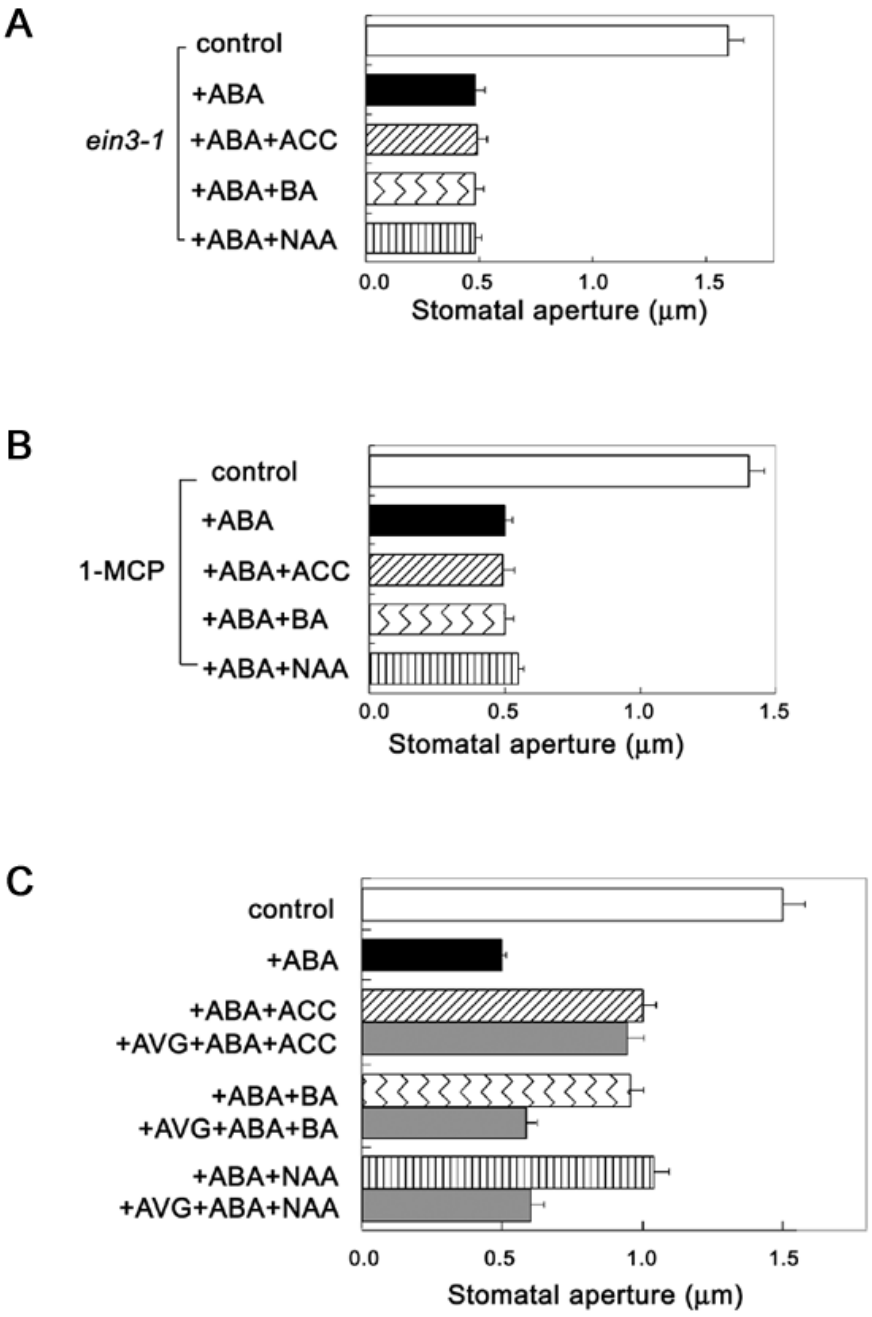


Figure II-2.
Negation of the antagonistic effects of ACC, BA or NAA
on stomatal closure in plants defective in ethylene signaling.

Stomatal apertures of ein3-1 plants (A), and WT plants treated with 1-MCP (B) or AVG (C) were measured in the preopened condition (control), after incubation with 10 μM ABA and with 10 μM ACC, 10 μM BA or 10 μM NAA in addition to ABA application for 2 h.
The data is representative of three independent experiments with the means of 100 stomata. Bars represent means ± SEs.

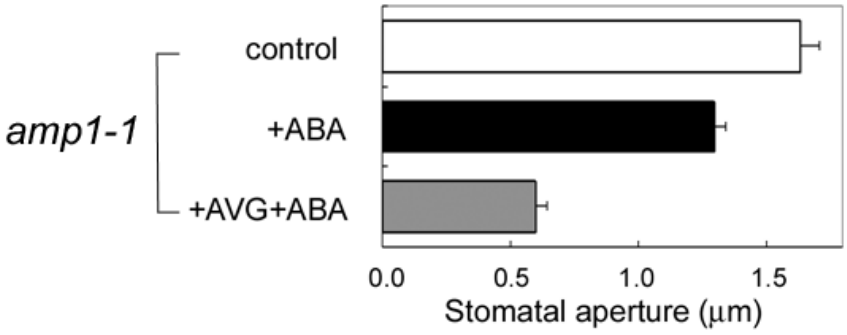


Figure II-3
ABA-induced stomatal closure is inhibited in the cytokinin over-expressing mutant, *amp1-1*.

Stomatal apertures of amp1-1 plants in the preopened condition (control),
after incubation with 10 μM ABA, and after 100 μM AVG in addition to ABA for 2 h.
The data is representative of three independent experiments with means of 100 stomata.
Bars represent means ± SEs.

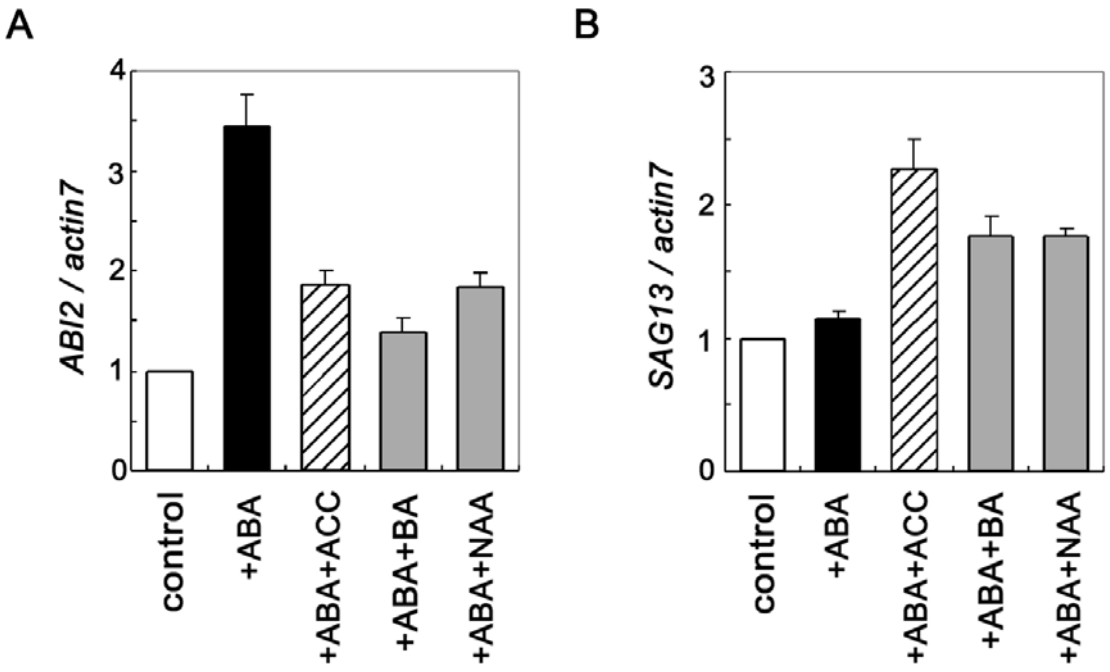


Figure II-4.
Gene expression analysis in guard cell protoplasts (GCPs)
in response to phytohormones.

Expression patterns of an ABA-induced gene, *ABI2* (A),
and an ethylene-response gene, *SAG13* (B), were examined after various phytohormone treatments. Real-time quantitative PCR was conducted with mRNAs extracted from GCPs without treatment (control), after incubation with 10 μM ABA, and with 10 μM ACC, 10 μM BA, or 10 μM NAA in addition to ABA application for 1 h.
The amounts of transcripts were normalized with the actin7 transcript levels.
Bars represent means ± SEs (n = 5).
Differences between +ABA and +ABA+ACC, +BA or +NAA were significant ($0.01 < P < 0.05$), as were those between control and +ABA+ACC, +BA or +NAA ($0.01 < P < 0.05$).

Chapter III

Summary

To examine the point at which ethylene effects ABA signaling during stomatal closure, I measured the changes in guard cell volume and vacuolar volume in the guard cells. Upon ABA application both the guard cell and vacuolar volumes decreased, but ACC application inhibited these responses. Although the inhibitory effect of ACC on the guard cells of epidermal peels was incomplete, ACC application completely inhibited the effect of ABA on volume changes in guard cell protoplasts lacking a cell wall. The above observations suggest that ethylene inhibited ABA-induced stomatal closure by inhibiting the ABA-induced regulation of osmotic pressure in guard cells, but did not affect the response to ABA in which the cell wall was concerned.

Introduction

In the previous Chapters, I described the antagonistic effects of ethylene, cytokinin and auxin on ABA-induced stomatal closure and suggested that these effects of cytokinin and auxin were due to an enhanced biosynthesis of ethylene. However, it is still unclear at which point in the ABA-signaling pathway ethylene has its effects, and so in this current Chapter, I addressed this question by examining the guard cell vacuolar volume and its structures as well as the volume of the guard cell itself.

The mechanism of ABA-induced stomatal closure, which has been the

subject of numerous studies, is known to be driven by a decrease in guard cell turgor as a result of effluxes of K^+ and associated anions, such as Cl^- and/or malate, that are triggered by an increase in cytoplasmic Ca^{2+} concentrations (Ward and Schroeder, 1994). These solutes that are stored in the vacuole are first transported into the cytoplasm and then to apoplasts by activation of the appropriate ion channels and transporters in the vacuolar and plasma membranes (MacRobbie, 2000). The reduced osmotic pressure by the efflux of the solutes releases water into the apoplasts so that the stomata close due to the decrease in guard cell volume. Thus, ABA-induced stomatal closure is mainly mediated by control of the osmotic pressure that results in the reduction of cell size.

The expansion of a plant body is predominantly accomplished by an increase in cell volume rather than through an increase in cell number due to cell division. The large central vacuole, which occupies a large part of the cell volume, is thought to play a major role in cell expansion (Marty 1999). The vacuoles of plant cells are multifunctional organelles that are involved in cellular responses to environmental and biotic factors that provoke stress. The central vacuole is considered to have a particularly important function in volume changes in mature guard cells, since cell expansion and shrinkage is required for the daily reversible and relatively rapid cell volume changes rather than the irreversible process of cell division.

To observe the tonoplast, I used a transgenic *Arabidopsis* line that stably expresses GFP-AtVam3p (Uemura et al. 2002). AtVam3p is one of several vacuolar t-SNARE proteins that mediate membrane fusions. The GFP fusion of AtVAM3p allows visualization of the vacuolar membrane in several *Arabidopsis* tissues, such as the mesophyll, root epidermis, root hair and guard cells (Saito et al. 1997, Uemura et al. 2002), as well as in tobacco BY-2 cells (Kutsuna and Hasezawa 2002). This use of this GFP-AtVam3p *Arabidopsis* line enabled us to perform time-lapse

observations of the dynamics of both stomatal apertures and vacuolar structures in living epidermal cells without the usual damage observed with conventional membrane staining methods.

In addition to the changes in the vacuolar volume and its structures, the change in volume of the guard cell itself is known to be involved in guard cell movement. To measure guard cell volumes, it is particularly convenient to use guard cell protoplasts (GCPs) since they possess simple, spherical forms. In addition to enabling volume measurements, the GCPs provide a unique means of studying the biochemical and physiological properties of guard cells since they are separated from the epidermal tissues that surround the guard cells. By removal of their resistant and thickened cell walls, the GCPs can respond to a wide variety of environmental signals, such as light and phytohormones, and the changes in their osmotic pressures are directly reflected into simple changes in their diameter.

Materials and Methods

Plant materials and culture conditions

A transgenic *Arabidopsis thaliana* line expressing GFP-AtVam3p (Col-0 background) was kindly provided by Dr. M. H. Sato (Kyoto University). The plant culture conditions were as described in Chapter I.

Observation of vacuolar structures and measurement of areas in vacuoles of guard cells and of stomatal aperture

Preparation of epidermal tissues and application of phytohormones were performed as described in Chapter I. Structures and areas in the vacuoles were determined

from images of guard cell cross-sections using the Arabidopsis GFP-AtVam3p transgenic line (Uemura et al. 2002) that allows the vacuolar membranes to be visualized. Images of the vacuolar membranes were observed with a fluorescence microscope (IX70, Olympus Co. Ltd., Tokyo, Japan) equipped with a cooled CCD camera head system (CoolSNAP HQ, PhotoMetrics Inc., Huntington Beach, Canada). Subsequently, the images were digitally processed using Photoshop software (Adobe Systems Inc., California, USA). The areas of the vacuolar regions were obtained by manual segmentation and the areas then calculated on the basis of the number of pixels. Stomatal apertures were measured from the pore widths and measured with a digital ruler in Adobe Photoshop 6 (Adobe systems, CA, USA).

Measurements of GCP volume changes

The response of the Arabidopsis GCPs was monitored by incubation for 1 h under light in the GCP reaction buffer containing 0.3 M mannitol with or without phytohormones. To increase or decrease the osmotic pressure in the suspension medium, saturated D-mannitol or distilled water, respectively, was added into the medium. GCPs were observed by light microscopy (Olympus BX51), photographed with a fitted camera (Olympus DP70 digital camera unit), and their diameters measured with a digital ruler in Adobe Photoshop 6 (Adobe systems, CA, USA). For treatment with aminoethoxyvinyl glycine (AVG), 100 μM AVG was added to the GCP reaction buffer during the experiment.

Results

Changes in structure and vacuolar volume are also inhibited by ACC application

When stomata close in response to ABA, the decreased turgor produces changes in the volume and shape of both the guard cell and its vacuole. As a recent report demonstrated the appearance of numerous vacuolar luminal structures, such as folding and bulb-like structures, as a result of the decrease in vacuolar volume and stomatal aperture by ABA application (Sano et al, manuscript submitted), I first observed the changes in the vacuolar structures of stomata in the *Arabidopsis* GFP-AtVam3p transgenic line (Uemura et al. 2002). To quantify the structural changes in the vacuole, I categorized the guard cells as follows; cells without luminal vacuolar structures (Type 1), cells with some folding structures (Type 2), and those with bulb-like structures (Type 3, Fig. III-1A to C). Prior to ABA application, about 65 % of the cells were Type 1, but this percentage decreased after ABA application and the cells were almost absent by 30 min. In contrast, the percentage of Type 3 cells increased to about 80 % after 30 min of ABA application (Fig. III-1D). The number of Type 2 cells first increased and then decreased, suggesting that the Type 2 cells were transient forms of the Type 1 and 3 cells. When ACC was applied in addition to ABA, the percentage of Type 1 cells decreased to about 15% and then remained at this level. The percentage of Type 2 cells increased to about 50 % whereas the Type 3 cells decreased to 35% even 30 min after ABA application (Fig. III-1E).

In addition to the structural changes of the vacuoles, I observed the changes in stomatal vacuolar volumes in response to ABA application. To simplify the measurements and calculations, I measured the vacuolar areas obtained from guard cell cross-sectional images of the *Arabidopsis* GFP-AtVam3p line. After ABA application, the value of the vacuolar region started to decrease in parallel with the stomatal closure (Fig. III-2A, B). Interestingly, although the stomata closed completely after 5 min of ABA application, the value of the area continued to

decrease until 25 min. When ACC was applied in addition to ABA, the value of the area started to decrease, although slower than in the condition without ACC, and almost ceased by 10 min. This retardation also paralleled the inhibition of stomatal closure by ACC treatment (Fig. III-2A, B). Within 30 min after removal of ABA, both the vacuolar volume and the stomatal aperture recovered to the states before ABA application (Fig. III-2A, B).

Inhibition of GCP size changes by ACC, BA or NAA application

Stomatal aperture is regulated by the changes in guard cell volume. Since the decrease in vacuolar volume was inhibited by ACC application (Fig. III-2A), I measured the changes in guard cell volume itself in response to phytohormone treatment. To measure guard cell volumes easily, I used GCPs. To determine whether the isolated GCPs were viable, I measured their diameters in response to changes in osmotic pressure of the medium. The GCP diameters were found to increase when I reduced the osmotic pressure in the medium (Fig. III-3A), whereas they decreased as the mannitol concentration was increased (Fig. III-3B). When ABA was introduced into the suspension medium, the GCP diameters decreased by 7 %, indicative of the reduction in guard cell volume during stomatal closure. Subsequently, to confirm the effect of ethylene, I applied ACC, BA or NAA into the suspension medium in addition to ABA; the reduced diameter following ABA application was compensated perfectly in WT plants (Fig. III-4A). In contrast, the compensational effects of BA and NAA were not observed in GCPs prepared from either the *ein3-1* mutant or WT plant treated with AVG (Fig. III-4B, C). ACC application were compensated the ABA-induced diameter reduction in GCPs from WT plant treated with AVG (Fig. III-4C), as was observed in epidermal peels (Fig. II-2C).

Discussion

Ethylene inhibits ABA-induced osmotic regulation but not cell wall metabolism

ABA regulates ion channel activities in the vacuolar and plasma membranes of guard cells and triggers effluxes of K^+, Cl^-, and malate across these membranes (MacRobbie, 2000). The reduction in these solutes lowers the osmotic pressure of the guard cells which therefore release water into the apoplasts.

ABA application was found to alter the vacuolar structures of the guard cells from a rather simple form to the more complicated form of the Type 3 cells. ABA application also reduced the vacuolar volume of the guard cells and that of the guard cells themselves. These observations suggest that the reduction in guard cell volume and its vacuolar volume, and the structural changes in the vacuole, are all involved in stomatal closure.

However, ACC application in addition to ABA increased the proportion of Type 2 cells and decreased the Type 3 cells. These observations suggest that ethylene could inhibit the response by ABA at some later stage, when luminal vacuolar structures changed from the folding to bulb-like structures, but not at the start of the structural changes. ACC application in addition to ABA began to decrease the vacuolar volumes, although to a lesser extent than that by ABA alone. These observations are in accordance with the mode of decrease of the stomatal apertures (Fig. III-2).

In the GCPs, however, ACC application completely inhibited the effects of ABA on the changes in volume (Fig. III-4). The differences observed between epidermal peels and GCPs suggest a particular role of the cell wall in stomatal closure. At the early stages of stomatal closure, K^+ was immediately released following ABA application. However, the extent of K^+ efflux was much less than the

decrease in stomatal aperture (MacRobbie 1981). In contrast, the swelling rate of GCPs under light illumination correlated with K^+ uptake (Gotow et al, 1985). It has also been reported that ABA caused an change the physical properties of the cell walls of guard cells in addition to the promotion of Cl^- and malate efflux from the guard cells (Kondo and Maruta, 1987), and that ABA affected cell wall metabolism, such as the synthesis of pectic substances and cellulose (Takeuchi and Kondo, 1988). These observations suggest that changes in the physical properties of the guard cell wall influence stomatal aperture (Meidner 1982) independently of the contribution of solute efflux by ABA.

Therefore, in ABA-induced stomatal closure, ethylene appears to inhibit the ABA-induced reduction of osmotic pressure in the guard cells rather than by affecting cell wall metabolism. The decreases in stomatal aperture and vacuolar volume observed in the presence of ACC might well result from the cell wall alterations induced by ABA.

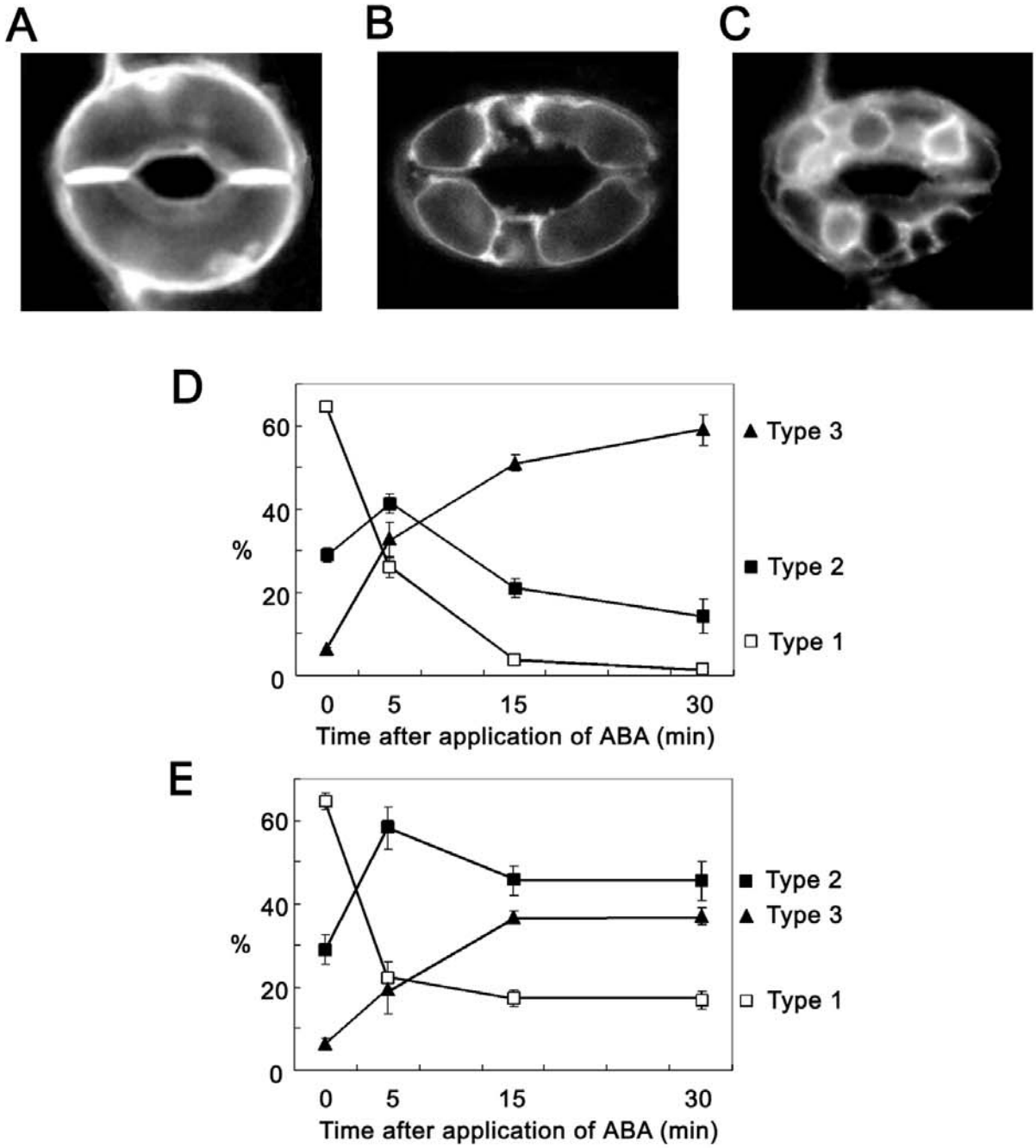


Figure III-1.
ACC inhibits the complicated stomatal vacuolar structures induced by ABA application.

Categorization of guard cells by their vacuolar luminal structures (A to C); guard cells without vacuolar luminal structures (Type 1, A), with some folding structures (Type 2, B), and with bulb-like structures (Type 3, C).

Time-course changes in stomatal vacuolar structures (D, E). The percentages of each cell type were determined after application of ABA (D) or ACC plus ABA (E). The data is representative of three independent experiments with means of 60 stomata. Bars represent means ± SEs.

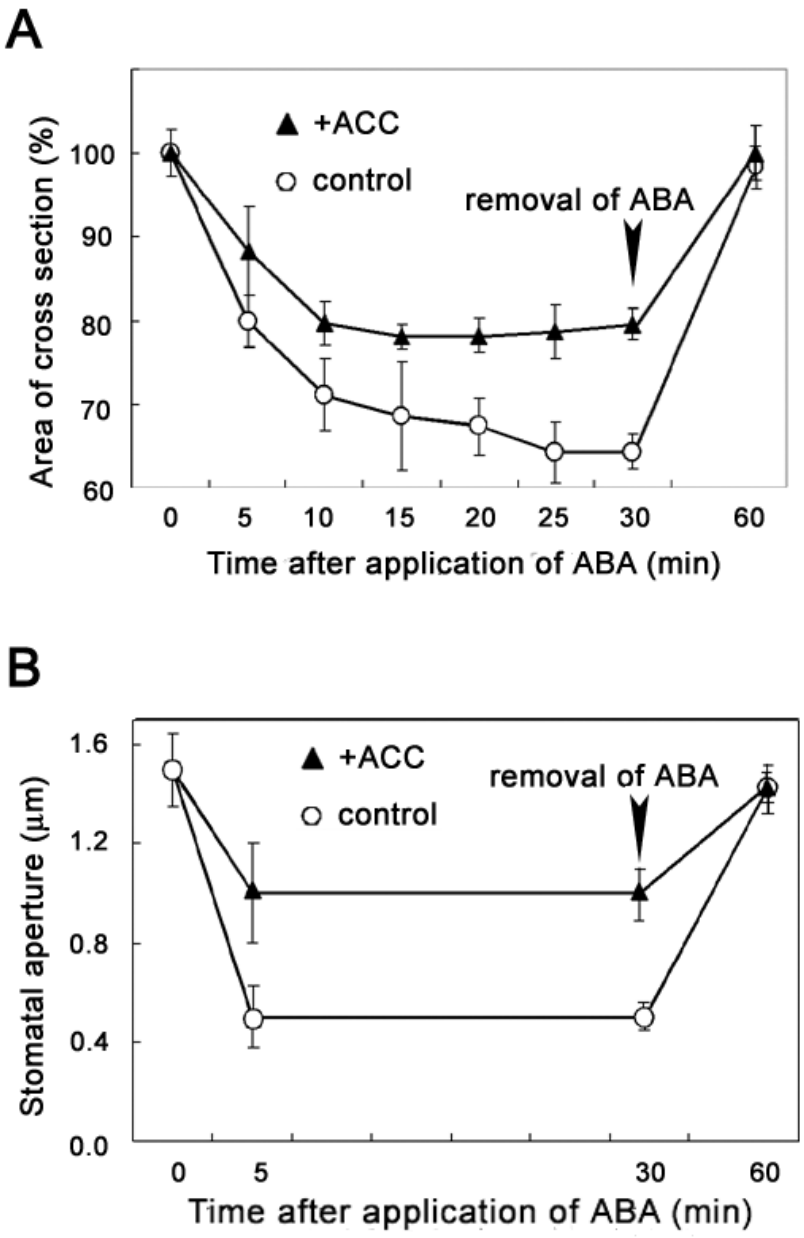


Figure III-2
ACC inhibits the reduction in stomatal vacuolar volume induced by ABA application.

Time-course changes in the stomatal vacuolar volumes (A) and stomatal apertures (B) after application of ABA (open circle) or ACC in addition to ABA (filled triangles).
Vacuolar areas were measured from images of guard cell cross-sections of the transgenic GFP-AtVam3p Arabidopsis line that allows visualization of vacuolar membranes.
The data is representative of three independent experiments with means of 20 stomata per sample time. Bars represent means ± SEs.
After 30 min, both ABA and ACC were removed by exchanging the buffer with fresh buffer.

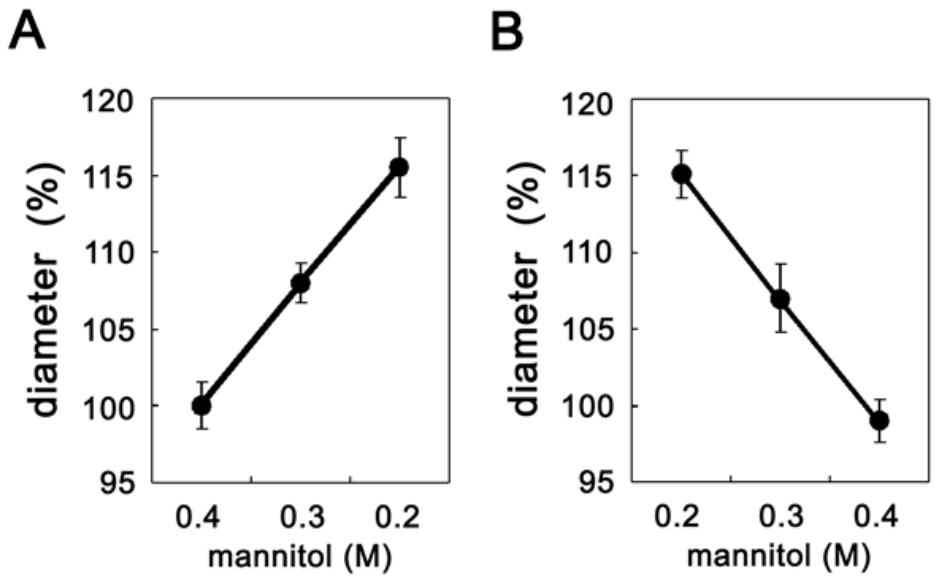


Figure III-3.
Changes in GCP diameter in response to changes in osmotic pressure of the medium.

Changes in the GCP diameter by reducing (A) and increasing (B) the osmotic pressure of the medium. The osmotic pressure of the medium was reduced by the addition of water to the medium (A), and increased by the addition of saturated D-mannitol to the medium (B).

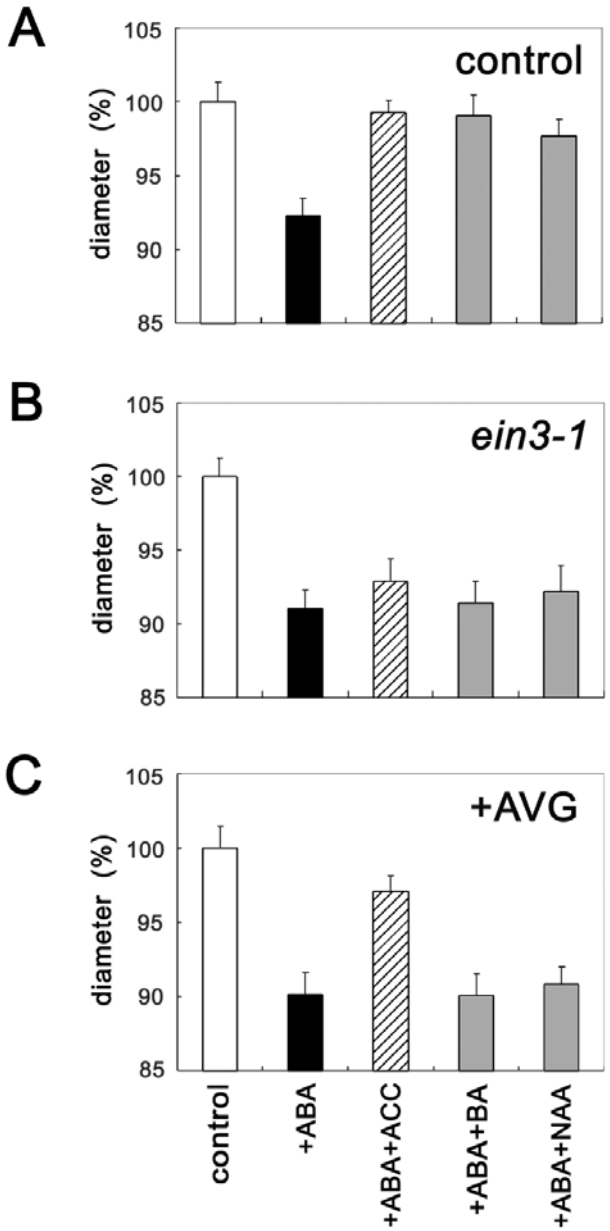


Figure III-4
ACC, BA and NAA cancel the ABA-induced reduction in GCP diameters only when GCPs can transmit the ethylene signal.

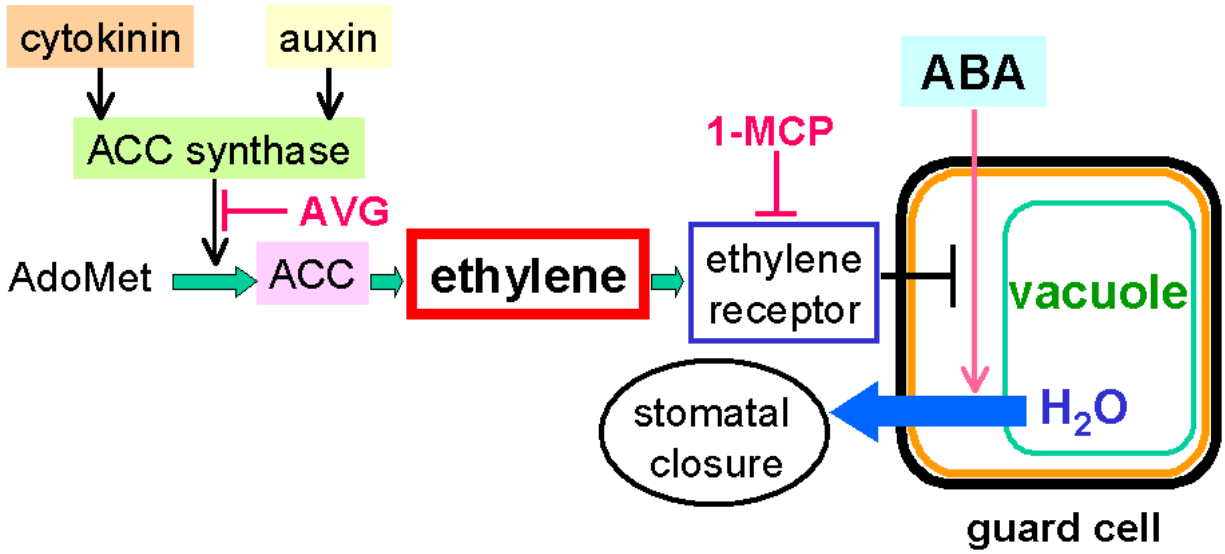
Changes in the diameters of GCPs from WT plants (A), *ein3-1* plants (B) and WT plants treated with AVG (C). The GCP diameters in response to 10 µM ABA and to 10 µM ACC, 10 µM BA or 10 µM NAA in addition to ABA application for 30 min, were compared to those without phytohormone treatment (control).
The data is representative of three independent experiments with means of 100 stomata. Bars represent means ± SEs.

General Conclusions and Prospects

I showed in this study mainly 2 aspects about ABA-induced stomatal closure. One is ethylene inhibits this process and the other is the antagonistic effect of cytokinins and auxins on this process is also the effect of ethylene (Chapter 1, 2 and see the Figure below). The cross talks between ethylene and ABA were already reported in other plant organs such as shoot and root and during other processes, for example, seed germination. It is not the first time that phenomena formally considered as an effect of cytokinins or auxins turn out to be caused by ethylene. In stomatal closure, the above 2 ideas were also true.

Concerning the inhibitive effect of ethylene on stomatal movement, ethylene inhibited almost completely the ABA-induced reduction of osmotic pressure in guard cells. In addition, ABA seemed to regulate the mechanical properties of the cell wall, however, the effect of ethylene on this process still uncertain. Since the efflux of the H^+ to the cell wall decreased its elastic modulus, investigation of the pH regulation in cell wall by ethylene will clarify its role in the cell wall metabolism.

Ethylene is estimated to play a “best supporting role” in plant growth regulation. Arabidopsis plants could provide so many kinds of ethylene mutants compared to other phytohormones mutants since both excess and shortage of ethylene is not lethal in plants. Using these mutants, it will be possible to clarify the mutual relationship between ethylene and ABA and their effect on the plant life. It would be also clarified why ethylene, a relatively simple substance which consists of two carbons and four hydrogens is involved in so many stress responses. In Arabidopsis plants, in spite of the difficulties in observation of the stomata because of its small size, the genetic analysis is a powerful tool to analyze the plant growth and development.



A possible scheme about the inhibitive effect of ethylene on ABA-induced stomatal closure.

The antagonistic effects of cytokinin and auxin against ABA-induced stomatal closure were induced by ethylene synthesis since this process was cancelled by treatment of AVG, which inhibits ACC synthase activity. The inhibitive effects of ethylene, cytokinin or auxin were also cancelled by 1-MCP, an inhibitor of ethylene receptor(s). During the ABA-induced stomatal closure, ethylene was supposed to inhibit the ABA-induced water efflux from the guard cells since the reduction of the guard cell and their vacuolar volume by ABA application was inhibited by ethylene treatment.

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