Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in Arabidopsis

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Abstract

Cytokinins and auxins are major phytohormones involved in various aspects of plant growth and development. These phytohormones are also known to antagonize the effects of abscisic acid (ABA) on stomatal movement, and to affect ethylene biosynthesis. As ethylene has an antagonistic effect on ABA-induced stomatal closure, the possibility that the antagonistic effects of these phytohormones on ABA were mediated through ethylene biosynthesis was investigated. Both the cytokinin, 6-benzyladenine (BA), and the auxin, 1-naphthaleneacetic acid (NAA), antagonized ABA-induced stomatal closure in a manner similar to that following application of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). However, these effects were negated when ethylene signalling, perception, or biosynthesis were blocked. As stomatal aperture is regulated by changes in guard cell volume, ABA application was found to reduce the volume of the guard cell protoplasts (GCP). It was found that BA, NAA, or ACC application compensated perfectly for the reduction in GCP volume by ABA application in WT plants. The above observations suggest that cytokinins and auxins inhibit ABA-induced stomatal closure through the modulation of ethylene biosynthesis, and that ethylene inhibits the ABA-induced reduction of osmotic pressure in the guard cells.

Key words: Abscisic acid, auxin, cytokinin, ethylene, guard cell, stomatal closure.

Introduction

Regulation of stomatal aperture by guard cells is crucial for minimizing water loss from leaf tissues and maximizing CO2 exchange for photosynthesis. In response to various environmental stresses, such as drought, cold, air pollutants, and high CO2 concentrations, stomata close in a process that involves the phytohormone, abscisic acid (ABA) (Schroeder et al., 2001).

Cytokinins and auxins 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 stomata, cytokinin inhibits the effects of ABA in isolated epidermal strips of Commelina benghalensis (Das et al., 1976). 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). With regard to stomatal movement, auxin is found to repress stomatal closure in the dark by CO2 exposure and ABA application in isolated epidermal peels of Commelina communis (Snaith and Mansfield, 1982; reviewed by Mansfield and McAinsh, 1995).

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; AVG, aminoethoxyvinyl glycine; BA, 6-benzyladenine; ein3-1, ethylene insensitive3-1; GCP, guard cell protoplast; 1-MCP, 1-methylcyclopropene; MES, 2-morpholinoethanesulphonic acid, monohydrate; NAA, 1-naphthaleneacetic acid; PCR, polymerase chain reaction; WT, wild type.

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In addition, 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 was found to induce ethylene biosynthesis markedly (Abeles and Rubinstein, 1964), and in a mutant plant with elevated endogenous auxin concentrations, phenotypic alterations suggested an increased response to auxin or ethylene (King et al., 1995). 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 catalysed by ACC synthase (ACS), is generally considered as the rate-limiting step of ethylene biosynthesis. An enhanced level of ACS gene expression by auxin has also been reported in guard cells (Nakagawa et al., 1991; Liang et al., 1992).

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 detached rice leaves, and this promotion was found to be due to enhanced ACC and/or ethylene production (McKeon et al., 1982). A recent study also revealed that cytokinins increased the stability of the ACS protein (Chae et al., 2003). Overall, these studies suggest that the various physiological responses to auxins and cytokinins could be modulated by ethylene biosynthesis.

In a previous paper, it was demonstrated that ABA-induced stomatal closure was inhibited by ethylene treatment (Tanaka et al., 2005). The mechanism of this 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 vacuole-stored solutes are first transported into the cytoplasm and then to the apoplasts by activation of the appropriate ion channels and transporters of the vacuolar and plasma membranes (MacRobbie, 2000). The reduced osmotic pressure following 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. However, the mechanism by which ethylene antagonizes ABA-induced stomatal closure is still not well understood. In this study, it was first investigated whether the effects of auxin and cytokinin on the inhibition of stomatal closure were modulated through ethylene biosynthesis using Arabidopsis leaf epidermal tissues. In the next step, the mechanism by which ethylene antagonizes the effects of ABA was investigated by measurement of the changes in guard cell volume following phytohormone treatment. For this, guard cell protoplasts were used which possess simple spherical forms, and in which changes in osmotic pressure become directly reflected into simple changes in their diameters.

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. 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 16/8 h light/dark cycles.

Measurement of stomatal aperture

The abaxial epidermis was peeled from the rosette leaves of 5–6-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. For the application of phytohormones, the epidermal peels with pre-opened stomata were transferred to the same buffer supplemented with 10 μM ABA (Sigma-Aldrich, MO, USA), with or without the addition of 10 μM of either BA, NAA, gibberellin acid (GA\(_3\)), or ACC. For dark conditions, preopened epidermal peels were incubated in the dark for 2 h with or without the addition of 10 μM of either BA, NAA, or fusicoccin (Sigma-Aldrich, MO, USA). Stomatal apertures were determined on the basis of their 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 glycin (AVG)

Treatment of samples with 1-MCP was performed by evaporating 5.6 mg 1-MCP, dissolved in 85 μl distilled water, in a closed chamber 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 glycin (AVG), 10 μM AVG was added to the opening buffer during the experiment.

Isolation of guard cell protoplasts (GCPs) and measurement of GCP volume changes

GCPs were isolated from leaves of 4–5-week-old Arabidopsis thaliana plants by overnight enzymatic digestion according to the method of Pandey et al. (2002). Collected GCPs were preincubated in the dark in GCP reaction buffer (0.3 M mannitol, 10 mM KCl, and 1 mM CaCl\(_2\)) prior to use. To increase or decrease the osmotic pressure in the suspension medium, saturated d-mannitol or distilled water, respectively, was added into the medium. The response of the Arabidopsis GCPs was monitored by incubation for 1 h under light in GCP reaction buffer containing 10 μM ABA with or without the addition of 10 μM of either BA, NAA, or ACC. GCPs were observed using 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 AVG, 1 μM AVG was added to the GCP reaction buffer during the experiment.
Gene expression analyses

To analyse the ABA responses of GCPs, total RNA was extracted from 1 ml of GCPs incubated for 1 h with or without phytohormones under light, using Trizol (Invitrogen, 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) using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer’s specifications. As an internal standard for cDNA amounts, a 143 bp fragment of the actin-7 cDNA was amplified with the PCR primers, 5’-GGAAT-TGTCCGTGACATAAAGGAG-3’ (upstream primer) and 5’-CTC-CTCGACTCCGGATTTGATGCTT-3’ (downstream primer). A 172 bp fragment of the ABI2 cDNA was amplified with the PCR primers, 5’-TTCTATCTCGCCGCTTTCAT-3’ (upstream primer) and 5’-ACCACACTTCTCTTGTGGA-3’ (downstream primer). A 180 bp fragment of the SAG13 cDNA was amplified with PCR primers, 5’-TCGTCAACAAATGTTGGAACG-3’ (upstream primer) and 5’-CGACTCCAGCAGCAGAGGAT-3’ (downstream primer).

Results

Cytokinin and auxin inhibit ABA-induced stomatal closure through ethylene biosynthesis

To examine the effects of cytokinin and auxin on ABA-induced stomatal closure, an in vitro system using isolated epidermal peels was used in which stomatal apertures could be measured. After light illumination of WT plants, the stomata opened and their apertures increased to approximately 2.94 μm. ABA application closed the stomata almost completely, with stomatal apertures decreasing to approximately 0.92 μm (Fig. 1A). When BA or NAA were applied together with ABA to the isolated epidermal peels, ABA-induced stomatal closure was suppressed; stomatal apertures decreased to only 1.8 or 2.0 μm, respectively, and the stomata remained in a half-opened state. By contrast, GA3 had no inhibitory effect on ABA-induced stomatal closure. The mode of suppression of ABA-induced stomatal closure by BA or NAA application was similar to that achieved by ACC application (Fig. 1A).

Under dark conditions, the stomata closed as with ABA application while fusicoccin application opened the stomata rather wider than that following light illumination (Fig. 1B). However, neither BA nor NAA application suppressed the dark-induced stomatal closure, nor did they enhance stomatal opening under light conditions (Fig. 1C).

The above results demonstrated that BA and NAA suppressed stomatal closure induced by ABA but not by dark conditions. Since these effects were similar to those following ethylene treatment (Tanaka et al., 2005), the mode of stomatal closure was examined in an ethylene signalling mutant and in WT plants treated with inhibitors of ethylene receptor(s) and biosynthesis. In the ein3-1 (ethylene insensitive3-1) mutant, which cannot transmit the ethylene signal (Roman et al., 1995), neither BA nor NAA application affected ABA-induced stomatal closure as also observed with ACC application (Fig. 2A; Tanaka et al., 2005). In addition, overnight treatment with 1-methylcyclopropene (1-MCP, Sisler and Serek, 1997), a competitive inhibitor of ethylene receptor(s), negated the effects of BA and NAA on stomatal responses, as also observed with ACC application (Fig. 2B; Tanaka et al., 2005). Furthermore, application of aminoethoxyvinyl glycone (AVG), which inhibits ACC synthase (ACS) activity and results in the inhibition of endogenous ethylene biosynthesis (Yoshii and Imaseki, 1982), inhibited

![Fig. 1. BA, NAA, and ACC applications impair ABA-induced stomatal closure. (A) Stomatal apertures of WT plants preopened by white light illumination (without ABA), and after incubation with 10 μM BA (control) and with 10 μM NAA, 10 μM GA3, or 10 μM ACC in addition to ABA application for 2 h. (B) Stomatal apertures of WT plants preopened by white light illumination (light), after incubation in the dark (control), and with 10 μM BA, 10 μM NAA, or 10 μM fusicoccin (+FC) for 2 h in the dark. (C) Stomatal apertures of WT plants preopened by white light illumination (control) and with 10 μM BA or 10 μM NAA for 2 h. The data are representative of three independent experiments with the means of 100 stomata. Bars represent means ±SEs. Differences between control and +BA, +NAA, or +ACC shown by asterisks in (A) are significant (P <0.01).](https://academic.oup.com/jxb/article-abstract/57/10/2259/472600)
the effects of BA or NAA, whereas exogenous ACC application still inhibited ABA-induced stomatal closure (Fig. 2C). To confirm these findings, the mode of stomatal closure in the cytokinin over-producing mutant, \textit{amp1-1} (Chaudhury \textit{et al.}, 1993) was analysed, and it was found that ABA-induced stomatal closure was inhibited, whereas AVG treatment in addition to ABA promoted stomatal closure (Fig. 3). These results suggest that the inhibitive effects of BA and NAA on ABA-induced stomatal closure result from the enhanced production of ethylene.

\textit{Inhibition of GCP volume reduction by BA, NAA or ACC application}

\textit{Arabidopsis} guard cells are surrounded by epidermal cells through 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, guard cell protoplasts (GCPs) isolated from the epidermal tissues were used. As stomatal aperture is regulated by changes in guard cell volume, the changes in GCP volume were examined in response to phytohormone treatment. To determine whether the isolated GCPs were responsive to changes in osmotic pressure in the medium, the subsequent changes in their diameters were measured. The GCP diameters increased with reduced osmotic pressure in the medium (Fig. 4A), whereas the diameters decreased with increased mannitol concentration in the medium (Fig. 4B). Addition of ABA into the suspension medium resulted in a 7\% reduction in the GCP diameters; equivalent to a 20\% reduction in guard cell volume during stomatal closure. However, addition of BA or NAA into the suspension medium in addition to ABA perfectly compensated for the reduced diameters following ABA application in WT plants, as also observed following ACC application (Fig. 5A). By contrast, these compensational effects of BA and NAA were not observed in GCPs prepared from the \textit{ein3-1} mutant or WT plants treated with AVG (Fig. 5B, C). Furthermore, ACC suppressed the ABA-induced reduction in the diameters of GCPs prepared from WT plants treated with AVG but not from the \textit{ein3-1} mutant (Fig. 5C), as was also observed in epidermal peels (Fig. 2C).

\textit{Differential expression of ethylene-responsive and ABA-induced genes in guard cell protoplasts}

To monitor the responses of GCPs to phytohormones at the molecular level, the expression patterns of an ABA-induced gene, \textit{ABI2}, and an ethylene-response gene, \textit{SAG13}, were examined by quantitative real-time PCR. Following ABA treatment, \textit{ABI2} expression was enhanced, whereas simultaneous treatment with ABA and BA, NAA or ACC decreased the \textit{ABI2} transcript levels (0.01 \( \leq P \leq 0.05 \))
By contrast, SAG13 transcript levels were elevated by ACC and also BA or NAA (0.01 \( < P < 0.05 \)), but not by ABA treatment (Fig. 6B). These results indicate that the guard cells could respond to phytohormones even in the absence of their cell walls.

Discussion

Cytokinin and auxin inhibit ABA-induced stomatal closure through acceleration of ethylene biosynthesis

In the leaf epidermis of Arabidopsis, BA or NAA application inhibits ABA-induced stomatal closure similar to that following ACC treatment (Fig. 1A; Tanaka et al., 2005). The same effect was observed upon application of kinetin, an alternative cytokinin, or IAA, an alternative auxin (data not shown). As both BA and NAA were unable to inhibit dark-induced stomatal closure or to increase stomatal opening under light illumination (Fig. 1B, C), it was concluded that cytokinin and auxin were only effective in suppressing ABA-induced stomatal closure. This was confirmed by the observed inhibition of stomatal closure in the cytokinin-over-producing amp1-1 mutant (Fig. 3), which has 7-fold higher zeatin levels than WT seedlings (Chaudhury et al., 1993). Indeed, the inhibitory effects of these phytohormones were suggested several years previously (Das et al., 1976; Snaith and Mansfield, 1982; Mansfield and Mc. Inish, 1995). Furthermore, the antagonistic effects of cytokinins on ABA-induced stomatal movement were indirectly implied by measurement of the delivery rates of these phytohormones in xylem sap of almond trees during the annual drying cycle or upon drought stress of sunflower plants (Fußeder et al., 1992; Shashidhar et al., 1996). The results of this current study, based on stomatal aperture measurements, demonstrate directly an antagonistic effect of cytokinin or auxin on ABA-induced stomatal closure.

The results from 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 signalling pathway, following cytokinin or auxin application, in stomatal movement (Figs 2, 3). The observation that ACC application compensated for the effects of AVG (Figs 2C, 5C) concurs with the inhibitory effects of AVG on the activity of ACS, which catalyses the formation of ACC from AdoMet (Chi et al., 1991). Furthermore, this compensation suggested limited side-effects of ACC application on stomatal closure. Taken together with previous findings (Tanaka et al., 2005), it is proposed that auxin and cytokinin inhibit ABA-induced stomatal closure by accelerating ethylene biosynthesis, especially upstream of ACS activity.

However, in aquatic plants, such as deepwater rice or Rumex palustris, submergence induced ethylene production and resulted in shoot elongation and concomitant stomatal closure (Bradford and Hsiao, 1982; Raskin and...
The responsiveness of GCPs to cytokinin and auxin application

Guard cell protoplasts (GCPs) provide a useful in vitro system since their volume changes in response to osmotic pressure can easily be determined by measuring the changes in their diameters. The responsiveness of GCPs isolated from Vicia faba to stimuli, such as light, fusicoccin, and ABA, was similarly examined by measurement of the changes in their diameters (Zeiger and Hepler, 1977; Schnabl et al., 1978). In the current study, the reduced diameters of GCPs isolated from Arabidopsis epidermal tissues suggested a reduction in guard cell volume during stomatal closure (Fig. 5A). Enhanced expression of the ABI2 and SAG13 genes also confirms GCP responsiveness to both ABA and ethylene (Fig. 6), even though the guard cells are surrounded by epidermal cells through which they exchange materials.

The observed inhibition of the ABA-induced reductions in volume by ACC application is indicative of the antagonistic effects of ethylene on stomatal closure, and is in accordance with previous findings using epidermal tissues (Fig. 5A; Tanaka et al., 2005). In this current study, this negation was also observed with BA or NAA application (Fig. 5A) which, together with the lack of effect of BA and NAA in GCPs from the ein3-1 mutant and those treated with AVG, suggest the involvement of ethylene signalling or ethylene production in the responses to cytokinin and auxin (Fig. 5B, C). Repression of ABI2 gene expression by BA or NAA as well as by ACC application also suggests the involvement of these phytohormones in the ABA-signalling pathway (Fig. 6A). Although the levels of ethylene produced were not measured directly, the increased levels of SAG13 gene expression upon BA or NAA application are suggestive of increased ethylene production (Fig. 6B). Increased ACS gene expression in Arabidopsis leaves and guard cells by application of the auxin, indole-3-acetic acid (Arteca and Arteca, 1999; Tsuichikawa and Theologis, 2004), supports this assumption. Thus, the GCP experiments confirm the conclusions using epidermal peels, and suggest that ethylene production in response to BA or NAA application occurs within the guard cells themselves.

Interestingly, ethylene almost completely inhibited the effects of ABA in the GCPs, whereas this inhibition was only partial in the epidermal peels (compare Figs 1A and 5A). This implies a particular role of the cell wall of guard cells 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). It has also been reported that ABA affected the physical properties and metabolism of the cell walls of guard cells in addition to the promotion of Cl− and malate efflux from these cells (Kondo and Maruta, 1987; Takeuchi and Kondo, 1988). Therefore, the partial inhibition of ABA-induced stomatal closure by ACC observed in the epidermal peels might well result from the cell wall alterations induced by ABA. During stomatal closure, ethylene would inhibit ABA-induced reductions in osmotic pressure in the guard cells rather than alter cell wall metabolism.

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