INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17F–010/2017/19–5–1220–1226
DOI: 10.17957/IJAB/15.0429
http://www.fspublishers.org

Full Length Article

Role of Cytoplasmic Alkalization and Nitric Oxide in Ethylene-induced Stomatal Closure in Arabidopsis

Chenyu Shi[1,2], Feifei Chen[1], Tian Peng[3] and Xiaoping She[1*]
[1]*School of Life Sciences, Shaanxi Normal University, Xi'an 710062, China*
[2]*School of Chemistry and Bioengineering, Hechi University, Yizhou 546300, China*
[3]*Xi'an Tie Yi High School, Xi'an 710054, China*
[*]For correspondence: shexiaoping@snnu.edu.cn

Abstract

Ethylene-induced stomatal closure is well-known. However, the mechanism by which ethylene closes stomata remains clear. This research was conducted to explore the roles of guard cell cytoplasmic alkalization and nitric oxide (NO) and the relationship between them during stomatal closure by ethylene. To achieve this goal, we used pharmacological approach, confocal laser scanning microscope and Arabidopsis mutant *etr1-1* and *etr1-3*, which have defect in ethylene perception and *Nia2-1*, *Nia1-2* and *Nia1-2/Nia2-5*, which are NO generation enzyme nitrate reductase (NR) mutant. Our data show that ethylene precursor ACC induced stomatal closure by promoting guard cell cytoplasmic alkalization and subsequent NO synthesis. ACC caused the rises in cytosol pH and NO level and promoted stomatal closing in the wild type but did not in mutant *etr1-1* and *etr1-3*. Furthermore, ACC failed to induce guard cells cytosol alkalization in the wild type in the presence of butyric acid and NO generation in *Nia1-2* and *Nia1-2/Nia2-5*, which is coincide with its effects on stomatal aperture in these plants. Cytosol alkalization and Nia1-dependent NO generation were vital for ethylene-led reduction of stomatal aperture. Moreover, the rise in cytosol pH was prerequisite for NO production by ethylene. Butyric acid prevented ACC-triggered NO synthesis in the wild type but ACC enhanced cytosol pH in *Nia1-2* and *Nia1-2/Nia2-5*. SNP rescued the defect of ACC-led stomata closing in the wild type in the presence of butyric acid but methylamine did not reverse the impairment of ACC-led stomata closing in *Nia1-2* and *Nia1-2/Nia2-5*. Taken together, the present study unambiguously reveals that ethylene induces guard cell cytosol alkalization, and then promotes Nia1-dependent NO synthesis and finally initiates stomata closing in Arabidopsis. © 2017 Friends Science Publishers

Keywords: Ethylene; Cytosol alkalization; NO synthesis; Stomatal closure

Introduction

Ethylene, as an important plant hormone, affects numerous aspects of plant live, such as seed germination, seedling growth, fruit ripening, leaves senescence and abscission, and so on (Guo and Ecker, 2004; Romera *et al*., 2017). Stomata control gas exchange and transpiration. Stomatal aperture or conductance is regulated by many external stimulu and internal factors (Mansfield *et al*., 1990; Iqbal *et al*., 2015; Ahmed *et al*., 2017). Ethylene also promotes stomata closing (Young *et al*., 2004; Desikan *et al*., 2006), although it has been known to prevent abscisic acid (ABA)-led stomata closing (Tanaka *et al*., 2005; Watkins *et al*., 2014). In Arabidopsis, ethylene is perceived by its receptors, including ETR1 (Hwang *et al*., 2002; Grefen and Harter, 2004). Constitutive triple response1 (CTR1) is a negative modulator of ethylene signaling pathway, while ethylene insensitive 2, 3, 5 and 6 are positive modulators of the pathway, which act downstream of CTR1. In the deficiency of ethylene, CTR1 depresses ethylene insensitive 2, 3, 5 and 6. Once ethylene is bound to its receptors, CTR1 is inactivated, thus relieve this repression of the pathway and final leads to the regulation of ethylene-controlled gene expression (Guo and Ecker, 2004).

Intracellular pH changes regulate a variety of plant biological processes, including tip growth (Gibbon and Kropf, 1994), gravitropism (Scott and Allen, 1999), nodulation (Felle *et al*., 1996), response to hormones such as ABA (Beffagna *et al*., 1997) and so on. A great deal of studies has shown that guard cells cytoplasmic pH alterations also affect plant stomatal movement. Weak alkalinizing agent methylamine or benzylamine raises guard cells cytosolic pH, also closes stomata. Butyrate, which is a weak acid, reduces guard cells cytoplasmic pH and induces stomata opening (Irving *et al*., 1992; Gonugunta *et al*., 2008). This relevance between cytosolic pH changes and stomatal movement suggests that guard cells cytosolic pH plays role in stomatal movement. Significantly, several studies have shown that guard cell cytosolic alkalization mediate stomata closing by ABA-, methyl jasmonate (MJ)- and light/darkness transition (Suhita *et al*., 2004; Ma *et al*., 2012), while cytosolic pH reduction is involved in stomata

To cite this paper: Shi, C., F. Chen, T. Peng and X. She, 2017. Role of cytoplasmic alkalization and nitric oxide in ethylene-induced stomatal closure in Arabidopsis. *Int. J. Agric. Biol*. 19: 1220–1226

opening caused by indole-3-acetic acid (IAA)-, kinetin- and fusicoccin (FC) (Irving *et al*., 1992; Suhita *et al*., 2004; Gonugunta *et al*., 2008). The data supports that cytoplasmic pH changes, as important signaling event are implicated in stomatal movement by different stimuli.

Nitric oxide (NO) has wide-ranging effects in plant. It mediates plant disease resistance (Delledonne *et al*., 1998), growth and development (He *et al*., 2004; Prado *et al*., 2004; Bethke *et al*., 2006) and response to abiotic stimuli (Siddiqui *et al*., 2011). NO has also been revealed to be a common signal during stomatal closing by ABA, MJ, salicylic acid (SA), elicitors or light/darkness transition (García-Mata and Lamattina, 2002; Liu *et al*., 2003; She *et al*., 2004; Munemasa *et al*., 2007; Srivastava *et al*., 2009). NO synthase (NOS) and nitrate reductase (NR) are two main the NO generation enzyme in plant cells. Mammalian NOS inhibitor prevention of ABA-led NO production and stomata closing (Neill *et al*., 2002a) suggests that NO sourced from NOS-type enzyme mediates stomatal closure by ABA. Furthermore, NO generated from NR is essential for ABA-led stomata closing in Arabidopsis (Desikan *et al*., 2002).

Considering the facts that hydrogen dioxide (H_2O_2), a form of reactive oxygen species is involved in ethylene-led stomata closing (Desikan *et al*., 2006) guard cell cytosolic alkalization mediates ABA-led stomata closing by stimulating H_2O_2 and NO accumulation (Suhita *et al*., 2004; Gonugunta *et al*., 2008) and NO generation is H_2O_2 synthesis-dependent during stomata closing by ABA (Bright *et al*., 2006). A previous study (Liu *et al*., 2010) also supports this postulation. This was hypothesized that ethylene causes guard cell cytosolic alkalization with promotes NO accumulation and stomata are closed. To examine this hypothesis, pharmacological approach and Arabidopsis mutant *etr1-1* and *etr1-3*, which have defect in ethylene perception and *Nia2-1*, *Nia1-2* and *Nia1-2/Nia2-5*, which are NO generation enzyme NR mutants, were used.

Materials and Methods

Chemicals

As previously described by Ma *et al*. (2012) and Shi *et al*. (2015), fluorescent indicator dye DAF-2 DA, ACC, butyric acid, methylamine, SNP, BCECF-AM, MES and c-PTIO were purchased from Sigma-Aldrich, whereas DMSO was from Amresco (Solon, OH and US). The remaining agents were from various suppliers of Chinese companies.

Plant Material

Seeds of Arabidopsis (*Arabidopsis thaliana*) wild type ecotype Columbia (Col-0) and Landsberg *erecta* (Ler) and the seeds of *etr1-3*, *Nia1-2*, *Nia2-1* and *Nia2-5*/*Nia1-2* mutant (background Col-O) were from Nottingham Arabidopsis Stock Center. Seeds of *etr1-1* mutant come from Arabidopsis Biological Resource Center. These seeds were sown in potting mix and grown in plant growth chambers with a 16 h light/8 h dark cycle and a 22°C/18°C day/night temperature cycle. Photon flux density during photophase was 0.1 mmol m^{-2} sec^{-1}. Fully expanded rosette leaves of four six-week-old seedlings were harvested and used immediately. Genotypes of various mutants were confirmed by PCR analysis.

Stomatal Bioassay

Stomatal aperture was monitored using the method of Desikan *et al*. (2006) with minor changes. Briefly the leaves from all seedlings were incubated in MES-KCl buffer (50 mM KCl, 10 mM MES-KOH and pH 6.15) for 3 h under a photon flux density of 0.1 mmol m^{-2} sec^{-1} and at 22°C. Once stomata were fully open different treatment was carried out. To know the effect of ethylene SNP or methylamine on stomata, the leaves from the wild type and mutant *etr1-1* or *etr1-3* were floated in MES-KCl buffer alone or containing 10 µM ACC, 100 µM SNP or 2 mM methylamine. To study the role and enzymatic source of NO in stomatal closure by ethylene, the leaves from the wild type and mutant *Nia1-2*, *Nia2-1* or *Nia2-5/Nia1-2* were floated in MES-KCl buffer without or with 200 µM c-PTIO or 100 µM tungstate alone or with 10 µM ACC. To study the role of guard cell cytoplasmic pH change in stomatal closure by ethylene, the leaves of the wild type were floated in MES-KCl buffer without or with 0.5 mM butyric acid alone or with 10 µM ACC. To study the relationship between guard cell cytoplasmic pH change and NO in stomatal closure by ethylene, the leaves of the wild type and mutant *Nia1-2*, *Nia 2-1* or *Nia1-2/Nia2-5* were floated in MES-KCl buffer without or with 200 µM c-PTIO or 100 µM tungstate alone or with 10 µM ACC, 2 mM methylamine or 10 µM ACC and 2 mM methylamine, or the leaves of the wild type were floated in MES-KCl buffer without or with 0.5 mM butyric acid alone or with 10 µM ACC, 100 µM SNP or 10 µM ACC and 100 µM SNP. The incubation time, temperature and light condition were 3 h, 22°C and 0.1 mmol m^{-2} sec^{-1}, respectively. After these treatments, epidermic strips were carefully peeled from abaxial surfaces of the treated leaves stomatal apertures were recorded with a light microscope.

In order to exclude possible rhythmic effects experimentations were always carried out at daily same time. In every one treatment, fifty randomly picked apertures were scored and each treatment contained three independent repetitions. The data shown are the means of 150 tests ± SE.

Measurement of Guard Cells Cytosolic pH and NO Level

Changes in pH were monitored in the strips by incubation with BCECF-AM as shown by Irving *et al*. (1992). NO accumulation in guard cells was measured by using DAF-2 DA as described by Kojima *et al*. (1998). Briefly, the

treatments were done as described those in stomatal bioassay section. Then, cuticles were striped and promptly loaded with 10 µM DAF-2 DA for 30 min or 20 µM BCECF-AM for 10 min in Tris-KCl loading buffer (Tris 10 mM and KCl 50 mM, pH 7.2) in darkness at 25°C. Excess dye was washed out with fresh Tris-KCl loading buffer in the darkness the detection of the peels was immediately carried out with TCS-SP5 confocal laser scanning microscope with the following settings: emission at 530 nm and excitation at 488 nm. Images obtained from the confocal microscope were processed with PHOTOSHOP and analyzed with Leica Image software. In each treatment, three strips derived from different plant were monitored and every treatment was repeated three times. The selected images represented the same results from nine time tests.

Statistical Analysis

Statistical analyses were performed using a one-way ANOVA followed by the least significant difference test.

Results

Rise in Guard Cell Cytoplasmic pH is Involved in Stomatal Closing by Ethylene

To evaluate whether cytosol pH alterations in guard cell are implicated in ethylene-led stomatal closing the effects of ACC, an immediate precursor of ethylene, on stomatal aperture and guard cell cytosol pH in the wild type and mutant *etr1-1* or *etr1-3* were measured. ACC raised guard cell cytosol pH also induced stomata to close in the wild type (Fig. 1A, D and E) the effects of ACC were abolished in *etr1-1* and *etr1-3* (Fig. 1A, D and E). However, methylamine, a weak alkalinizing agent closed stomata in *etr1-1* and *etr1-3*, like in the wild type (Fig. 1A). The results unequivocally indicate that guard cell cytosol alkalization is an essential signal in stomatal closure by ethylene and a functional ETR1 protein is necessary for ethylene-led cytosol alkalization and subsequent stomatal closure.

To continue to probe the role of guard cell cytosol alkalization during ethylene-led stomatal closure, the influences of butyric acid, a weak acid, on ACC-led stomatal closing and alters in guard cells cytosol pH in the wild type were measured. Butyric acid fully prevented ACC-induced guard cell cytosol alkalization and completely inhibited stomata closing by ACC (Fig. 2A, B and C). The data support that guard cells cytosol alkalization has a vital role in ethylene-led stomatal closing.

NO Participates in Stomatal Closing by Ethylene

To assess whether NO mediates guard cell ethylene signaling, Arabidopsis wild type and mutant *etr1-1* and *etr1-3* were used and the effects of ACC on stomatal behavior and NO production were measured.

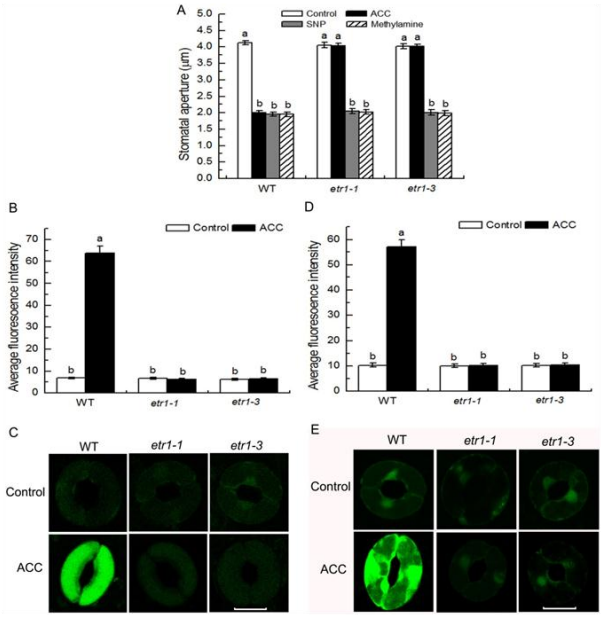

Fig. 1: Effects of ethylene on cytosol alkalization, NO production and stomatal closure are *etr1*-dependent. Leaves of the WT (Col-0) or *etr1*mutants with open stomata were incubated in MES buffer alone (Control) or containing 10 µM ACC, 100µM SNP or 2 mM methylamine for 3 h then epidermal strips were peeled from abaxial surfaces of the treated leaves. A, Stomatal apertures were measured in epidermal strips. B to E, Fluorescence pixel intensities (B) and images (C) in guard cells preloaded with 10 µM DAF-2 DA for 30 min and pixel intensities (D) and fluorescence images (E) in guard cells preloaded with 20 µM BCECF-AM for 10 min, in darkness were recorded. Each assay was repeated at least three times. Data of stomatal aperture are displayed as means ± SE (n = 150) and means with different letters are significantly different at P <0.01. Data of fluorescence pixel intensities are displayed as means ± SE (n = 60) and means with different letters are significantly different at P<0.05. Bars in C and E= 10 µm for all images

ACC stimulated NO synthesis, also reduced the aperture of stomata in the wild type, these effects of ACC were abolished in *etr1-1* and *etr1-3*. However, SNP which is a NO-releasing compound, reduced the aperture of stomata not only in the wild type but also in *etr1* mutants (Fig. 1A, B and C). The data confessedly show that NO, as a signal, mediates ethylene-led stomatal closing in Arabidopsis and a functional ETR1 protein is required for ethylene-led NO accumulation and stomatal closing.

To further study the role and the enzymatic source of NO in ethylene-led stomatal closing, the effects of NO scavenger c-PTIO and NR inhibitor tungstate on ACC-led guard cell NO accumulation and stomatal closing in the wild type were tested. c-PTIO obviously reduced ACC-triggered NO accumulation in guard cells and largely

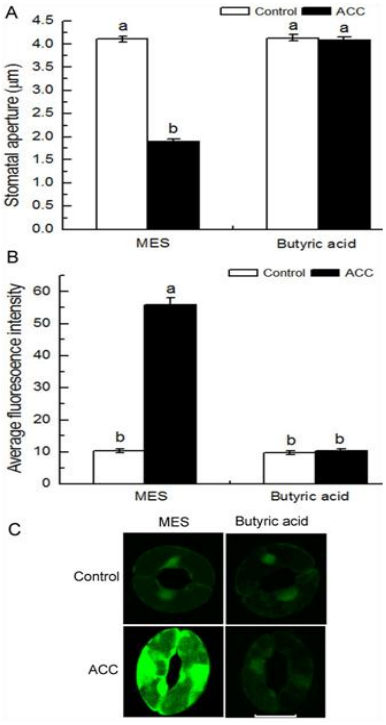

Fig. 2: Cytosol alkalization mediates ethylene-induced stomatal closure. Arabidopsis leaves of wild-type (WT) Col-0 with open stomata were incubated in MES buffer without or with 0.5 mM butyric acid alone (Control) or with 10 μM ACC for 3 h. A, Stomatal apertures were measured in epidermal strips. B and C Fluorescence pixel intensities (B) and images (C) in guard cells preloaded with 20 μM BCECF-AM for 10 min in darkness were recorded. Bar in C = 10 μm for all images. Other explanations are the same as in Fig. 1

inhibited ACC-led stomatal closing (Fig. 3A, B and C), which suggests that NO plays in ethylene-caused stomatal closing. Further, tungstate greatly prevented ACC-led stomatal closuring (Fig. 3A), which is in accordance with the effect of tungstate on NO generation (Fig. 3B and C). The data show that NO sourced from NR is an intermediate signal molecular in ethylene-caused stomatal closing. To confirme the pharmacological results, mutants *Nia1-2*, *Nia2-1* and *Nia1-2/Nia2-5* were used. Like in the wild type, ACC promoted NO production also closed stomata in *Nia2-1*. However, the effects of ACC in the wild type and *Nia2-1* were largely prevented in *Nia1-2* and *Nia1-2/Nia2-5* (Fig. 3A, B and C). The responses of *Nia1-2* and *Nia1-2/Nia2-5* to ACC are analogous to those of the wild type in the presence of tungstate (Fig. 3A, B and C). The results not only provide persuasive evidence that NO plays in ethylene-led Arabidopsis stomatal closing but also show that ethylene-caused NO synthesis in guard cells is Nia1-dependent.

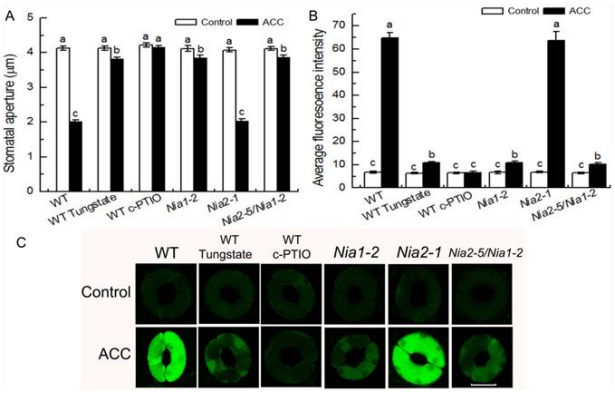

Fig. 3: Nia1-catalyed NO synthesis is required for ethylene-induced stomatal closure. Arabidopsis leaves of wild-type (WT) Col-0 or mutants *Nia1-2*, *Nia2-1* or *Nia2-5/Nia1-2* with open stomata were incubated in MES buffer without or with 200 μM c-PTIO or 100 μM tungstate alone (Control) or with 10 μM ACC for 3 h. A, Stomatal apertures were measured in epidermal strips. B and C Fluorescence pixel intensities (B) and images (C) in guard cells preloaded with 10 μM DAF-2DA for 30 min in darkness were recorded. Bar in C = 10 μm for all images. Other explanations are the same as in Fig. 1

Cytosol Alkalization Induces NO Generation in Stomatal Closure by Ethylene

Having known that both NO and cytosol alkalization are involved in ethylene-led stomatal closing, the relationship between them during ethylene-led stomatal closing was studied. SNP largely rescued the inhibitory effect of butyric acid on ACC-induced stomatal closure in the wild type (Fig. 4D) but methylamine did not reverse the deficiencies of ACC-led stomatal closing in the wild type in the presence of tungstate or c-PTIO and in *Nia1-2* or *Nia1-2/Nia2-5* (Fig. 4A). Similarly, SNP closed stomata in the wild type in the presence of butyric acid (Fig. 4D) but methylamine did not or less reduced stomatal aperture in the wild type in the presence of tungstate or c-PTIO and in *Nia1-2* or *Nia1-2/Nia2-5* (Fig. 4A). The data propose that cytosol alkalization has a regulatory effect on NO generation in stomatal closure by ethylene. To further confirm this conclusion, guard cells cytosol pH and NO content in the plants described above were measured. ACC did not induce NO generation in the wild type in the presence of butyric acid (Fig. 4E and F) but it raised cytosol pH in the wild type in the presence of tungstate or c-PTIO and in *Nia1-2* or *Nia1-2/Nia2-5* (Fig. 4B and C). Together, the interrelation between stomatal aperture and cytosol pH alteration or NO level reveals that cytosol alkalization regulates NO generation in stomatal closure by ethylene.

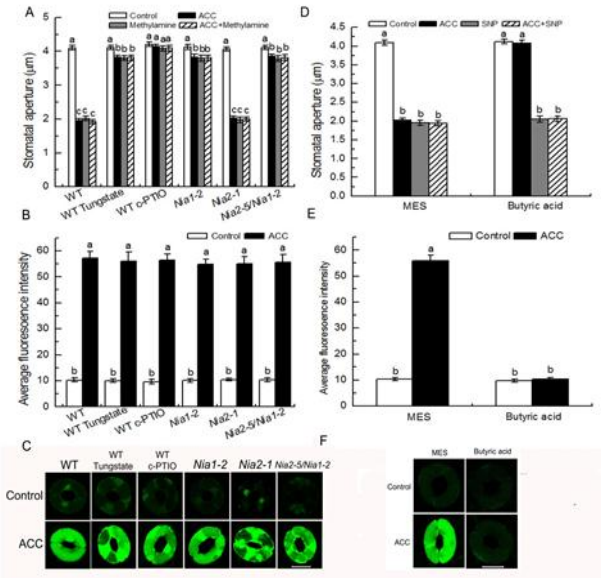

Fig. 4: Cytosol alkalization is necessary for NO synthesis in ethylene-induced stomatal closure. A. Leaves of wild-type Col-0 and mutants *Nia1-2*, *Nia 2-1* and *Nia1-2/Nia2-5* with open stomata were incubated in MES buffer without or with 200 μM c-PTIO or 100 μM tungstate in the absence (Control) or presence of 10 μM ACC, 2 mM methylamine or 10 μM ACC and 2 mM methylamine for 3 h then stomatal apertures were measured in epidermal strips. B and C Leaves of wild-type Col-0 and mutants *Nia1-2*, *Nia 2-1* and *Nia1-2/Nia2-5* with open stomata were incubated in MES buffer without or with 200 μM c-PTIO or 100 μM tungstate in the absence (Control) or presence of 10 μM ACC for 3 h fluorescence intensities (B) and images (C) of guard cells preloaded with 20 μM BCECF-AM for 10 min in darkness were recorded. D. Leaves of wild-type Col-0 with open stomata were incubated in MES buffer without or with 0.5 mM butyric acid in the absence (Control) or presence of 10 μM ACC, 100 μM SNP or 10 μM ACC and 100 μM SNP for 3 h then stomatal apertures were measured in epidermal strips. E and F Leaves of wild-type Col-0 with open stomata were incubated in MES buffer without or with 0.5 mM butyric acid in the absence (Control) or presence of 10 μM ACC for 3 h fluorescence intensities (E) and images (F) of guard cells preloaded with 10 μM DAF-2DA for 30 min in darkness were recorded. Bars in D and F = 10 μm for all images. Other explanations are the same as in Fig. 1

Discussion

Although the ethylene promotion of stomatal closure has been confirmed (Young *et al*., 2004; Desikan *et al*., 2006), the mechanism by which it triggers stomata closure, including the role of guard cell cytosol alkalization and NO synthesis, remains incomplete clear. Our results here provide convincing evidence that guard cell cytosol alkalization and NO synthesis mediate ethylene-led stomata closing in Arabidopsis. Butyric acid prevention of ACC-led cytosol alkalization and stomata closing (Fig. 2) the disability of ACC to induce NO synthesis and close stomata in the wild type in the presence of NO scavenger or NR inhibitor and in *Nia1-2* or *Nia1-2/Nia2-5* (Fig. 3), indicating that cytosol alkalization and NO synthesis are vital for ethylene-led stomata closing. The failure of ACC to cause cytosol alkalization, NO synthesis and stomata closing in *etr1* mutants, methylamine and SNP promotion of stomata closing in *etr1* like in the wild type (Fig. 1), further provide genetic evidence for the vital role of cytosol alkalization and NO in stomata closing by ethylene. Cytosol alkalization and NO synthesis participate in ABA-, MJ- and light/darkness transition-led stomata closing (García-Mata and Lamattina, 2002; Suhita *et al*., 2004; Ma *et al*., 2012) our results here suggest that cytosol alkalization and NO are common signal transduction event during stomatal closure by multiple stimuli, including ethylene.

The NO origin in plant guard cells contains NOS and NR (Neill *et al*., 2002, 2008; Qiao and Fan, 2008); however, this is not clear whether plant contains mammalian-type NOS remains or not (Guo *et al*., 2003; Crawford, 2006). However, a large number of study indicate that NR catalyzes NO synthesis in plants (Crawford, 2006; Neill *et al*., 2008; Wilson *et al*., 2008). Thus, whether or not NR is responsible for NO synthesis in ethylene-led stomata closing remains to be studied. Here, our genetic evidence indicates that NO mainly sourced from Nia1 mediates ethylene-induced stomatal closing. ACC triggered NO generation and the reduction of stomatal aperture in *Nia2-1* and the wild type. However, the effects of ACC were obviously prevented in the wild type in the presence of NR inhibitor tungstate and in *Nia1-2* and *Nia1-2/Nia2-5* (Fig. 2). The results are similar to a previous study, which indicates that Nia1 mediates ABA-stimulated NO synthesis (Bright *et al*., 2006). However, Hao *et al*. (2010) reported that both Nia1 and Nia2 are responsible for salicylic acid-caused NO accumulation in guard cells and stomatal closing. These differences suggest that the activation mechanisms of Nia1 and Nia2 may be different.

By means of comparing the time course of cytosolic pH and ROS or NO alterations in guard cells, cytoplasmic alkalization has been shown to be an essential prerequisite for ROS and NO production during ABA-, MJ- and light/darkness transition-induced stomatal closing (Suhita *et al*., 2004; Gonugunta *et al*., 2008; Ma *et al*., 2012, 2013). NO detection requires the formation of DAF-2 from DAF-2 DA a frequently used method of pH detection is based on BCECF protonation/deprotonation (Islam *et al*., 2010). Because the rate of alteration in BCECF fluorescence is faster than that of DAF-2 formation, the result of the time course study needs to be carefully analyzed. Thus, in this study, the effect of pH alteration on NO generation and the effect of the NO change on pH fluctuation were studied.

The data presented here show that ACC-induced NO production was fully depressed by butyric acid but ACC normally caused guard cell cytoplasmic alkalization in *Nia1-2* and *Nia1-2/Nia2-5* like in the wild type (Fig. 4). The data support the conclusion of time course study (Suhita *et al.*, 2004; Gonugunta *et al.*, 2008; Ma *et al.*, 2012, 2013). Together with the data that cytosolic alkalization and NO mediate stomata closing by ethylene (Fig. 1, 2 and 3), the results clearly show that the rise in cytosolic pH as an essential signal transduction event mediates ethylene-induced NO accumulation and stomatal closure. Linked with that cytoplasmic alkalization is an essential prerequisite for ROS and NO production during ABA-, MJ- and light/darkness transition-induced stomatal closure (Suhita *et al.*, 2004; Gonugunta *et al.*, 2008; Ma *et al.*, 2012, 2013; Ma and Niu, 2017), the results here support a postulation that the rise in cytosol pH maybe a common event in plant hormones- and abiotic or biotic stimuli-led stomata closing. However, the mechanism by which plant hormones and environmental stimuli regulate the changes in guard cell cytosol pH remains unclear, how cytosolic alkalization induces NO production also need to be further studied.

Acknowledgements

The first author acknowledges the financial grant from the research center for the development and function of plant resources in northwest of Guangxi, Scientific research project of Hechi university (Grant No. XJ2017KQ02) and National Natural Science Foundation of China (Grant No. 21563010).

References

Ahmed, Z., E.A. Waraich, R. Ahmad and M. Shahbaz, 2017. Morpho-physiological and biochemical responses of *camelina* (*Camelina sativa crantz*) genotypes under drought stress. *Int. J. Agric. Biol*., 19: 1–7

Beffagna, N., G. Romani, G. Meraviglia and S. Pallini, 1997. Effects of abscisic acid and cytoplasmic pH on potassium and chloride efflux in Arabidopsis thaliana seedlings. *Plant Cell Physiol*., 38: 503–510

Bethke, P.C., I.G.L. Libourel and R.L. Jones, 2006. Nitric oxide reduces seed dormancy in *Arabidopsis*. *J. Exp. Bot.*, 57: 517–526

Bright, J., R. Desikan, J.T. Hancock, I.S. Weir and S.J. Neill, 2006. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. *Plant J*., 45: 113–122

Crawford, N.M., 2006. Mechanisms for nitric oxide synthesis in plants. *J. Exp. Bot.*, 57: 471–478

Delledonne, M., Y. Xia, R.A. Dixon and C. Lamb, 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature*, 394: 585–588

Desikan, R., K. Last, R. Harrett-Williams, C. Tagliavia, K. Harter, R. Hooley, J.T. Hancock and S.J. Neill, 2006. Ethylene-induced stomatal closure in *Arabidopsis* occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant J*., 47: 907–916

Desikan, R., R. Griffiths, J.T. Hancock and S.J. Neill, 2002. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. *Proc. Natl Acad. Sci. USA*, 99: 16314–16318

Felle, H.H., E´. Kondorosi, A. Kondorosi and M. Schultze, 1996. Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. *Plant J*., 10: 295–301

García-Mata, C. and L. Lamattina, 2002. Nitric oxide and abscisic acid crosstalk in guard cells. *Plant Physiol*., 128: 790–792

Gibbon, B.C. and D.L. Kropf, 1994. Cytosolic pH gradients associated with tip growth. *Science*, 263: 1419–1421

Gonugunta, V.K., N. Srivastava, M.R. Puli and A.S. Raghavendra, 2008. Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid. *Plant Cell Environ.*, 31: 1717–1724

Grefen, C. and K. Harter, 2004. Plant two-component systems: principles, functions, complexity and cross-talk. *Planta*, 219: 733–742

Guo, F.Q., M. Okamoto and N.M. Crawford, 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science*, 302: 100–103

Guo, H. and J.R. Ecker, 2004. The ethylene signalling pathway: new insights. *Curr. Opin. Plant Biol*., 7: 40–49

Hao, F., S. Zhao, H. Dong, H. Zhang, L. Sun and C. Miao, 2010. Nia1 and Nia2 are involved in exogenous salicylic acid-induced nitric oxide generation and stomatal closure in Arabidopsis. *J. Integr. Plant Biol*., 52: 298–307

He, Y.K., R.H. Tang, Y. Hao, R.D. Stevens, C.W. Cook, S.M. Ahn, L.F. Jing, Z.G. Yang, L.E. Chen, F.Q. Guo, F. Fiorani, R.B. Jackson, N.M. Crawford and Z.M. Pei, 2004. Nitric oxide represses the *Arabidopsis* floral transition. *Science*, 305: 1968–1971

Hwang, I., H.C. Chen and J. Sheen, 2002. Two-component signal transduction pathways in *Arabidopsis*. *Plant Physiol*., 129: 500–515

Iqbal, M.M., G. Murtaza, Z.A. Saqib and R. Ahmad, 2015. Growth and physiological responses of two rice varieties to applied lead in normal and salt-affected soils. *Int. J. Agric. Biol*., 17: 901–910

Irving, H.R., C.A. Gehring and R.W. Parish, 1992. Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *Proc. Natl. Acad. Sci. USA*, 89: 1790–1794

Islam, M.M., M.A. Hossain, R. Jannat, S. Munemasa, Y. Nakamura, I.C. Mori and Y. Murata, 2010. Cytosolic alkalization and cytosolic calcium oscillation in Arabidopsis guard cells response to ABA and MeJA. *Plant Cell Physiol*., 51: 1721–1730

Kojima, H., N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata and T. Nagano, 1998. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.*, 70: 2446–2453

Liu, J., G.H. Liu, L.X. Hou and X. Liu, 2010. Ethylene-induced nitric oxide production and stomatal closure in *Arabidopsis thaliana* depending on changes in cytosolic pH. *Chin. Sci. Bull*., 55: 2403–2409

Liu, X., S.Q. Zhang and C.H. Lou, 2003. Involvement of nitric oxide in the signal transduction of salicylic acid regulating stomatal movement. *Chin. Sci. Bull*., 48: 449–452

Ma, Y.L., X.P. She and S.S. Yang, 2012. Sphingosine-1-phosphate (S1P) mediates darkness-induced stomatal closure through raising cytosol pH and hydrogen peroxide (H_2O_2) levels in guard cells in *Vicia faba*. *Sci. Chin. Life Sci*., 11: 974–983

Ma, Y.L., X.P. She and S.S. Yang, 2013. Cytosolic alkalization-mediated H_2O_2 and NO production are involved in darkness-induced stomatal closure in *Vicia faba*. *Can. J. Plant Sci*., 93: 119–130

Ma, Y.L. and J. Niu, 2017. The role of phytosphingosine-1-phosphate (Phyto-S1P) and its relationships with cytosolic pH and hydrogen peroxide (H_2O_2) during stomatal closure by darkness in broad bean. *S. Afr. J. Bot*., 108: 237–242

Mansfield, T.A., A.M. Hetherington and C.J. Atkinson, 1990. Some aspects of stomatal physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 41: 55–75

Munemasa, S., K. Oda, M. Watanabe-Sugimoto, Y. Nakamura, Y. Shimoishi and Y. Murata, 2007. The *coronatine-insensitive 1* mutation reveals the hormonal signaling interaction between abscisic acid and methyljas-monate in Arabidopsis guard cells. Specific impairment of ion channel activation and second messenger production. *Plant Physiol*., 143: 1398–1407

Neill, S., R. Barros, J. Bright, R. Desikan, J. Hancock, J. Harrison, P. Morris, D. Ribeiro and I. Wilson, 2008. Nitric oxide, stomatal closure, and abiotic stress. *J. Exp. Bot*., 59: 165–176

Neill, S.J., R. Desikan, A. Clarke and J.T. Hancock, 2002a. Nitric oxide is a novel component of abscisic acid signalling in stomatal guard cells. *Plant Physiol*., 128: 13–16

Neill, S.J., R. Desikan, A. Clarke, R.D. Hurst and J.T. Hancock, 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot*., 53: 1237–1247

Prado, A.M., D.M. Porterfield and J.A. Feijó, 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development*, 131: 2707–2714

Qiao, W. and L.M. Fan, 2008. Nitric oxide signaling in plant responses to abiotic stresses. *J. Integr. Plant Biol*., 50: 1238–1246

Romera, F.J., C. Lucena, M.J. García, E. Alcántara and R. Pérez-Vicente, 2017. The role of ethylene and other signals in the regulation of Fe deficiency responses by dicot plants. *Stress Signaling in Plants: Genomics and Proteomics Perspective*, 2: 277–300

Scott, A.C. and N.S. Allen, 1999. Changes in cytosolic pH within Arabidopsis root columella cells play a key role in the early signaling pathway for root gravitropism. *Plant Physiol.*, 121: 1291–1298

She, X.P., X.G. Song and J.M. He, 2004. Role and relationship of nitric oxide and hydrogen peroxide in light/dark-regulated stomatal movement in *Vicia faba*. *Acta Bot. Sin.*, 46: 1292–1300

Shi, C.Y., C.Qi, H.Y. Ren, A.X. Huang, S.M. Hei and X.P. She, 2015. Ethylene mediates brassinosteroid-induced stomatal closure via Gα protein-activated hydrogen peroxide and nitric oxide production in Arabidopsis. *Plant J*., 82: 280–301

Siddiqui, M.H., M.H. Al-Whaibi and M.O. Basalah, 2011. Role of nitric oxide in tolerance of plants to abiotic stress. *Protoplasma*, 248: 447–455

Srivastava, N., V.K. Gonugunta, M.R. Puli and A.S. Raghavendra, 2009. Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum*. *Planta*, 229: 757–765

Suhita, D., A.S. Raghavendra, J.M. Kwak and A. Vavasseur, 2004. Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. *Plant Physiol*., 134: 1536–1545

Tanaka, Y., T. Sano, M. Tamaoki, N. Nakajima, N. Kondo and S. Hasezawa, 2005. Ethylene inhibits abscisic acid-induced stomatal closure in Arabidopsis. *Plant Physiol*., 138: 2337–2343

Watkins, J.M., P.J. Hechler and G.K. Muday, 2014. Ethylene-induced flavonol accumulation in guard cells suppresses reactive oxygen species and moderates stomatal aperture. *Plant Physiol*., 164: 1707–1717

Wilson, I.D., S.J. Neill and J.T. Hancock, 2008. Nitric oxide synthesis and signalling in plants. *Plant Cell Environ*., 31: 622–631

Young, T.E., R.B. Meeley and D.R. Gallie, 2004. ACC synthase expression regulates leaf performance and drought tolerance in maize. *Plant J*., 40: 813–825

(Received 31 March 2017; Accepted 10 July 2017)