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Involvement of Ethylene and Hydrogen Peroxide in Induction of Alternative Respiratory Pathway in Salt-Treated *Arabidopsis* Calluses

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The role of ethylene and hydrogen peroxide (H_2O_2) in the induction of the alternative respiratory pathway (AP) in calluses from wild-type (WT) *Arabidopsis* and ethylene-insensitive mutant *etr1-3* under salt stress was investigated. The capacity and the contribution of the AP to the total respiration were significantly induced by 100 mM sodium chloride (NaCl) in WT calluses but only slightly induced in *etr1-3* calluses. Ethylene emission was enhanced in WT calluses under salt stress. Application of 1-aminocyclopropane-1-carboxylic acid (an ethylene precursor) further increased the AP capacity in WT calluses but not in *etr1-3* calluses under salt stress. Reduction of ethylene production by aminooxyacetic acid (AOA, an ethylene biosynthesis inhibitor) in WT calluses eliminated the NaCl-induced increase of ethylene emission and inhibited AP induction under salt stress, suggesting that ethylene is required for AP induction. H_2O_2 enhanced ethylene production while ethylene reduced H_2O_2 generation in WT calluses under salt stress. In addition, ethylene and H_2O_2 modulated NaCl-induced alternative oxidase gene (*AOX1a*) expression and the increase in pyruvate content in WT calluses. Inhibition of the AP by salicylhydroxamic acid in WT calluses under salt stress resulted in severe cellular damage as indicated by the high content of H_2O_2, malondialdehyde and more electrolyte leakage. Taken together, ethylene and H_2O_2 are involved in the salt-induced increase of the AP, which plays an important role in salt tolerance in WT calluses, and ethylene may be acting downstream of H_2O_2.

Keywords: Alternative respiratory pathway • *Arabidopsis* callus • Ethylene • Hydrogen peroxide • Salt stress.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, aminooxyacetic acid; AOX, alternative oxidase; AP, alternative respiratory pathway; APX, ascorbate peroxidase; CAT, catalase; CP, cytochrome respiratory pathway; FW, fresh weight; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NO, nitric oxide; POD, peroxidase; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; SOD, superoxide dismutase; TCA, trichloroacetic acid; V_{alt}, capacity of alternative respiratory pathway; V_{cyt}, capacity of cytochrome respiratory pathway; WT, wild type.

Introduction

Respiration plays a pivotal role in the metabolism of plants by providing adequate energy and carbon sources to drive the cellular metabolism and transport processes. Plant mitochondria possess two different pathways of electron transport branching at the ubiquinone: the main, cyanide-sensitive cytochrome pathway (CP) and the cyanide-resistant alternative pathway (AP). It has been confirmed that alternative oxidase (AOX) is used as the terminal oxidase in the AP (Siedow and Umbach 1995, Vanlerberghe and McIntosh 1997). AOX is localized to the inner membrane of mitochondria (Millenaar and Lambers 2003). The AP contributes to the whole respiration of plant cells to greater or lesser degrees depending on the environmental and physiological conditions. It is well known that the AP plays an important role in plant thermogenesis, fruit ripening and responses to environmental stresses, including wounding, low temperature, ozone and pathogen invasion (Vanlerberghe and McIntosh 1992, Chivasa and Carr 1998, Simons et al. 1999, Ederli et al. 2006, Feng et al. 2008).

In many areas of the world, salinity of cropland has increased and has become the main constraint for agriculture (Munns 2002). Salt stress leads to the production of reactive oxygen species (ROS), such as the superoxide anion radical ($O_2^{-}\cdot$), hydroxyl radical ($OH\cdot$), and hydrogen peroxide (H_2O_2) (Hasegawa et al. 2000, Liu et al. 2007). Singha and Choudhuri (1990) reported that H_2O_2 and $O_2^{-}\cdot$ were mainly responsible for NaCl-induced injury in *Vigna catjang* and *Oryza sativa* leaves. To minimize the ROS damage, plants have evolved the antioxidant defense system, comprised of enzymes that are responsible for scavenging excessively accumulated ROS under stress conditions such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) (Jung et al. 2000).

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Regulation of these antioxidant constituents by some exogenous substances (e.g. abscisic acid, nitric oxide) might mediate plant tolerance to salt stress. It has been shown that the AP may participate in the adaptation to salt stress since salt stress increased the activity of the AP (Costa et al. 2007, Smith et al. 2009). Mitochondria have a potential function in avoiding ROS damage by regulating the electron shift between the AP and CP (Maxwell et al. 1999). However, whether respiration could be involved in the prevention of ROS formation under salt stress is not reported, despite the fact that mitochondria is an important source of ROS, especially under stress conditions (Bartoli et al. 2004, Taylor et al. 2004).

Ethylene has long been regarded as a stress hormone (Morgan and Drew, 1997). It is not only involved in plant growth and development, but also in plant responses to biotic stress such as pathogen attack and abiotic stresses such as wounding, ozone and salt (Abeles et al. 1992, Vahala et al. 2003, Cao et al. 2007, Wang et al. 2009). ETR1 and EIN2 are two well-characterized ethylene signaling molecules (Buer et al. 2006). It has been reported that plant responses to salt stress are modulated by changes in the expression level of ethylene receptor ETR1 (Zhao and Schaller 2004). Mutations in the *ETR1* gene resulted in reduced ethylene responses (O'Malley et al. 2005). The *ein2* mutant is also ethylene insensitive, but the biochemical function of EIN2 has not been demonstrated (Alonso and Stepanova 2004). Cao et al. (2007) reported that ethylene signaling might be required for plant salt tolerance, as evidenced by the fact that ethylene-insensitive mutants *etr1-1* and *ein2-1* were more sensitive to salt stress. The essential role of ethylene for AP induction was also confirmed by Simons et al. (1999) in an *Arabidopsis* ethylene-insensitive mutant *etr1-1*. AOX is affected in the *Arabidopsis ctr-1* mutant, indicating AP operation may be ethylene dependent (Simons et al. 1999).

Survival of plants under stress conditions depends on their adaptability or tolerance to environmental perturbations, including the ability to perceive the stimulus, generate and transmit signals, and initiate various physiological and biochemical changes (Shinozaki and Yamaguchi-Shinozaki 1997, Feng et al. 2008). In addition to functioning as an endogenous oxidant, H_2O_2 has been suggested as a diffusible signal for selective induction of defense mechanisms in plant cells (Chen et al. 1993, Prasad et al. 1994). It was reported that H_2O_2 induced by elicitors initiated programmed cell death in *Arabidopsis* suspension cells, but H_2O_2 itself induced the expression of defense-related genes (Desikan et al. 1998). In several studies, H_2O_2 is considered as the second messenger in inducing AOX activity by directly oxidizing transcription factors or by modulating phosphorylation processes (Wagner 1995, Neill et al. 2002). Under stress conditions, ROS levels may induce AOX expression (Maxwell et al. 2002, Vanlerberghe et al. 2002). Although H_2O_2 and ethylene have been found to be possibly involved in AP induction, the interaction of H_2O_2 and ethylene in the induction of the AP during environmental stresses remains unclear. Furthermore, the mechanism of AOX regulation affected by salinity remains unknown.

Recent studies have demonstrated that salt tolerance was induced by exogenous 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor) in *Arabidopsis* (Cao et al. 2007, Wang et al. 2009). However, whether the AP is involved in the ethylene-initiated mechanism of salt tolerance needs clarification. Few reports in the literature have used the mutant that is altered in ethylene signaling to examine the role of ethylene in the regulation of the AP under salt stress. In this study, the relationship between H_2O_2 and ethylene in AP induction in *Arabidopsis* calluses from wild type (WT) and ethylene-insensitive mutant *etr1-3* under salt stress was investigated. An effort was also made to demonstrate the possible regulation and physiological function of the AP under salt stress.

Results

Changes of electrolyte leakage and malondialdehyde content under NaCl stress

Electrolyte leakage and malondialdehyde (MDA) are considered to be indicators of stress-induced cell damage. Under different NaCl concentrations, the electrolyte leakage and the MDA content in WT *Arabidopsis* calluses increase dramatically as the NaCl concentration increases (**Fig. 1**). At 50 mM NaCl

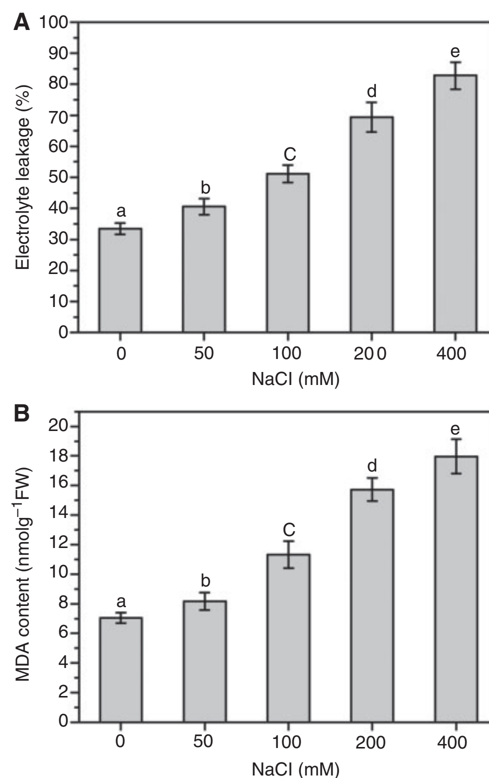

Fig. 1 Effects of NaCl concentration (0–400 mM) on electrolyte leakage (A) and MDA content (B) in WT calluses for 48 h suspension treatment. Data are mean values ± SE for three independent experiments. Within each set of experiments, different letters were significantly different at the 0.01 level.

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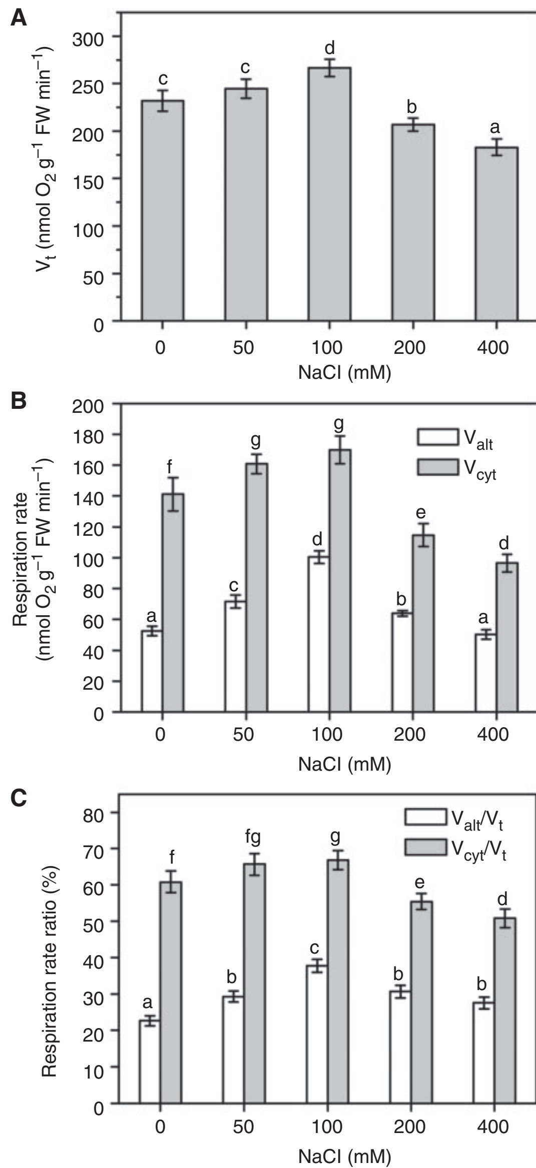

Fig. 2 Effects of NaCl concentration (0–400 mM) on V_t (A), V_{alt} and V_{cyt} (B), V_{alt}/V_t and V_{cyt}/V_t (C) in WT calluses for 48 h suspension treatment. Data are mean values ± SE for three independent experiments. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

treatment, the electrolyte leakage and the MDA content only slightly increased. However, both the electrolyte leakage and the MDA content increased markedly in the presence of 100 mM NaCl (**Fig. 1A, B**). With 200 mM or higher NaCl concentration treatment, the cells were severely damaged, as the electrolyte leakage was close to that of dead cells.

Effect of salt stress on alternative pathway (AP) and cytochrome pathway (CP)

Total respiration rate (V_t), the capacity of alternative respiratory pathway (V_{alt}) and cytochrome respiratory pathway (V_{cyt}), the ratio of V_{alt} to V_t and V_{cyt} to V_t were measured and calculated in WT calluses under different NaCl concentrations (0–400 mM) treatment for 48 h. The results showed that V_t increased in the presence of NaCl up to 100 mM and then decreased at higher NaCl concentrations (200–400 mM) (**Fig. 2A**). Concomitantly, V_{alt} increased under salt stress and reached a maximum at 100 mM NaCl while V_{cyt} slightly increased in the presence of NaCl up to 100 mM (**Fig. 2B**). Consequently the ratio of V_{alt}/V_t increased and reached the maximum at 100 mM NaCl (**Fig. 2C**), indicating an increased contribution of the AP to total respiration under salt stress. In contrast, the ratio of V_{cyt}/V_t changed little in the presence of NaCl from 0 to 100 mM, suggesting small changes in the contribution of the CP to total respiration under salt stress (**Fig. 2C**). V_{alt} and V_{cyt} declined markedly as did V_t when NaCl concentration was further increased (**Fig. 2B**). Therefore we used 100 mM NaCl for the treatment in the following experiments.

The effects of salt stress on AP and CP were also determined in ethylene-insensitive mutant *etr1-3* in the presence of 100 mM NaCl for 48 h. As shown in **Table 1**, V_{alt} and the ratio of V_{alt}/V_t did not exhibit a significant elevation under salt stress in the *etr1-3* calluses. To further confirm the role of ethylene signaling in the induction of AP, another ethylene-insensitive mutant, *ein2-1*, was investigated. Results showed that a similar pattern was observed in *ein2-1* calluses as seen in *etr1-3* calluses under salt stress (**Table 1**). These findings indicate that induction of the AP by salt stress is dependent on ethylene signaling. Since the two ethylene-insensitive mutants displayed similar responses to salt stress, we focused further studies exclusively on *etr1-3* calluses.

Table 1 Effects of salt stress on total respiration rate (V_t), the capacity of the alternative respiratory pathway (V_{alt}) and the cytochrome respiratory pathway (V_{cyt}), V_{alt}/V_t and V_{cyt}/V_t in the calluses from WT, *etr1-3* and *ein2-1*

	Treatment	V_t	V_{alt}	V_{cyt}	V_{alt}/V_t (%)	V_{cyt}/V_t (%)
WT	Control	233.2 ± 9.3a	52.2 ± 2.8a	141.1 ± 8.7a	22.4 ± 1.6a	60.8 ± 3.1a
	NaCl	266.5 ± 11.2b	98.8 ± 4.7b	169.8 ± 9.4b	37.1 ± 2.4b	66.4 ± 3.4b
etr1-3	Control	226.8 ± 6.7a	48.6 ± 2.8a	134.9 ± 7.7a	21.3 ± 1.4a	59.5 ± 2.2a
	NaCl	234.8 ± 7.8a	51.7 ± 3.1a	161.2 ± 7.4b	23.4 ± 1.2a	68.3 ± 3.2b
ein2-1	Control	219.7 ± 8.2a	48.3 ± 2.8a	126.8 ± 6.9a	21.9 ± 1.8a	57.9 ± 3.1a
	NaCl	227.3 ± 7.9a	49.7 ± 3.4a	152.9 ± 7.1b	21.8 ± 1.7a	66.5 ± 3.7b

100 mM NaCl was added in the medium for salt stress. After 48 h, the calluses were collected for determination of respiration rate (nmol O_2 g^{-1} FW min^{-1}). Data are mean values ± SE of three independent experiments. Within each set of experiments, different letters were significantly different at the 0.01 level.

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Ethylene-induced AP under salt stress in WT calluses

In order to further investigate the effect of ethylene on the AP and CP under salt stress, ethylene emission was also determined. Results showed that ethylene emission increased in a similar pattern as did V_{alt} in WT calluses under salt stress (**Fig. 3A, B**). Application of exogenous ACC to WT calluses drastically increased ethylene emission and, as expected, also induced the AP in the presence or absence of NaCl. On the other hand, NaCl-induced increase in ethylene emission and V_{alt} was inhibited by AOA in WT calluses. In contrast, NaCl-induced ethylene content was less in *etr1-3* calluses than in WT calluses (**Fig. 3A**). Moreover, ACC or AOA had little effect on the AP in *etr1-3* calluses in the presence or absence of NaCl (**Fig. 3B**), suggesting ethylene signaling is required for AP induction. In both WT and *etr1-3* calluses under salt stress, ACC had almost no effect on the CP (**Fig. 3C**).

H_2O_2-induced AP under salt stress in WT calluses

In order to investigate the effect of H_2O_2 on the AP and CP under salt stress, H_2O_2 content, V_{alt} and V_{cyt} were determined. H_2O_2 production and V_{alt} were obviously increased in WT calluses under salt stress (**Fig. 4A, B**). Application of exogenous H_2O_2 to WT calluses also induced the AP in the presence or absence of NaCl. Exogenous H_2O_2 application and NaCl had an additive effect on AP induction in WT calluses (**Fig. 4B**). NaCl-induced H_2O_2 production and V_{alt} were inhibited by CAT (a H_2O_2 scavenger). In contrast, H_2O_2 application had little effect on the AP in *etr1-3* calluses in the presence or absence of NaCl (**Fig. 4B**), suggesting that H_2O_2-induced AP requires ethylene signaling. Furthermore, H_2O_2 had almost no effects on the CP in both WT and *etr1-3* calluses under salt stress (**Fig. 4C**). We further examined the relationship between ethylene and H_2O_2 under salt stress. As shown in **Fig. 4A**, increasing ethylene by applying ACC to WT calluses under salt stress reduced NaCl-induced H_2O_2, while reducing ethylene by AOA application enhanced H_2O_2 production under salt stress. In contrast, application of ACC or AOA to *etr1-3* calluses had no effect on H_2O_2 production under salt stress (**Fig. 4A**). On the other hand, application of exogenous H_2O_2 to WT calluses under salt stress enhanced ethylene production, while CAT application decreased ethylene production (**Fig. 3A**). However, both H_2O_2 and CAT had little effect on ethylene emission in *etr1-3* calluses under salt stress (**Fig. 3A**). Taken together, it can be concluded that H_2O_2-induced AP resulted from enhanced ethylene production in WT calluses.

Expression analysis of the AOX encoding gene in WT *Arabidopsis*

In higher plants, AOX is encoded by a small gene family. Recent studies have indicated that AOX gene transcription can be modulated by exposure to a range of biotic and abiotic factors (Yoshida et al. 2007, Giraud et al. 2008). *AOX1a* is the major isoform in *Arabidopsis*. Microarray studies and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis in *Arabidopsis* have revealed up-regulation of *AOX1a* transcripts in many different types of stress treatments (Clifton et al. 2006). Given that ethylene and H_2O_2 are involved in AP

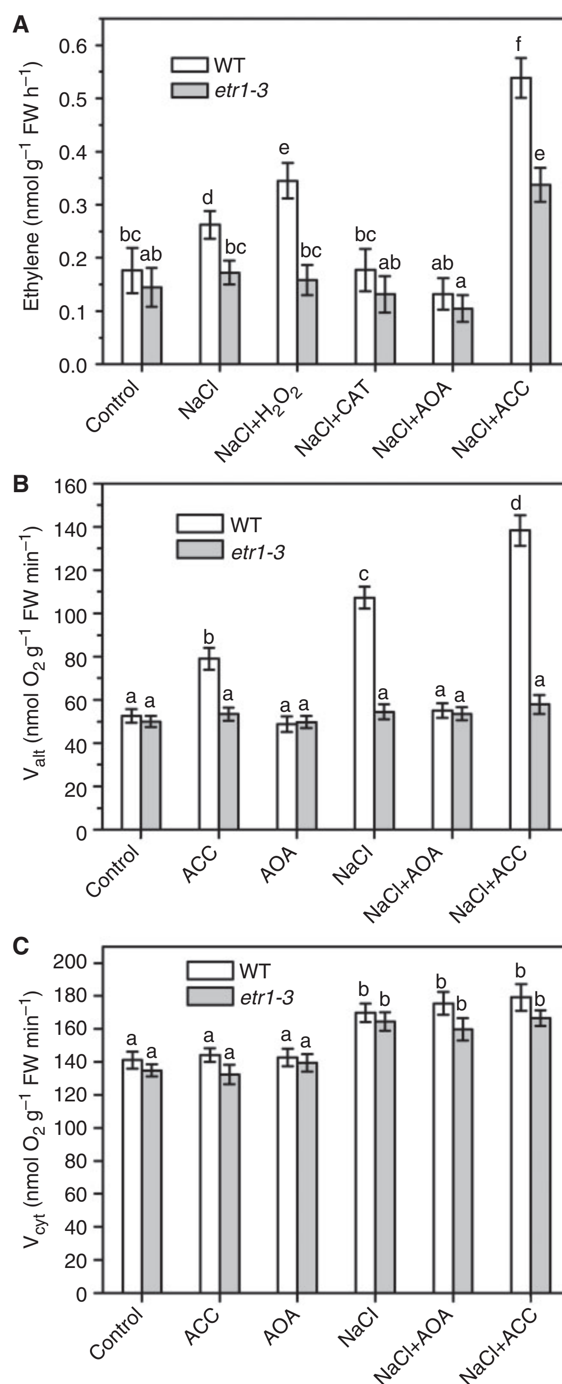

Fig. 3 Changes in ethylene content (A), V_{alt} (B) and V_{cyt} (C) in the calluses from WT and *etr1-3* under 100 mM NaCl for 48 h. Added in the medium for treatments were 50 U/ml CAT, 100 μM AOA, 100 μM ACC, and 1 mM H_2O_2. Data are mean values ± SE for three independent experiments. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

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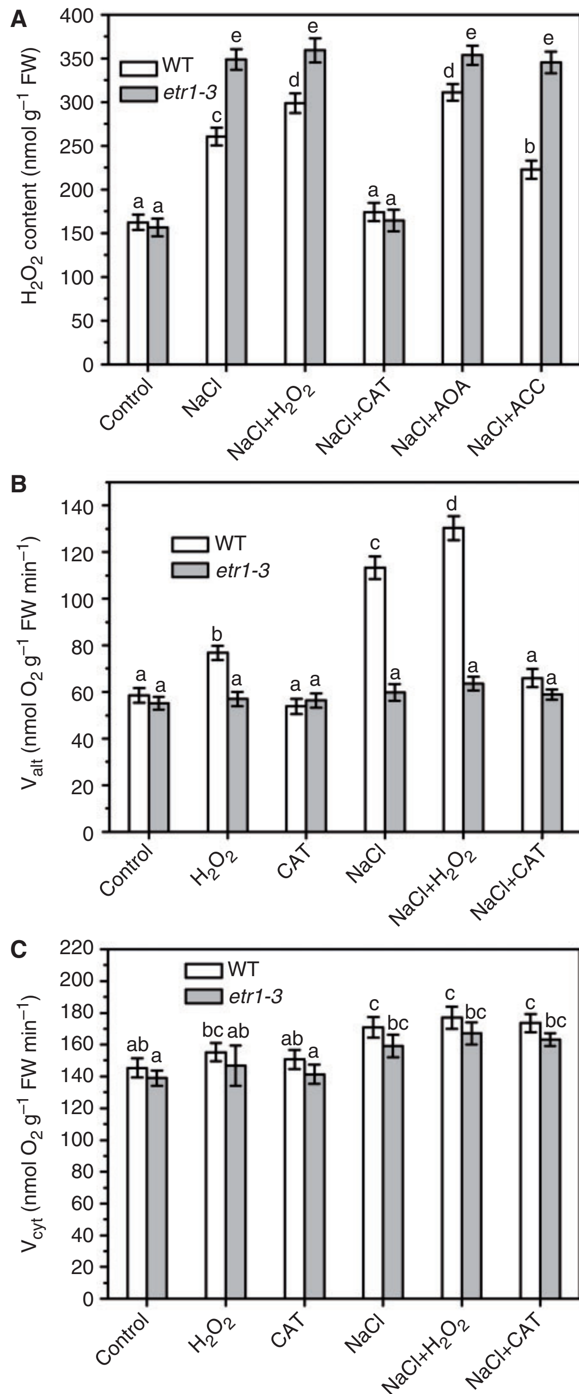

Fig. 4 Changes in H_2O_2 content (A), V_{alt} (B) and V_{cyt} (C) in the calluses from WT and *etr1-3* under 100 mM NaCl for 48 h. The calluses were treated as described in **Fig. 3**. Data are mean values ± SE for three independent experiments. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

induction under salt stress (**Figs. 3, 4**), we investigated if ethylene, H_2O_2 or NaCl can alter the expression of the AOX gene. Specific transcripts were determined by semi-quantitative RT-PCR. Results showed that treatment of WT calluses with NaCl enhanced *AOX1a* expression; exogenous ACC or H_2O_2 application further enhanced the *AOX1a* expression level in WT calluses under salt stress (**Fig. 5A, B**). Moreover, *AOX1a* mRNA accumulation induced by NaCl was completely destroyed by AOA and CAT treatments in WT calluses (**Fig. 5A, B**). In addition, *AOX1a* expression was also analyzed in *Arabidopsis* seedlings. The results showed that a similar phenomenon was found in *Arabidopsis* seedlings as observed in *Arabidopsis* calluses (**Fig. 5C, D**), suggesting that the regulation of *AOX1a* expression under salt stress was not limited to specific tissues or organs. These findings indicate that ethylene and H_2O_2 mediate the NaCl-induced *AOX1a* expression in *Arabidopsis*.

Effect of ethylene and H_2O_2 on the pyruvate content under salt stress

To further explain the observed changes in the activity of the AP under salt stress, we attempted to determine factors known to affect AOX activity in WT calluses. The activity of AOX can be stimulated by short-chained α-keto acids such as pyruvate, which is an allosteric activator of AOX. We investigated if ethylene, H_2O_2 or NaCl can modulate the pyruvate content. Results showed that treatment of WT calluses with NaCl increased the pyruvate content. Exogenous ACC or H_2O_2 application further enhanced the pyruvate content in WT calluses under salt stress (**Fig. 6A**). Furthermore, pyruvate accumulation induced by NaCl was eliminated by AOA and CAT treatments in WT calluses (**Fig. 6A**). We next investigated the effect of pyruvate at different concentrations on the AP in our system. As shown in **Fig. 6B**, the capacity of the AP (V_{alt}) was enhanced by exogenous pyruvate in a dose-dependent manner. When pyruvate concentrations ranged from 0.5 to 2 mM, the V_{alt} increased significantly. The V_{alt} further increased slightly with an increase in pyruvate concentration greater than 2 mM. These results indicate that the increased pyruvate content, which is mediated by ethylene and H_2O_2 under salt stress, may contribute to AP induction.

The enhanced AP under salt stress contributes to antioxidant protection

Salicylhydroxamic acid (SHAM) has long been used to inhibit AOX activity in intact tissues (Chivasa and Carr 1998, Naylor et al. 1998). In order to investigate the role of AP in NaCl-induced oxidative damage, 1 mM SHAM was used in this study. Application of SHAM to WT and *etr1-3* calluses had no significant effects on H_2O_2 generation, MDA content and electrolyte leakage in the absence of NaCl (**Fig. 7**). However, under salt stress, H_2O_2 generation, MDA content and electrolyte leakage were significantly enhanced in both WT and *etr1-3* calluses (**Fig. 7**). Application of SHAM to WT calluses under salt stress elevated H_2O_2 generation, MDA content and electrolyte leakage. However, application of SHAM to *etr1-3* calluses had almost no effect on H_2O_2 generation, MDA content and electrolyte leakage in the presence of NaCl since H_2O_2, MDA and electrolyte leakage were already at a high level under salt stress (**Fig. 7B, C**),

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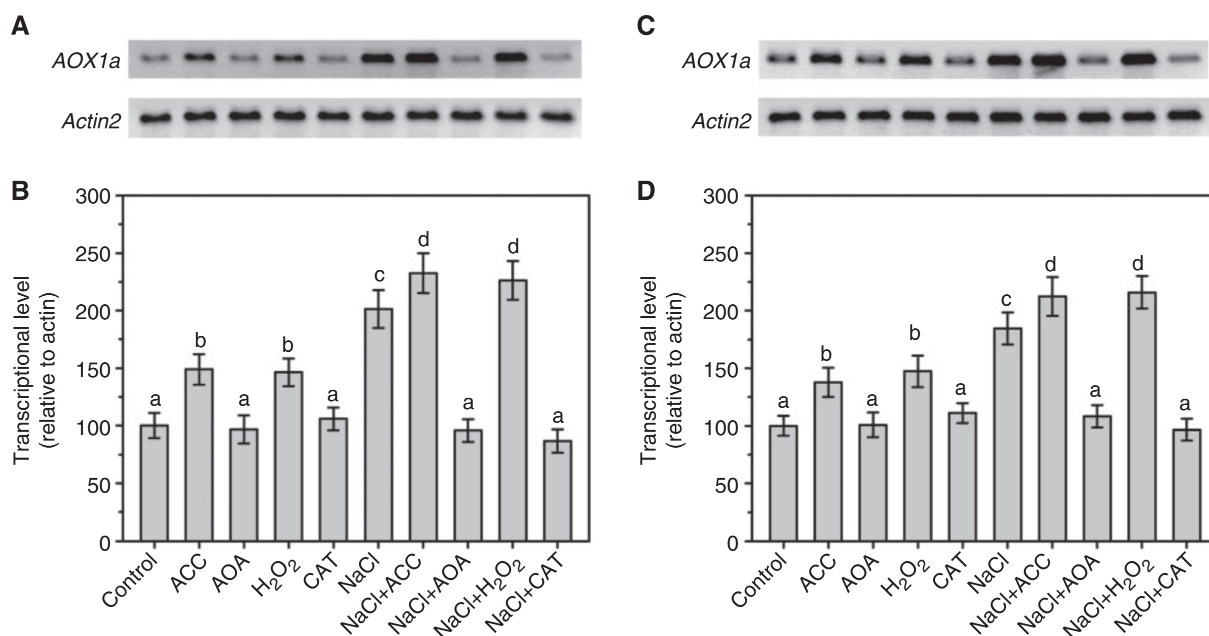

Fig. 5 Effects of ethylene and H_2O_2 on the expression of *AOX1a* in WT calluses (A, B) and seedlings (C, D) under 100 mM NaCl for 48 h. The calluses were treated as described in **Fig. 3**. Two-week-old seedlings grown on 0.5×MS solid medium were transferred to 0.5×MS liquid medium containing various reagents for treatment. Semiquantitation of mRNA levels loaded in each lane was performed by coamplification and normalization with an internal standard (*actin*). Under the blot (A, C), graphs with relative intensities of the signals ± SE are shown for *AOX1a* (B, D), respectively. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

suggesting that calluses lacking ethylene signaling suffer more damage from ROS under salt stress and ethylene-induced AP protects calluses from severe ROS damage. Further investigation of AP's role under salt stress was also determined in *Arabidopsis* seedlings. As shown in **Table 2**, the data obtained in seedlings were consistent with the data obtained in calluses.

Effect of ethylene on antioxidant enzymes under salt stress

The previous results indicate that ethylene plays a role in AP induction to avoid damage from ROS under salt stress. We further examined whether ethylene had effects on antioxidant enzymes. **Fig. 8** shows that SOD activity decreased slightly, while the activity of CAT, APX and POD increased under salt stress. Application of ACC or AOA to WT calluses under salt stress had little effect on the activities of antioxidant enzymes (**Fig. 8**). These results indicate that ethylene has no effects on antioxidant enzyme activity under salt stress.

Discussion

Salt-stressed WT calluses had higher levels of the AP than unstressed WT calluses (**Fig. 2, Table 1**), suggesting that salt stress induced AP activity. However, the phenomenon of salt-induced AP in WT calluses was not found in the ethylene-insensitive mutants *etr1-3* and *ein2-1* calluses (**Table 1**), indicating ethylene might be involved in AP induction under salt stress.

It is postulated that H_2O_2 may be a secondary messenger in the signal transduction pathway to induce the AP (Wagner 1995, Neill et al. 2002). Feng et al. (2008) also confirmed H_2O_2 could induce AP and *AOX1* expression under chilling. It has been reported that addition of 2 mM H_2O_2 into *Petunia hybrida* cells resulted in increased cyanide (CN)-resistant respiration and expression of AOX protein, both lasting for 4 to 5 days. It was suggested that the exogenous H_2O_2 could initiate the signaling pathway for induction of the AOX pathway (Wagner 1995). Our results showed that H_2O_2 could induce the AP in WT calluses but not in *etr1-3* calluses under salt stress (**Fig. 4A, B**), suggesting that H_2O_2 and ethylene might function in AP induction under salt stress.

In this study, we showed that the CP was not affected by either H_2O_2 or ethylene under salt stress (**Figs. 3C, 4C**). Thus it seemed that the CP was not a H_2O_2 or ethylene-inducible respiratory pathway under salt stress. These findings were consistent with previous studies in *Petunia hybrida* cells, in which application of 2 mM H_2O_2 did not change the activity level of the CP (Wagner 1995). However, H_2O_2 or ACC treatment alone significantly enhanced the AP in WT calluses under salt stress (**Figs. 3B, 4B**), suggesting that the AP may be involved in H_2O_2- and ethylene-induced changes of metabolism. As shown in **Fig. 3**, AP activity had little effect in WT and *etr1-3* calluses under controlled conditions. In addition, treatment with AOA alone also had almost no effect on AP activity in WT calluses. These data may suggest the possibility that ethylene- or ETR-independent pathways contribute to AP activity under

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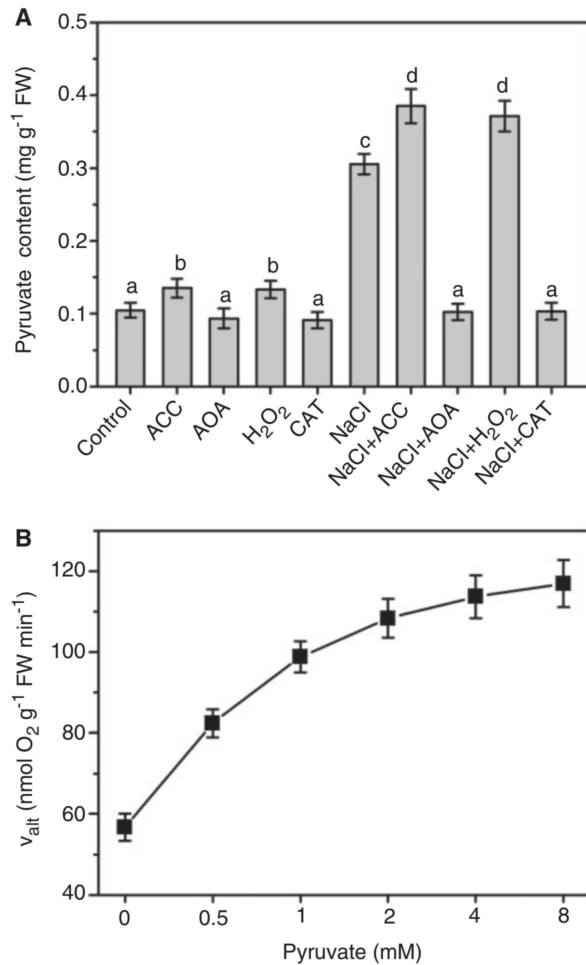

Fig. 6 Effects of ethylene and H_2O_2 on the pyruvate content (A) and effect of exogenous pyruvate on V_{alt} (B) in WT calluses under salt stress. The calluses were treated as described in **Fig. 3**. Data are mean values±SE for three independent experiments. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

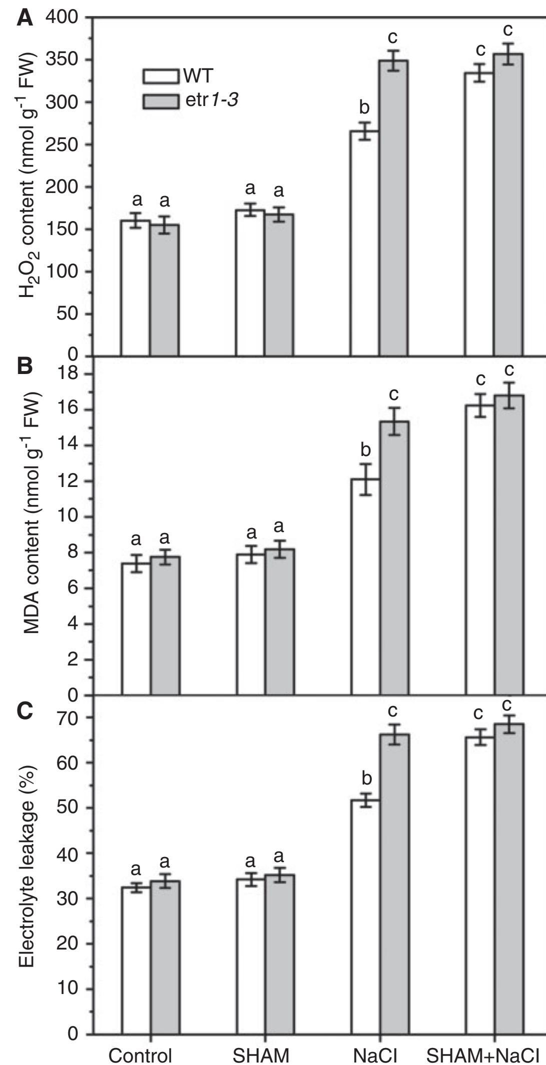

Fig. 7 Effects of SHAM on H_2O_2 content (A), MDA content (B), and the value of electrolyte leakage (C) in the calluses from WT and *etr1-3* under 100 mM NaCl for 48 h. Treatments used 1 mM SHAM. Data are mean values±SE for three independent experiments. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

controlled conditions. Ethylene emission and AP activity were low in WT under controlled conditions (**Fig. 3**). Some changes induced by ethylene may require ethylene emission (at a relative high level) to be effective: when ethylene content is low under controlled conditions, ethylene is not sufficient in itself to induce AP activity. In contrast, when ethylene content is at a high level under salt stress, the salt-induced ethylene signal in *Arabidopsis* is able to activate downstream targets that induce AP activity. The lower AP activity of *etr1-3* in response to salt stress than the WT could be explained by the salt-induced ethylene signal in the callus being unable to activate downstream targets that induce the AP. Further investigation will be required to clarify at what level ethylene exerts its fundamental effect.

In the present work, we found that ethylene could significantly induce the AP in WT calluses but not in *etr1-3* calluses under salt stress (**Fig. 3A, B**). Further investigations were

Table 2 Effects of SHAM on H_2O_2 content, MDA content and the value of electrolyte leakage in WT *Arabidopsis* seedlings under 100 mM NaCl for 48 h

Treatment	H_2O_2 content (nmol g^{-1} FW)	MDA content (nmol g^{-1} FW)	Electrolyte leakage (%)
Control	14.7±2.1a	19.8±2.3a	20.6±1.2a
SHAM	15.5±2.7a	19.6±3.5a	22.3±1.5a
NaCl	20.5±2.6b	27.2±3.7b	42.6±2.4b
NaCl+SHAM	29.7±3.4c	35.0±4.3c	52.7±2.5c

Two-week-old seedlings grown on 0.5×MS solid medium were transferred to 0.5×MS liquid medium containing various reagents for treatment. 1 mM SHAM was used for treatment. Data are mean values±SE of three independent experiments. Within each set of experiments, different letters were significantly different at the 0.01 level.

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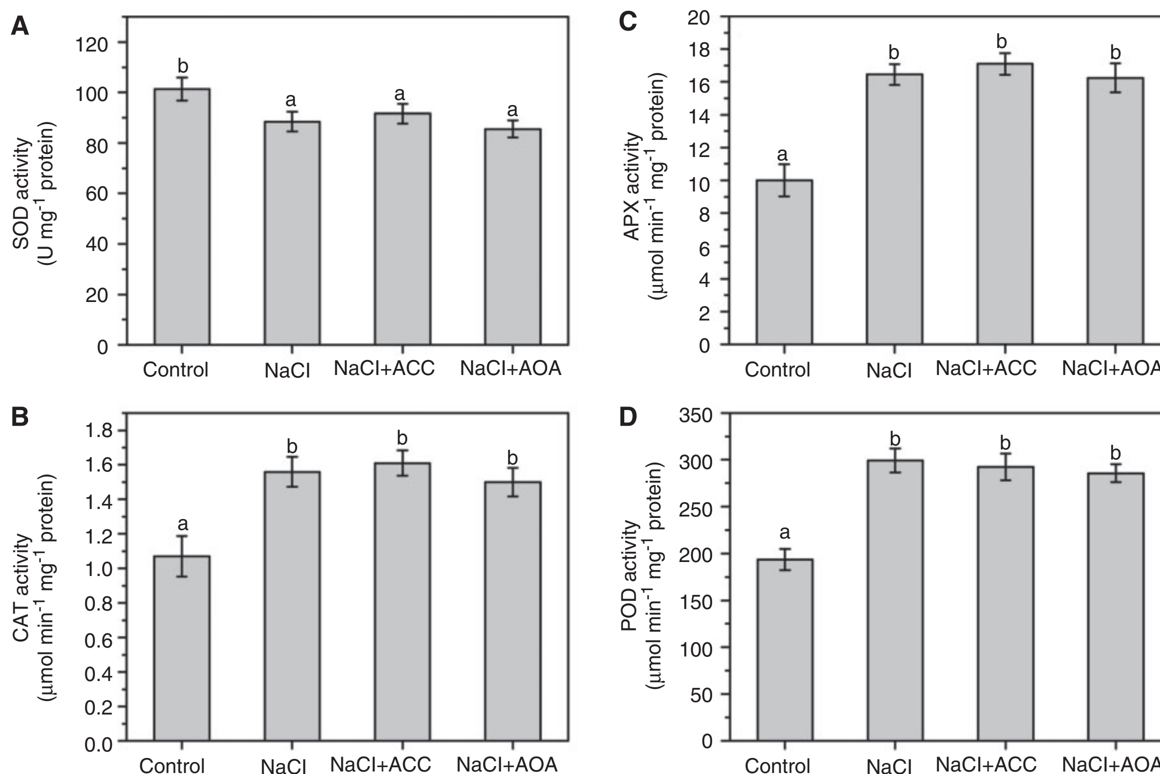

Fig. 8 Effect of ethylene on the activities of antioxidant enzymes (A, SOD; B, CAT; C, APX; D, POD) in WT calluses under 100 mM NaCl for 48 h. Treatments used 100 µM AOA and 100 µM ACC. Data are mean values ± SE for three independent experiments. Within each set of experiments, bars with different letters were significantly different at the 0.01 level.

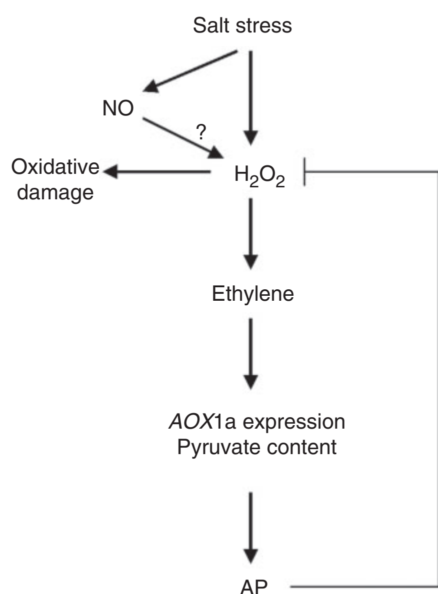

Fig. 9 Model illustrating the hypothetical function of ethylene, H_2O_2 and NO in AP induction in *Arabidopsis* under salt stress.

conducted to elucidate the relationship between ethylene and H_2O_2 in AP induction under salt stress. The results showed that H_2O_2 could induce ethylene production, and ethylene could reduce H_2O_2 generation in WT calluses under salt stress (**Figs. 3A, 4B**). In *etr1-3* calluses, the perception of ethylene is greatly reduced, thus the H_2O_2-induced AP in WT calluses was not observed in *etr1-3* calluses (**Fig. 4**). These results indicated that ethylene might be acting downstream of H_2O_2 in AP induction under salt stress.

The role of AOX in alleviating ROS production is well documented in mitochondria as well as at the cellular and even the tissue level, particularly in response to abiotic stresses such as low temperature (Maxwell et al. 1999, Umbach et al. 2005, Feng et al. 2008). To investigate the molecular mechanism of how ethylene and H_2O_2 enhance AP activity under salt stress, the effects of NaCl, ACC and H_2O_2 on the transcriptional regulation of AOX were examined in WT calluses. Our results showed that ethylene and H_2O_2 play a pivotal role in the response of *Arabidopsis* calluses to NaCl, and in particular, that ethylene is indispensable for activation of the AP and AOX gene (*AOX1a*) in the scavenging of mitochondrial ROS (**Figs. 3, 4, 5**). To corroborate this result, the transcriptional regulation of AOX was also analyzed in *Arabidopsis* seedlings. As shown in **Fig. 5**, similar phenomena in two different biological systems (cultured cells and seedlings) were observed. This result strengthens the validity of the observation in calluses, suggesting a broader occurrence of AOX regulation not limited to specific tissues or organs. It has been reported that induction of *AOX1a* expression in *Arabidopsis* cell cultures resulted in increased respiration through the AP (Huang et al. 2002),

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which was consistent with our results (**Fig. 5**), suggesting that the regulation of AP activity might occur at the transcription level.

In addition to transcriptional regulation, AOX activity is also influenced by the presence of pyruvate, an allosteric activator of AOX (Vanlerberghe and McIntosh 1997). In this study, a significant increase in pyruvate content was found in WT calluses under salt stress, and ethylene and H_2O_2 mediated a salt-induced increase in pyruvate content in WT calluses (**Fig. 6**). A higher pyruvate content in salt-stressed calluses might function to increase AP activity and enhance salt tolerance. Hu et al. (2006) reported that pyruvate content significantly increased in tolerant roots of cucumber but not in sensitive leaves under chilling stress. Kumar et al. (2007) also found that plants exhibited greater AP activity at high altitude as compared to plants grown at low altitude, meanwhile pyruvate content increased with an increase in altitude.

What might be the biological context in which salt induction of the AP plays a role? In general, the AP may dampen the generation of ROS during periods of rapid respiration (Maxwell et al. 1999). It has been reported that AOX inhibition stimulates H_2O_2 production in plant mitochondria (Popov et al. 1997), whereas overexpression of AOX results in lower ROS levels (Maxwell et al. 1999). In the present study, the salt-stressed WT *Arabidopsis* (calluses and seedlings) treated with SHAM generated more H_2O_2 relative to *Arabidopsis* either subjected to salt stress or SHAM application alone (**Fig. 7A, Table 2**). It suggests that inhibition of the AP by SHAM leads to additional H_2O_2 generation under salt stress. Consequently, as we observed, application of SHAM to WT calluses and seedlings under salt stress increased MDA content and the value of electrolyte leakage (**Fig. 7B, C, Table 2**). In contrast, SHAM application to *etr1-3* had almost no effect on cellular damage under salt stress (**Fig. 7B, C**), suggesting that calluses lacking ethylene signaling suffer more damage from ROS since H_2O_2, MDA and electrolyte leakage were already at a high level under salt stress. SHAM decreased the tolerance of WT calluses under salt conditions (**Fig. 7**). In the present study, we did not further investigate other potential targets of SHAM. It has been reported that SHAM is an inhibitor of peroxidases and xanthine oxidase (Able et al. 2000). However, according to Bartoli et al. (2005), 1 mM SHAM is sufficiently low to minimize possible side effects. Furthermore, these reactions require higher concentrations of SHAM. So the decreased protection to salt stress in SHAM-treated calluses is most likely a consequence of the inhibited AP. These results indicated that AP enhancement under salt stress might play an important role in limiting the production of ROS.

It has been reported that ROS accumulates under salt stress and high salinity induces oxidative stress (Hasegawa et al. 2000, Liu et al. 2007). The increase in the activity of antioxidant enzymes enhanced plant salt tolerance (Jung et al. 2000, Liu et al. 2007). CAT, POD and APX have been regarded as the most predominant H_2O_2-scavenging enzymes in plant systems (Puntarulo et al. 1988). In this study, we investigated the effects of ethylene on antioxidant enzymes under salt stress. The results showed that ethylene had no significant effects on the activity of antioxidant enzymes under stress (**Fig. 8**).

There are reports that nitric oxide (NO) greatly induces *AOX1a* expression in *Arabidopsis* cell cultures (Huang et al. 2002) and in tobacco plants (Ederli et al. 2006). More recently, Wang et al. (2009) also reported that NO accumulation was found in *Arabidopsis* calluses under salt stress. These observations imply that NO may also be involved in AP induction under salt stress. On the basis of the results presented here, as well as those reported previously (Simons et al. 1999, Huang et al. 2002, Vanlerberghe et al. 2002, Ederli et al. 2006, Giraud et al. 2008, Wang et al. 2009), we present a hypothetical model in *Arabidopsis* calluses and seedlings describing the interrelationships among ethylene, H_2O_2, NO, the AP and salt stress (**Fig. 9**). In this model, salt stress induces H_2O_2 generation, which subsequently causes ethylene emission. This increased ethylene induces *AOX1a* expression and pyruvate content, thus resulting in enhanced AP activity. The enhanced AP can dampen H_2O_2 generation to avoid ROS damage in plant cells. In addition, this putative pathway might be mediated by NO, whose biosynthesis was stimulated by salt stress (Wang et al. 2009); salt-induced NO production was involved in H_2O_2 generation in *Populus euphratica* calluses (Zhang et al. 2007). Thus NO may be acting upstream of H_2O_2 and ethylene. The ROS can act as important signaling molecules in plant cells, but in excess can also cause oxidative damage. Therefore plants need to make adjustments between these two situations to better survive stress conditions.

In conclusion, our results showed that ethylene and H_2O_2 both significantly induced the AP in WT calluses but not in *etr1-3* calluses under salt stress. The AP was induced under salt stress and played a role in salt tolerance. Enhancement of the AP under salt stress is mediated, at least in part, by H_2O_2 and then by ethylene. Furthermore, these findings provide additional degrees of complexity in the cross talk between ethylene and other signaling molecules (H_2O_2 and NO), which modulate AP induction under salt stress. Future investigations should focus on the multilevel interactions between NO, H_2O_2 and ethylene.

Materials and Methods

Plant material and chemical treatments

Calluses of the wild-type *Arabidopsis* (*Arabidopsis thaliana*; ecotype Col-0, WT) and ethylene-insensitive mutants *etr1-3* and *ein2-1* were induced as described by May and Leaver (1993). Following 4-month subcultures, 0.50 ± 0.05 g of callus was maintained on 30 ml of Gamborg B5 solid medium (Gamborg et al. 1968). After 16-day subcultures, the calluses were transferred to suspension medium for various treatments on a rotary shaker at 110 rpm. Sodium chloride was added to the medium for salt stress. Different concentrations of ACC, AOA, CAT, H_2O_2 or SHAM were added to the medium for various

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treatments after filter sterilization. The calluses were collected at the selected time points, washed for 2 min with distilled water, and then collected onto filter paper by vacuum aspiration (May and Leaver 1993). The samples were used immediately for the following parameter assay.

Arabidopsis seeds were surface sterilized and grown on agar plates containing 0.5×Murashige and Skoog (MS) medium (Murashige and Skoog 1962) under 14-h light (120 $m^{-2} s^{-1}$)/10-h dark cycles, at 23°C. *Arabidopsis* plants were treated as described previously (Parre et al. 2007). Two-week-old seedlings were gently removed from 0.5×MS agar plates to avoid damaging the roots and transferred into 0.5×MS liquid medium for 12 h. The seedlings were then transferred to Petri dishes containing various reagents in a 0.5×MS liquid medium. The nutrient medium was changed every 12 h. After indicated incubation times, seedlings were collected immediately for parameter analysis.

O_2 consumption assays

Total respiration (V_t) was measured using a Clark-type oxygen electrode with 50 mM phosphate buffer (pH 6.8) at 25°C in a dark room as described by Bingham and Farrar (1989). The capacity of the alternative respiratory pathway (V_{alt}) was calculated as the difference between respiration in the presence of 1 mM potassium cyanide (KCN) and residual respiration (V_{res}). The capacity of the cytochrome respiratory pathway (V_{cyt}) was calculated as the difference between respiration in the presence of 2 mM SHAM and residual respiration (V_{res}). V_{res} was determined as the respiration in the presence of 1 mM KCN and 2 mM SHAM.

Determination of H_2O_2 content

The H_2O_2 content was determined by the peroxidase-coupled assay according to Veljovic-Jovanovic et al. (2002). Calluses (0.5 g) or seedlings (0.2 g) were extracted in 2 ml 1 M perchloric acid ($HClO_4$) in the presence of insoluble polyvinylpyrrolidone (5%). The homogenate was centrifuged at 12,000 *g* for 10 min at 4°C, and the supernatant was neutralized with 5 M potassium carbonate (K_2CO_3) to pH 5.6 in the presence of 100 ml 0.3 M phosphate buffer (pH 5.6). The mixture was centrifuged at 12,000 *g* for 1 min and the extracts were incubated for 10 min with 1 U ascorbate oxidase to oxidize the ascorbate prior to assay. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 3.3 mM 3-(dimethylamino) benzoic acid, 0.07 mM 3-methyl-2-benzothiazoline hydrazone and 0.3 U peroxidase. The reaction was initiated by the addition of the oxidized extracts. The absorbance change at 590 nm was monitored at 25°C.

Determination of ethylene emission

Calluses (0.5 g) were placed in 10 ml gas-tight glass vessels and incubated at room temperature for 10 h. For ethylene determination, a 1 ml sample of gas was removed and analyzed with a flame ionizing gas chromatograph (model 3700, Varian Medical Systems, Palo Alto, CA, USA) with a Porapak Q column (80–100 mesh, 1 m×3.2 mm). Oven, injector and detector temperatures were 50°C, 150°C and 200°C, respectively.

Isolation and analysis of RNA

RNA was isolated from 100 mg tissue samples using Trizol solution (Invitrogen, Carlsbad, CA, USA). Total RNA (2 µg) was used for the first-strand cDNA synthesis with the Thermoscript RT-PCR system (Invitrogen). The yield of cDNA was measured according to the PCR signal generated from the internal standard, the housekeeping gene β-*actin*, amplified from 18 to 24 cycles starting with 2 µl of the cDNA solution. The *AOX1a* gene specific primers were 5′-CCGACGATTGGAGGT ATGAG-3′ and 5′-CCATTCCAGGTACTGCTGCTAC-3′, and the β-*actin* primers were 5′-GTTGGGATGACCCAGAAGAG-3′ and 5′-CTTACATTTCCCGATATGC-3′. The amplification steps were initial heating at 94°C for 30 s, denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were electrophoresed in 1% agarose stained with ethidium bromide. For quantification, filters were scanned and band intensities were determined with image analysis software.

Determination of pyruvate content

Pyruvate content was analyzed according to the method of Millar et al. (1998) with some modification. Callus samples (1 g) were snap frozen in liquid nitrogen (N_2), ground to a powder, thoroughly mixed with 5 ml of 2 M $HClO_4$ with 10 mM phosphate buffer (pH 3.5), and kept on ice for 10 min. Extracts were then centrifuged for 10 min at 10,000 *g*. The supernatant was then filtered and pH was adjusted to 7.2 with potassium hydroxide (KOH). After removal of precipitate, aliquots were assayed for lactate dehydrogenase (10 units)–dependent nicotinamide adenine dinucleotide (NADH) oxidation at 340 nm in a solution of 0.2 mM NADH in 0.5 M triethanolamine with 0.05 M ethylenediaminetetraacetic acid (EDTA) (pH 7.5).

Electrolyte leakage assay

Electrolyte leakage (EL) was determined according to Sairam and Srivastava (2002). Tissue samples were collected and washed in deionized water and placed in test tubes with 10 ml of deionized water at 25°C for 2 h. After the incubation, the conductivity in the bathing solution was determined (C_1). The samples were then heated at 95°C for 30 min and the conductivity in the bathing solution (C_2) was read again. EL was expressed as a percentage of the total conductivity ($EL = (C_1/C_2) \times 100$).

Determination of lipid peroxidation

The MDA content was measured according to Liu et al. (2007) with minor modifications. Calluses (0.5 g) or seedlings (0.2 g) were homogenized in 3 ml of 10% trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 10 min. A 1 ml aliquot of the supernatant was incubated with 1 ml of 0.5% thiobarbituric acid in 10% TCA at 95°C for 30 min. After centrifugation at 10,000 *g* for 10 min, the absorbance of the supernatant was read

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at 532 nm and the value for nonspecific absorption at 600 nm was corrected.

Antioxidant enzyme assay

Calluses (0.5 g) were homogenized with a mortar and pestle in 3 ml 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 5 mM cysteine and 1% (w/v) polyvinylpyrrolidone at 4°C. The homogenate was filtered and centrifuged at 15,000 *g* for 20 min. The supernatant was used for determination of enzyme activity.

Catalase activity was assayed following the decomposition of H_2O_2 at 240 nm (Aebi 1984). APX activity was measured in the presence of 0.25 mM ascorbic acid and 0.5 mM H_2O_2 by monitoring the decrease in absorption at 290 nm (Janda et al. 1999). SOD activity was determined as described by Prochazkova et al. (2001). POD activity was determined according to Adam et al. (1995). All reactions were measured at 25°C in 3 ml reaction mixtures. Measurements were made with a spectrophotometer (DU640, Beckman) with no lag period.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as mean ± SE. All comparisons were performed using one-way analysis of variance (ANOVA) and Duncan's multiple range test for independent samples. In all cases, the confidence coefficient was set at 0.01.

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