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Regulation of soybean seed germination through ethylene production in response to reactive oxygen species

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• *Background and Aims* Despite their toxicity, reactive oxygen species (ROS) play important roles in plant cell signalling pathways, such as mediating responses to stress or infection and in programmed cell death, at lower levels. Although studies have indicated that hydrogen peroxide (H_2O_2) promotes seed germination of several plants such as *Arabidopsis*, barley, wheat, rice and sunflower, the role of H_2O_2 in soybean seed germination is not well known. The aim of this study therefore was to investigate the relationships between ROS, plant hormones and soybean seed germination.
• *Methods* An examination was made of soybean seed germination, the expression of genes related to ethylene biosynthesis, endogenous ethylene contents, and the number and area of cells in the root tip, using *N*-acetylcysteine, an antioxidant, to counteract the effect of ROS.
• *Key Results* H_2O_2 promoted germination, which *N*-acetylcysteine suppressed, suggesting that ROS are involved in the regulation of soybean germination. H_2O_2 was produced in the embryonic axis after imbibition. *N*-Acetylcysteine suppressed the expression of genes related to ethylene biosynthesis and the production of endogenous ethylene. Interestingly, ethephon, which is converted to ethylene, and H_2O_2 reversed the suppression of seed germination by *N*-acetylcysteine. Furthermore, morphological analysis revealed that *N*-acetylcysteine suppressed cell elongation at the root tip, and this suppression was also reversed by ethephon or H_2O_2 treatments, as was the case in germination.
• *Conclusions* In soybean seeds, ROS produced in the embryonic axis after imbibition induce the production of endogenous ethylene, which promotes cell elongation in the root tip. This appears to be how ROS regulate soybean seed germination.

Key words: Ethylene, reactive oxygen species, seed germination, *Glycine max*.

INTRODUCTION

Seed germination is an important process in plant development and the process is complicated by several factors. Recently, as one of such factors, the relationship between seed germination and reactive oxygen species (ROS) in species such as *Arabidopsis thaliana* (Liu *et al.*, 2010; Leymarie *et al.*, 2012), sunflower (Oracz *et al.*, 2007), wheat (Ishibashi *et al.*, 2008), cress (Müller *et al.*, 2009*a*) and barley (Ishibashi *et al.*, 2010*a*; Bahin *et al.*, 2011) has been reported.

In general, ROS such as O_2^-, hydrogen peroxide (H_2O_2) and $^{\bullet}OH$ cause oxidative damage to lipids, proteins and nucleic acids. Indeed, seed deterioration is due in part to peroxidation of membrane lipids by ROS and the resulting leakiness of the membranes (Sung and Jeng, 1994; Bailly *et al.*, 1998). Seed longevity is enhanced through elimination of ROS by overaccumulated ROS scavengers in transgenic seeds (Lee *et al.*, 2010; Zhou *et al.*, 2012). However, they also play various important roles in cellular signalling in plants, notably acting as regulators of growth and development, programmed cell death, hormone signalling, and responses to biotic and abiotic stresses (Mittler *et al.*, 2004). In seed physiology, several studies have reported that exogenous H_2O_2 promotes seed germination in many plants (Chien and Lin, 1994; Fontaine *et al.*, 1994). Furthermore, the production of H_2O_2 during the early imbibition period has been demonstrated in seeds of soybean (Puntarulo *et al.*, 1988), maize (Hite *et al.*, 1999), wheat (Caliskan and Cuming, 1998) and *Zinnia elegans* (Ogawa and Iwabuchi, 2001). On this basis, ROS produced after imbibition appear to regulate seed germination. Indeed, in barley seeds, NADPH oxidase, which is one of the major sources of ROS, acts as a key enzyme in germination and subsequent seedling growth (Ishibashi *et al.*, 2010*a*). In pea seeds, H_2O_2 accelerates germination and stimulates the early growth of seedlings (Barba-Espin *et al.*, 2010). In contrast, exogenous antioxidants, which act as ROS scavengers, significantly suppressed seed germination in several species (Ogawa and Iwabuchi, 2001; Ishibashi and Iwaya-Inoue, 2006).

Plant hormones, which are one of such factors, are important in the regulation of seed dormancy and germination (Koornneef *et al.*, 2002; Finkelstein, 2004). The interactions among abscisic acid (ABA), gibberellins, ethylene, brassinosteroids, auxins and cytokinins in regulating the interconnected molecular processes that control dormancy release and germination have been reported (Kucera *et al.*, 2005). There are many reports on the interaction of ROS with plant hormones in plant. In guard cells, ROS are considered second messengers in the ABA transduction pathway (Wang and Song, 2008), and

exogenous ABA leads to an increase in H_2O_2 in guard cells (Pei *et al.*, 2000) regulating ion channels leading to stomatal closure (Schroeder *et al.*, 2001). In addition, ethylene receptor ETR1 plays an important role in guard cell ROS signalling and stomatal closure (Desikan *et al.*, 2005). In seed physiology, exogenous H_2O_2 increased ABA catabolism by enhancing the expression of *CYCP707A* genes, played a major role in ABA catabolism and enhanced gibberellic acid (GA) biosynthesis genes in *Arabidopsis* dormant seeds (Liu *et al.*, 2010). ROS regulated the expression of ethylene response factor *ERF1*, a component of the ethylene signalling pathway in sunflower seed germination (Oracz *et al.*, 2009). In barley dormant seed, H_2O_2 enhanced GA synthesis genes such as *GA20ox1* rather than repression of ABA signalling in embryo (Bahin *et al.*, 2011). Recently, we have also shown that ROS regulate the induction of α-amylase through gibberellin–ABA signalling in barley aleurone cells (Ishibashi *et al.*, 2012).

In soybean seeds, ROS are produced in the embryonic axis during germination (Puntarulo *et al.*, 1988, 1991), and the production and scavenging of ROS during ageing were related to vigour and cell death, respectively, during accelerated ageing of the embryonic axis (Tian *et al.*, 2008). In addition, low temperatures led to oxidative stress and lipid peroxidation caused by ROS in the embryonic axis (Posmyk *et al.*, 2001). Although the negative role of ROS in soybean seed is now well documented, there have been fewer studies of a positive role of ROS in soybean seed. We therefore investigated the effects of ROS and antioxidants on soybean seed germination to clarify the role of ROS in germination.

MATERIALS AND METHODS

Plant material

Soybean (*Glycine max* 'Fukuyutaka') seeds were obtained from the Nakahara Seed Product Co. Ltd (Fukuoka, Japan), and only intact seeds were used.

Germination test

For each replicate, 20 soybean seeds between two filter papers were placed in a Petri dish (diameter: 9 cm). Then, 12 mL distilled water, 100 mM H_2O_2, 100 mM mannitol (added as an osmotic solute), *N*-acetylcysteine (NAC, at 10, 25 or 50 mM), or ethephon (at 1, 10, 100 or 300 p.p.m.) were added to each plate. The plates were incubated at 25 °C in the dark, and the number of germinating seeds was counted for 3 d. Seeds were considered to have germinated when the radicle protruded through the seed coat. The results presented are the means of the germination percentages obtained for five replicates per treatment.

Localization and content of H_2O_2

Hand-cut longitudinal sections of seeds treated with distilled water or 25 mM NAC for 24 h were incubated in 1 mg L^{-1} 3,3′-diaminobenzidine stain at room temperature for 1 h. H_2O_2 was visualized as deposits of dark brown stain under a stereomicroscope (Stemi DV4; Zeiss, Oberkocken, Germany). H_2O_2 content was measured according to the method of Oracz *et al.* (2007) using a peroxidase-based assay with 3-dimethylaminobenzoic acid and 1·3 mM 3-methyl-2-benxothiazolidone hydrazine (O'Kane *et al.*, 1996). The results presented are the means of the H_2O_2 contents obtained using three replicates.

Ethylene production

For each treatment, 20 soybean seeds were placed in a 9-cm-diameter Petri dish and germinated as described above. Each plate was then sealed with cling film, and a 1·0-mL gas sample after imbibition for 24 and 48 h was removed from the headspace using a gas-tight syringe. The samples were assayed on a gas chromatograph (GC-4000; GL Science, Tokyo, Japan) with a flame ionization detector and a column packed with Porapak-Q (GC-4000; GL Science, Tokyo, Japan). Temperature was maintained at 150 and 50 °C for infector/detector and oven, respectively. Ethylene was quantified by comparison of peak areas with those produced by known amounts of ethylene. Ethylene production was normalized by dividing the content by the number of seeds in each plate.

RT-PCR analysis

Embryonic axis samples were collected after imbibition for 12 or 24 h and frozen in liquid nitrogen. The frozen materials were ground to a fine powder in liquid nitrogen using a mortar and pestle, and total RNA was extracted by using the SDS–phenol–LiCl method (Chirgwin *et al.*, 1979). cDNA was synthesized from total RNA (1 μg) with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's protocol. cDNA (1 μL) was amplified in a reaction mixture containing 10 μL of Go Taq Green Master Mix (Promega, Madison, WI, USA), 0·1 μL each of 100 μM forward and reverse primers (Supplementary Data Table S1) and 8·8 μL of water. The amplification was conducted using a PC-320 Program Temp Control System (Astec, Fukuoka, Japan) as follows: 1 min at 94 °C; 25–30 cycles of 15 s at 94 °C, 30 s at 58–60 °C and 30 s at 72 °C; and a final 5 min at 72 °C. The resulting PCR products were visualized by FluorChem (Cell Bioscience, Santa Clara, CA, USA) after electrophoresis in 1·5 % agarose gels and staining with ethidium bromide.

Embryonic axis growth and longitudinal sections

The embryonic axis of seedlings was photographed with a stereomicroscope with a built-in digital camera (Stemi DV4), and we then measured the distance from the root tip to the hypocotyl base using the linear measurement tool of Adobe Acrobat Reader (http://get.adobe.com/reader/) (Adobe Systems Inc., San Jose, CA, USA). The segments of embryonic axis tissue were immediately placed in formalin–acetic acid–alcohol fixative solution. Serial longitudinal sections, 20 μm thick, were cut using a cryostat microtome (Cryostat HM 505 E; Microm GmbH, Walldorf, Germany). The sections were stained for 1 min in 0·1 % (w/v) toluidine blue and then gently washed for 2 min with distilled water.

Cell number and size in the embryonic axis

The contrast and tone of the digital images of the longitudinal sections were adjusted in the GIMP image editing software (http://www.gimp.org/). Each cell edge was manually drawn on a tracing desk. These traces were scanned on a digital scanner and then trimmed in GIMP. The size and number of the cells in the embryonic axis were determined with ImageJ software (http://rsb.info.nih.gov/ij/). Cell areas were calculated by using the 'Analyse Particles' function with a particle size cut-off threshold of 100 pixels (a pixel has an image area of 1·197 μm^2).

RESULTS

Effect of H_2O_2 and NAC on soybean seed germination and localization of H_2O_2 during soybean seed germination

NAC, which acts as an antioxidant, suppressed soybean seed germination, and the effect increased with increasing concentration (Fig. 1A). In contrast, H_2O_2 significantly promoted germination compared with the control (Fig. 1B). This result was in accordance with results of pea and barley seeds (Barba-Espin *et al.*, 2010; Ishibashi *et al.*, 2010*a*). The germination of seeds treated with distilled water (the control) reached approx. 70 % after 24 h of imbibition, whereas that of seeds treated with 25 mM NAC was approx. 20 %. Because the suppression of germination by 25 mM NAC was significant after 18 and 24 h, we used 25 mM NAC in the subsequent experiments. The germination of seeds treated with 100 mM mannitol was almost the same as that in the control, suggesting that the suppression or promotion caused by NAC or H_2O_2 was not caused by their osmolality. In addition, germination of seeds treated with a solution containing both NAC and H_2O_2 showed a significant reversal of the inhibitory effects caused by NAC alone (Fig. 1B).

We stained the seeds with 3,3′-diaminobenzidine to assay the accumulation of H_2O_2. Seeds treated with distilled water clearly showed H_2O_2 accumulation in the embryonic axis but not in the cotyledon (Fig. 2A, B), as reported previously (Puntarulo *et al.*, 1988, 1991). On the other hand, NAC-treated seeds accumulated little H_2O_2. In addition, the H_2O_2 content in embryonic axis in the control was significantly higher than that in the NAC treatment (Fig. 2C).

NAC suppresses ethylene production during soybean seed germination

We investigated the relationship between the stimulation of ethylene production by ROS and seed germination (Fig. 3), because both ROS and ethylene can stimulate germination and overcome dormancy in many species (Kępczyński and Kępczyńska, 1997; Bailly *et al.*, 2008). 1-Aminocyclopropane-1-carboxylic acid (ACC) is a direct precursor of ethylene during biosynthesis (Wang *et al.*, 2002). ACC synthase is a key enzyme in ethylene biosynthesis, and the *ACS* gene belongs to a multi-gene family whose members are regulated by a complex network of developmental and environmental signals that respond to both internal and external stimuli (Johnson and Ecker, 1998). Tucker *et al.* (2010) reported 21 *ACS*-like sequences

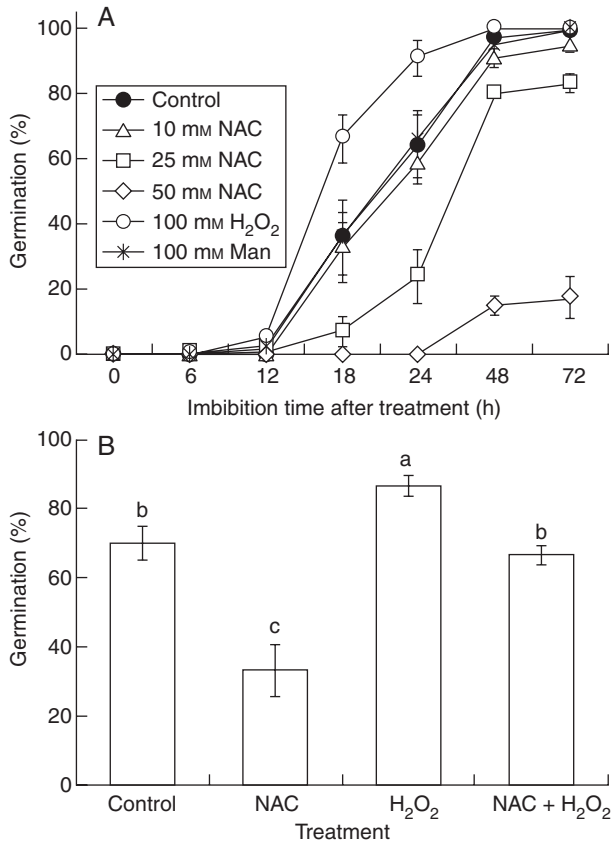

FIG. 1. The effects of hydrogen peroxide (H_2O_2) and *N*-acetylcysteine (NAC) on soybean seed germination. (A) Change of germination with time of soybean seeds treated with distilled water (control), and with 10, 25, or 50 mM NAC, 100 mM H_2O_2 or 100 mM mannitol (Man). (B) Germination of soybean seeds treated with distilled water (control), 25 mM NAC, 100 mM H_2O_2 or 25 mM NAC + 100 mM H_2O_2 for 24 h. Bars with different letters differ significantly ($P < 0{\cdot}05$, Tukey's test, $n = 5$).

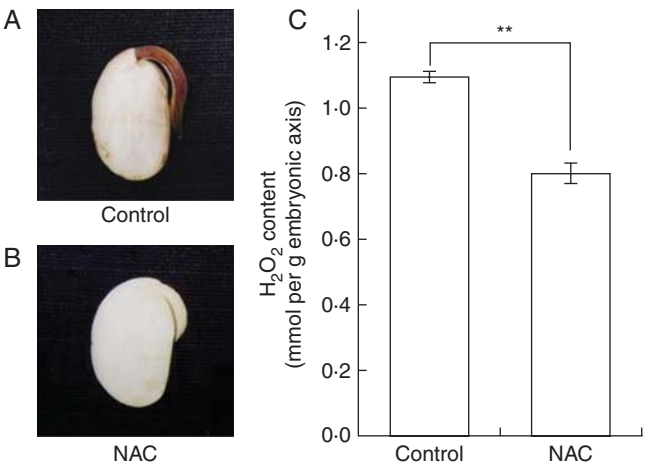

FIG. 2. The production of hydrogen peroxide (H_2O_2) in the soybean embryonic axis after imbibition. (A, B) ROS accumulation monitored by staining with 1 mg mL^{-1} 3,3′-diaminobenzidine. (C) H_2O_2 contents in the embryonic axis of germinating seeds treated with distilled water or 25 mM *N*-acetylcysteine (NAC) for 24 h. (**$P < 0{\cdot}05$, Student's test, $n = 3$).

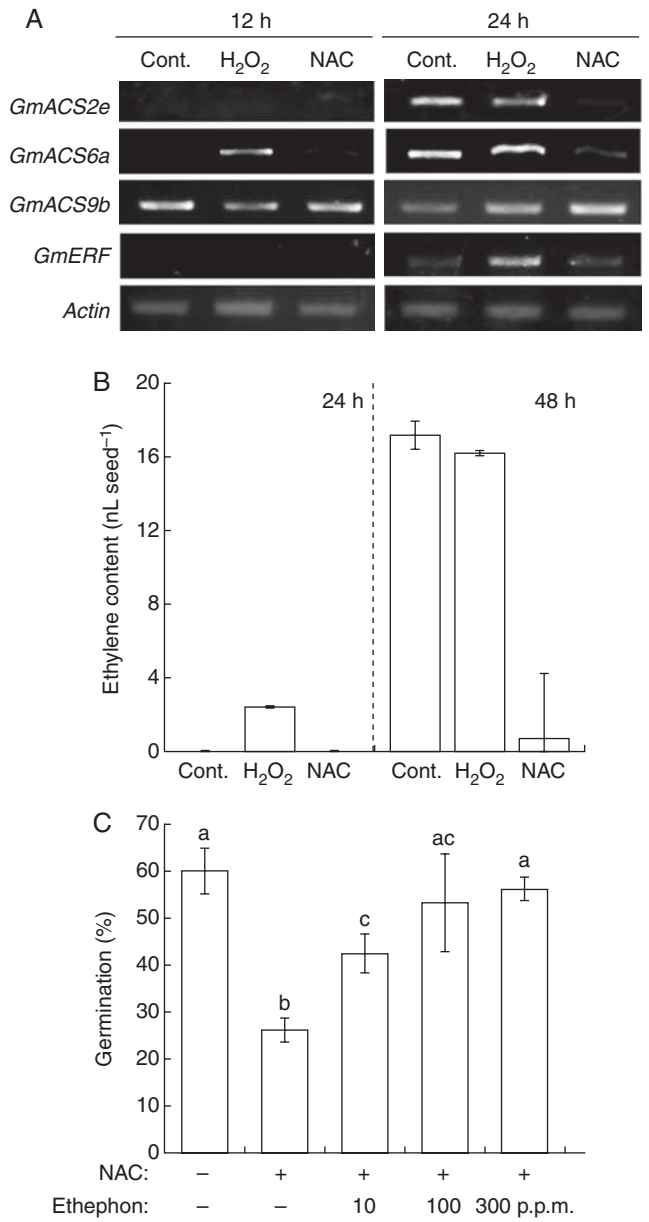

FIG. 3. Hydrogen peroxide (H_2O_2) promotes biosynthesis of ethylene during soybean seed germination. (A) Effect of H_2O_2 (100 mM) and *N*-acetylcysteine (NAC; 25 mM) on the expression of genes related to ethylene biosynthesis in embryonic axis and (B) production of ethylene in soybean seed after imbibition. (C) Germination of seeds treated with distilled water (Cont.), 25 mM NAC and 25 mM NAC + 10, 100 or 300 p.p.m. ethephon for 24 h. Bars with different letters differ significantly ($P < 0{\cdot}05$, Tukey's test, $n = 5$).

(the *GmACS* family) in the soybean genomic sequence database. Of these sequences, we focused on *GmACS2e*, *GmACS6a* and *GmACS9b*. *GmACS2e* is a homologue of the injury-induced *AtACS* genes in *Arabidopsis* (Broekaert *et al.*, 2006). *GmACS6a* and *GmACS9b* show high homology to *VrACS1* and *VrACS6*, whose expression is promoted by H_2O_2 or auxin in mungbean (*Vigna radiata*) hypocotyls (Song *et al.*, 2007).

In the expression of *GmACS* genes, *GmACS9b* was hardly affected by any of the treatments (Fig. 3A). Control increased the expression of *GmACS2e* and *GmACS6a* at 24 h but not at 12 h after imbibition. H_2O_2 treatment induced the expression of *GmACS6a* within 12 h of imbibition. On the other hand, NAC treatment suppressed the expression of *GmACS2e* and *GmACS6a* at both 12 and 24 h after imbibition. It also induced the expression of *GmERF*, which acts downstream of ethylene signals, after 24 h. Ethylene production increased significantly in the H_2O_2 treatment but not in the control and NAC treatments after 24 h (Fig. 3B). It did not differ significantly between the control and H_2O_2 treatments after 48 h (17·2 and 16·2 nL per seed, respectively). However, the ethylene content in soybean seeds treated with NAC was much lower after 48 h (0·69 nL per seed). Treatment of seeds with NAC plus ethephon (which is converted to ethylene) significantly increased germination compared with treatment with NAC alone, and the two higher levels of ethephon increased germination in the presence of NAC to levels that did not differ significantly from those in the control (Fig. 3C).

NAC suppresses cell elongation in the root tip after imbibition

The length of the embryonic axis in seeds treated with NAC was significantly less than the control (Fig. 4). However, the length in seeds treated with NAC + H_2O_2 or NAC + ethephon did not differ significantly from the control, indicating that H_2O_2 and ethephon reversed the inhibitory effect of NAC on the growth of the embryonic axis. Konings and Jackson (1979) reported that a certain amount of endogenous ethylene is necessary for root growth, but that excess ethylene decreased cell elongation in the roots. We therefore carried out a morphological analysis of the embryonic axis (Supplementary Data Fig. S1). In the morphological analysis, we found major structural differences among the treatments at the root tip (Fig. 5A, B). The total area of the root tip in seeds treated with NAC was significantly smaller than the control (Fig. 5C). However, the ethephon treatment fully counteracted the effects of NAC, producing a root tip area that was not significantly different from the control, but the H_2O_2 treatment could not fully counteract the effects of NAC; although the root area was significantly greater in the H_2O_2 treatment than in the NAC treatment, the root tip area remained significantly smaller than in the control. Moreover, the number of cells in the root tip did not differ significantly among the four treatments (Fig. 5D). The size of cells in root tips treated with NAC was significantly lower (by approx. 42 %) than the control. Ethephon completely counteracted the effect of NAC, but although H_2O_2 significantly decreased the adverse effects of NAC, cell size remained significantly smaller than the control (Fig. 5E). It seems likely that there was insufficient H_2O_2, not that something about the experimental conditions prevented this ROS from stimulating ethylene production because H_2O_2 completely counteracted the effect of NAC on expression of *GmACS2e* and *GmACS6a* (Supplementary Data Fig. 2S).

DISCUSSION

Recent studies of seed physiology have described the relationships between ROS and seed germination, dormancy and afterripening (Bailly *et al.*, 2008; Müller *et al.*, 2009*b*; Oracz *et al.*, 2009; Liu *et al.*, 2010; Ishibashi *et al.*, 2010*a*; Bahin *et al.*, 2011; Leymarie *et al.*, 2012). Here, we confirmed that

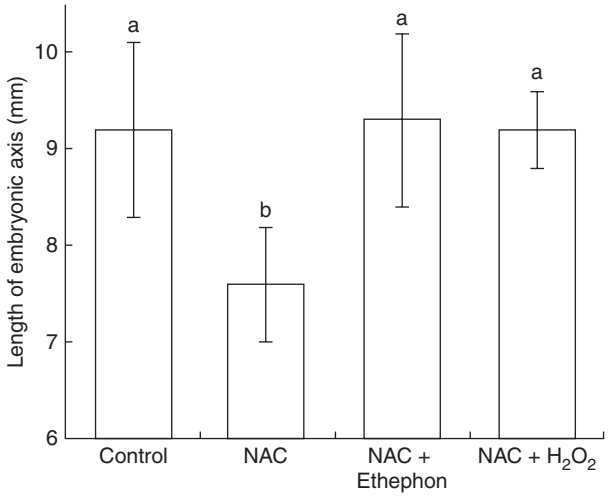

FIG. 4. Effect of *N*-acetylcysteine (NAC) on the length of the embryonic axis during soybean seed germination. Seeds were treated with distilled water (control), 25 mM *N*-acetylcysteine (NAC), 25 mM NAC + 300 p.p.m. ethephon, or 25 mM NAC + 100 mM H_2O_2 for 24 h. Bars with different letters differ significantly ($P < 0{\cdot}01$, Tukey's test, $n = 10$).

soybean seed germination was regulated through ethylene production in response to ROS. The first line of evidence supporting this conclusion was the suppression of germination by NAC, an antioxidant that suppressed H_2O_2 content in the embryonic axis (Fig. 2) and decreased seed germination (Fig. 1A). In addition, exogenous H_2O_2 counteracted the suppression of germination by NAC (Fig. 1B). These results suggest that NAC suppressed the germination of soybean seed by inhibiting H_2O_2 production in the embryonic axis after imbibition. Puntarulo *et al.* (1988) reported the production of H_2O_2 during early imbibition in soybean seeds, and subsequently found that the activities of the enzymes involved in H_2O_2 metabolism (e.g. superoxide dismutase, catalase, peroxidase, and glutathione and ascorbate peroxidases) changed markedly in the soybean embryonic axis during germination (Puntarulo *et al.*, 1991). However, the role of H_2O_2 in soybean seed germination remained unclear.

Ethylene was originally regarded as a plant stress hormone, because its synthesis is induced by a variety of stress signals, such as mechanical wounding (Kende, 1993), exposure to a range of chemicals and metals, drought, extreme temperatures and pathogen infection (Johnson and Ecker, 1998). In seed biology, ethylene clearly participates in the germination of certain seeds (Matilla and Matilla-Vazquez, 2008) and increases radicle emergence under unfavourable conditions (Abeles, 1986; Kozarewa *et al.*, 2006). In addition, there have been numerous reports of a relationship between ROS and ethylene: in legumes, ROS and ethylene are part of a Nod factor-induced signal cascade that is important for the initiation of nodule primordia (D'Haeze *et al.*, 2003). In winter squash (*Cucurbita maxima*), *CmACS1* is inhibited by diphenylene iodonium, which blocks the superoxide-generating enzyme NADPH oxidase (Watanabe *et al.*, 2001). In mungbean hypocotyls, exogenous ACC (a precursor that is easily converted to ethylene) did not affect ROS production, but hypocotyls exposed to H_2O_2 showed high ethylene

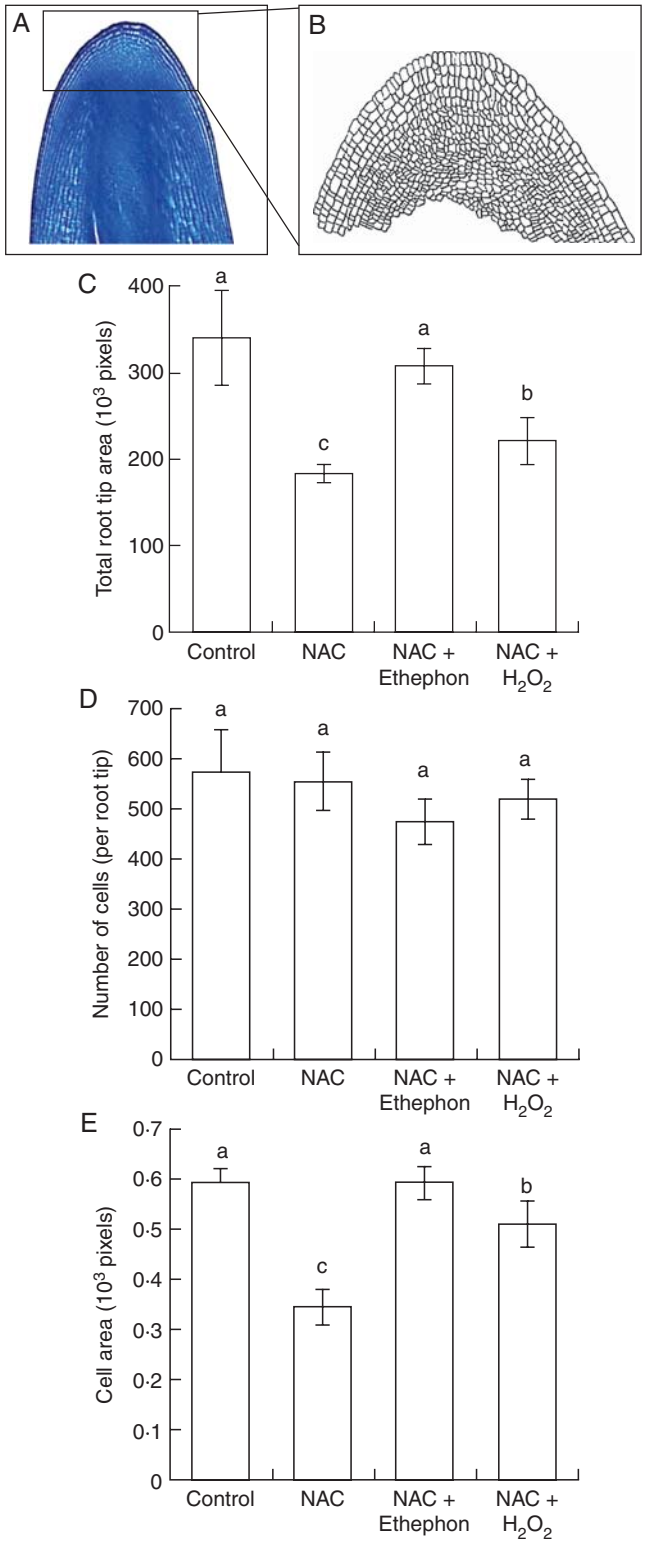

FIG. 5. Number and area of cells in the root tip during soybean seed germination. Seeds were treated with distilled water, 25 mM *N*-acetylcysteine (NAC), 25 mM NAC + 300 p.p.m. ethephon, or 25 mM NAC + 100 mM H_2O_2. (A) Staining of the embryonic axis by toluidine blue. (B) Trace of cells in the root tip. (C) Total root tip area, (D) number of cells and (E) cell area of the root tip, based on traces that were measured in ImageJ software. Bars with different letters differ significantly ($P < 0{\cdot}05$, Tukey's test, $n = 5$).

accumulation as a result of activation of ethylene biosynthesis enzymes (Song *et al.*, 2007). Thus, we hypothesized that ethylene production in soybean seeds would be affected by ROS generated after imbibition, as our results confirmed (Fig. 3).

Although expression levels of *GmACS9b* remained high in all treatments, those of *GmACS6a* and *GmACS2e* were very low in the NAC treatment but increased in the control and H_2O_2 treatments, especially *GmACS6a*, expression of which was promoted within 24 h after imbibition in the H_2O_2 treatment (Fig. 3A). In *Arabidopsis* and tomato, expression of specific members of the *ACS* gene family is rapidly induced by O_3 (Vahala *et al.*, 1998; Overmyer *et al.*, 2000; Nakajima *et al.*, 2001; Moeder *et al.*, 2002; Tamaoki *et al.*, 2003). Although the stimulation of ethylene synthesis by environmental stress involves the generation of ROS, there is also a regulatory mechanism by which ROS regulate ethylene biosynthesis (Surplus *et al.*, 1998; Qin *et al.*, 2008). We found that the production of endogenous ethylene in soybean seeds after imbibition was promoted by H_2O_2 and suppressed by NAC (Fig. 3B). Other researchers have reported that ethylene sensitivity is a major factor in the germination of dormant *Arabidopsis* seeds (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Gallardo *et al.*, 2002; Siriwitayawan *et al.*, 2003). In our study, expression of *GmERF* (which acts downstream of ethylene signalling) was induced by H_2O_2, but only after 24 h (Fig. 3A), and the suppression of seed germination by NAC could be mitigated or eliminated by ethephon treatment (Fig. 3C). These results indicate that ethylene production in response to the ROS produced after imbibition was involved in the germination of soybean seeds.

Several hypotheses have been proposed to explain the mechanisms of ethylene action in germinating seeds (Esashi, 1991; Kępczyński and Kępczyńska, 1997; Matilla and Matilla-Vazquez, 2008). For example, the promotion of radial cell expansion is a primary response to ethylene during seed germination (Kucera *et al.*, 2005). In addition, ROS are involved in cell elongation; for example, the requirement for ROS during elongation of growing apical cells in root hairs has been demonstrated through the use of NADPH oxidase mutants (Foreman *et al.*, 2003; Carol and Dolan, 2006). The production of ROS such as O_2^- has been detected in the expansion zone of maize leaf blades, and results in cell-wall loosening (Rodríguez *et al.*, 2002). In our study, NAC reduced the length of the embryonic axis, but the addition of ethephon or H_2O_2 counteracted the reduction by NAC (Fig. 4), suggesting that ethylene produced in response to ROS after imbibition regulated the length of the embryonic axis. Furthermore, morphological analysis revealed that the ethylene produced in response to ROS in the embryonic axis (root tip and zone of elongation) induced cell hypertrophy (Fig. 5E, Supplementary Data Fig. S3B) but not hyperplasia (Fig. 5D, Supplementary Data Fig. S3A). Our results suggest that the ethylene produced in response to ROS regulates the length of the embryonic axis by increasing the size of root tip cells without increasing their number, and thereby regulated soybean seed germination.

Konings and Jackson (1979) proposed that a certain amount of endogenous ethylene is necessary to maintain root growth. Increasing the concentration of endogenous ethylene leads to increased growth until a threshold concentration is reached; thereafter, further increases in the ethylene concentration reduce growth. We previously reported that pre-treatment with H_2O_2 promoted the germination of soybean seeds but delayed emergence (Ishibashi *et al.*, 2010*b*). The emergence was delayed by lateral growth of the hypocotyls in response to ethylene produced in response to the H_2O_2. Furthermore, Zheng and Inoue (1989) reported that when hypocotyl elongation during emergence was inhibited by soil particles and crust under field conditions, the seedlings produced ethylene, and their hypocotyls thickened and became able to break the crust. In soybean, the production of H_2O_2 in seeds after imbibition might govern the growth from seed germination to emergence through the production of ethylene.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: gene-specific oligo-DNA primers used in the RT-PCR analysis. Figure S1: morphology of the embryonic axis (root tip + elongation zone) of seeds treated with either distilled water (control), 25 mM NAC, 25 mM NAC + 300 p.p.m. ethephon, or 25 mM NAC + 100 mM H_2O_2 for 24 h. Figure S2: promotion of expression of genes for ethylene biosynthesis in embryonic axis during soybean seed germination by H_2O_2. Figure S3: number and area of cells in the zone of elongation in the root of seeds treated with distilled water or NAC.

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