African Journal of Biotechnology Vol. 9(11), pp. 1578-1586, 15 March, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1819
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Abscisic acid (ABA)-mediated inhibition of seed germination involves a positive feedback regulation of ABA biosynthesis in *Arachis hypogaea* L.

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Accepted 26 February, 2010

Abscisic acid (ABA) plays an important role in seed dormancy, embryo development and adaptation to environmental stresses. We found that imbibition of exogenous ABA by peanut seeds led to a significant increase in the levels of both *AhNCED1* gene [a key gene encoding nine-*cis*-epoxycarotenoid dioxygenase (NCED) involved in ABA biosynthesis in peanut] transcript and endogenous ABA in germinating seeds, and also led to a marked decrease in α-amylase activity, germination rate and viability index of germinating seeds. This was associated morphogenetically with inhibited plumule apex growth and reduced leaf primordium elongation, a decreased number and length of axial and lateral buds, and shorter length of compound leaves during germination. Imbibition by peanut seeds of naproxen (a potent ABA biosynthesis inhibitor specifically targeting to NCED) significantly decreased the levels of endogenous ABA and *AhNCED1* gene transcript in germinating seeds, and markedly increased α-amylase activity, germination rate and viability index of germinating seeds. This was associated morphogenetically with increased plumule apex growth and leaf primordium elongation as well as increased number and length of axial and lateral buds, but without a significant change in the length of compound leaves during seed germination. These observations suggest the involvement of a positive feedback regulation of ABA biosynthesis in ABA-mediated inhibition of seed germination in peanut.

Key words: Abscisic acid, *AhNCED1* gene, biosynthesis, feedback regulation, peanut (*Arachis hypogaea* L.), seed germination.

INTRODUCTON

Seed germination is regulated by dormancy and environmental factors such as light, oxygen and temperature, and it is thought that the key to this is the balance of the negative and positive regulative effects of abscisic acid (ABA) and gibberellin (GA). Gonai et al. (2004) suggested that exogenously applied GA_3 counteracted thermionhibition of lettuce (*Lactuca sativa*) seeds by enhancing the catabolism of ABA. Seo et al. (2006) indicated that endogenous ABA suppressed GA biosynthesis in developing seeds, and infra-red light-treated mature seeds during imbibition, through suppression of GA 20-oxidase (GA20ox; EC 1.14.11.-) and GA 3-oxidase (GA3ox; EC 1.14.11.15) genes in *Arabidopsis thaliana*. Zentella et al. (2007) showed that the transcript levels of *GA20ox1* in *Arabidopsis* plants were significantly reduced by application of exogenous ABA. Recently, Toh et al. (2008) showed that high temperature stimulated ABA biosynthesis and repressed GA biosynthesis and signaling, through the action of ABA, in *Arabidopsis* seeds during germination.

ABA is a plant hormone ubiquitously present in higher plants; it plays a vital role in seed dormancy regulation,

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Abbreviations: ABA, Abscisic acid; **NCED,** nine-*cis*-epoxycarotenoid dioxygenase; **GA,** gibberellins; **C40,** carotenoids; **vp14,** viviparous 14; **FAA,** formalin-glacial acetic acid-alcohol.

#These authors contributed equally to this paper

embryo development, and adaptation to various environmental stresses, most notably drought (Qin et al., 2008). Endogenous ABA level is a determinant of these physiological processes, and ABA-deficient mutants exhibit reduced seed dormancy and reduced drought tolerance (McCarty, 1995). Conversely, exogenous application of ABA resulted in delayed germination (Guo et al., 2008) and increased tolerance to a variety of abiotic stresses (Li and Pan, 1996). It is now well established that in higher plants, ABA is synthesized from carotenoids (C40) which is an apo-carotenoid compound derived from oxidative cleavage of the 11,12 double bond of nine-*cis*-epoxycarotenoids (Schwartz et al., 2003). Biochemical (Kende and Zeevaart, 1997) and genetic evidence (Koornneef et al., 1998) has demonstrated that the cleavage of nine-*cis*-epoxycarotenoids is the rate-limiting step in the ABA biosynthetic pathway, which is catalyzed by nine-*cis*-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51). The NCED enzyme was first identified by analysis of the maize *viviparous 14 (vp14)* mutant (Tan et al., 1997; Schwartz et al., 1997). *NCED* genes have subsequently been identified in several species, including tomato (Burbidge et al., 1997), bean (Qin and Zeevaart, 1999), cowpea (Iuchi et al., 2000), avocado (Chernys and Zeevaart, 2000), *Arabidopsis* (Iuchi et al., 2001), grape (Soar et al., 2004), orange (Rodrigo et al., 2006), potato (Destefano-Beltran et al., 2006), *Stylosanthes guianensis* (Yang and Guo, 2007), *Gentiana lutea* (Zhu et al., 2007), cleavers (Kraft et al., 2007), *Cuscuta reflexa* (a parasitic plant lacking neoxanthin) (Qin et al., 2008), *Cistus creticus* (a Mediterranean shrub) (Munne-Bosch et al., 2009), and persimmon (Leng et al., 2009). We characterized a dehydration-inducible *NCED* gene, *AhNCED1* (GenBank accession no. AJ574819), from dehydrated peanut (*Arachis hypogaea* L.) plants (Wan and Li, 2005). The *AhNCED1* gene expressed typically and significantly as a response to dehydration, i.e. water stress; the *AhNCED1* gene was further found to play an important role in the regulation of ABA biosynthesis in peanut (Wan and Li, 2006).

Many biosynthetic pathways are regulated by their end products. For example, ethylene biosynthesis is subjected to both positive and negative feedback regulation (Inaba, 2007; Kende, 1993). Positive feedback regulation characteristically occurs in ripening fruits and senescing flowers in which ethylene biosynthesis escalates. On the other hand, negative feedback regulation occurs mostly in instances of auxin- or stress-induced ethylene production in various plant organs. Ross et al. (1999) showed that GA biosynthetic steps were negatively feedback-regulated by GA_1 in pea. However, the feedback regulation of ABA biosynthesis in higher plants needs to be investigated further. We found that ABA inhibited lateral root development of peanut plants by up-regulation of *AhNCED1* gene causing accumulation of endogenous ABA (Guo et al., 2009). Szepesi et al. (2009) suggested a positive feedback regulation of ABA synthesis by salicylic acid-induced ABA accumulation in tomato plants, and Barrero et al. (2006) showed that accumulated ABA levels exerted a positive feedback on its own biosynthetic pathway in *Arabidopsis*, inspite of some earlier reports where no feedback regulation was found in ABA biosynthesis in cowpea (Iuchi et al., 2000) and tomato (Thompson et al., 2000).

In the present study, the effects of exogenous application of ABA and naproxen [a potent ABA biosynthesis inhibitor specifically targeting to NCED (Han et al., 2004)] on seed germination in peanut were investigated physiologically, biochemically and morphogenetically, with a view to gain further insight into the possible feedback regulation mechanism of ABA biosynthesis.

MATERIALS AND METHODS

Seed germination assay

Seeds of peanut plants (*A. hypogaea* L. cv. Yueyou 7) were soaked overnight in solutions containing increasing concentrations of ABA (0, 0.01, 0.1 and 1 mmol/l) or sodium 6-methoxy-α-methyl-2-naphthaleneacetate (naproxen) (0, 0.2, 0.5 and 1 mmol/l), respectively. The fully imbibed seeds were transferred to petri dishes containing wringing ABA-free Whatman 3 mm filter paper and then incubated in a growth chamber at 26°C with a 16 h light/8 h dark photoperiod and an irradiance of 50 µmol m^{-2} s^{-1}. At day 2 and 6 after the onset of imbibition, seed germination was assayed by obtaining germination rate scored from those seeds whose radicles had emerged through the seed coat; and viability index calculated as the product of germination rate and mean radicle length. The germination assay was carried out in three independent experiments with three replicates in each replicate dish containing 20 seeds.

RNA gel blot analysis

Total RNA was extracted from the frozen samples using the modified phenol-chloroform method as described in Wan and Li (2005). RNA gel blot analysis was carried out according to the instructions of the Digoxigenin nucleic acid detection kit (Roche, USA) to determine the expression pattern of *AhNCED1* gene in germinating peanut seeds in response to ABA or naproxen treatment. Each total RNA (20 µg) was separated by 1.5% agarose gel electrophoresis and then blotted onto the Hybond N membrane (Amersham, USA). The PCR digoxigenin probe synthesis kit (Roche) was used to generate the *AhNCED1* gene specific probe with peanut cDNA (GenBank accession no. AJ574819) as a template according to the manufacturer's instructions. The gene-specific primers GSP1 (5'-GTT CAC GCC GTG AAA TTC CAC-3') and GSP2 (5'-GCG CTT CAA TCC ACC GGA TAC CA-3') were used to amplify the probe specific to *AhNCED1* gene. The PCR product was confirmed by sequencing. Hybridization and detection were performed according to standard procedures as specified by the manufacturer (Roche). For the analysis of the *AhNCED1* gene expression, three rounds of Northern blot analysis were conducted with three independently isolated total RNA samples.

Determination of ABA level and α-amylase activity

ABA was extracted as described by Xiong et al. (2001) from germinating peanut seeds at day 0, 2 and 6 after the onset of

imbibition. For ABA determination, extraction in 80% (v/v) aqueous methanol, prepurification through Sep-Pak C18 cartridges (Waters, USA), and high performance liquid chromatography (HPLC) fractionation in a Kromasil C18 column (150 × 4.6 mm, 5 µm, Chenhang company, Shenzhen, China) were conducted as reported previously (Chen and Wang, 1992; Wan and Li, 2006). The α-amylase (EC 3.2.1.1) activity in germinating peanut seeds was determined by the production of β-limit dextrin catalyzed by the enzyme as described by Svensson et al. (1987). The ABA level and the α-amylase activity were determined from three independent seed batchs with three replicates for each sample.

Light microscopy and scanning electron microscopy

For light microscopy, the embryos of germinating seeds were fixed with formalin-glacial acetic acid-alcohol (FAA, 2:1:25 by volume) reagent at 4°C for 24 h, dehydrated in a graded ethanol series (30 - 100%, v/v) , embedded in paraffin and then cut into thin sections using a microtome. The sections were stretched on glass slides and dried in an incubator at 30 - 4°C for 1 h. After removal of wax in toluene, the sections were stained with 1% safranin (w/v) and 0.5% fast blue (w/v), followed by dehydration with ethanol, rinsed with xylene and finally mounted in balata for general histological examination with a light microscope (Leica DMLB). For scanning electron microscopy, the embryos of germinating seeds were fixed with FAA reagent and dehydrated in a graded ethanol series. Specimens in 100% ethanol were critical point-dried with liquid CO_2 in a Balzer CPD-020 dryer (Balzers Union Ltd) according to the method described by Anderson (1951). The dried specimens were mounted on an aluminum planchette, and coated with approximately 10 nm of 60/40% Au/Pd using an Edwards S150B sputter coater (Edwards High Vacuum Ltd). Examination was performed using a FEI Philips XL 30 scanning electron microscope (FEI, Philips, Holland).

Statistical analysis

All data presented below are mean values of three independent, pooled experiments. Data were subjected to an analysis of variance using the conditional maximum likelihood estimation (CMLE) from winsteps scientific software implemented in statistical analysis system (SAS) software (SAS System for Windows, version 8.02). The statistical significance of the results was analyzed by the Student's *t*-test at the 5% probability level.

RESULTS

Effects of ABA and naproxen on germination rate and viability index of germinating peanut seeds

To investigate the physiological effects of ABA and naproxen on germination, increasing concentrations of ABA or naproxen were applied to peanut seeds during imbibition. As shown in Figure 1, at day 2 and 6 after the start of imbibitions, the germination rate significantly decreased with increasing concentrations of ABA and respectively dropped to one sixth and one ninth of the control in the 1 mmol/l ABA treatment (Figure 1A); simultaneously, the viability index also declined significantly with increasing ABA concentration and dropped, respectively, to one thirteenth and one twenty-seventh of the control in the 1 mmol/l ABA treatment (Figure 1B). The ABA-mediated inhibition of seed germination in peanut thus exhibited a dose-dependency (Figures 1A and B). Naproxen, the potent ABA biosynthesis inhibitor specifically targeting to *NCED* (Han et al., 2004) increased the germination rate and viability index (Figures 1C and D). At day 2 and 6 after the onset of imbibition, the germination rate of germinating seeds treated with 0.5 mmol/l naproxen were respectively, 1.13 and 1.50 times as high as that of the control germinating seeds (Figure 1C); accordingly, the viability index of seeds treated with 0.5 mmol/l naproxen was respectively, 1.35 and 1.92 times as much as that of control seeds (Figure 1D). Due to the severe effects of high concentration of ABA or naproxen on germination and seedling development of germinating peanut seeds, 0.1mmol/l ABA and 0.5mmol/l naproxen were used in all subsequent experiments.

Effects of ABA and naproxen on the levels of *AhNCED1* transcript and ABA in peanut seeds during germination

The *AhNCED1* gene expression in seeds was strongly up-regulated by imbibition of 0.1 mmol/l ABA and, conversely, down-regulated by imbibition of 0.5 mmol/l naproxen (Figure 2A); in parallel, the ABA level was markedly increased by the presence of ABA and decreased by the presence of naproxen (Figure 2B). As shown in Figure 2B, the endogenous ABA level in control seeds slightly decreased over germination time. At days 2 and 6, the ABA levels in germinating seeds increased by 109 and 234%, respectively, in ABA treatments, and decreased by 74.1 and 44.6% respectively in the naproxen treatments, compared with control seeds (Figure 2B). These results suggest a positive feedback regulation of ABA biosynthesis in peanut seeds during germination.

Effects of ABA and naproxen on α-amylase activity in germinating peanut seeds

To evaluate the effects of ABA and naproxen on α-amylase activity during germination, the enzymatic activity was estimated as the production of β-limit dextrin. As shown in Figure 2C, the α-amylase activity in control germinating seeds increased with germination time, rising by 11.1 and 26.6%, respectively, at day 2 and 6 after the start of imbibition. The α-amylase activity was slightly decreased by imbibition of 0.1mmol/l ABA and significantly increased by imbibition of 0.5mmol/l naproxen. At day 2 and 6 of germination, the α-amylase activity decreased by 22.7 and 53.9%, respectively, as a result of ABA treatment, and increased by 155 and 232%, respectively, in the naproxen treatment, compared with control seeds (Figure 2C).

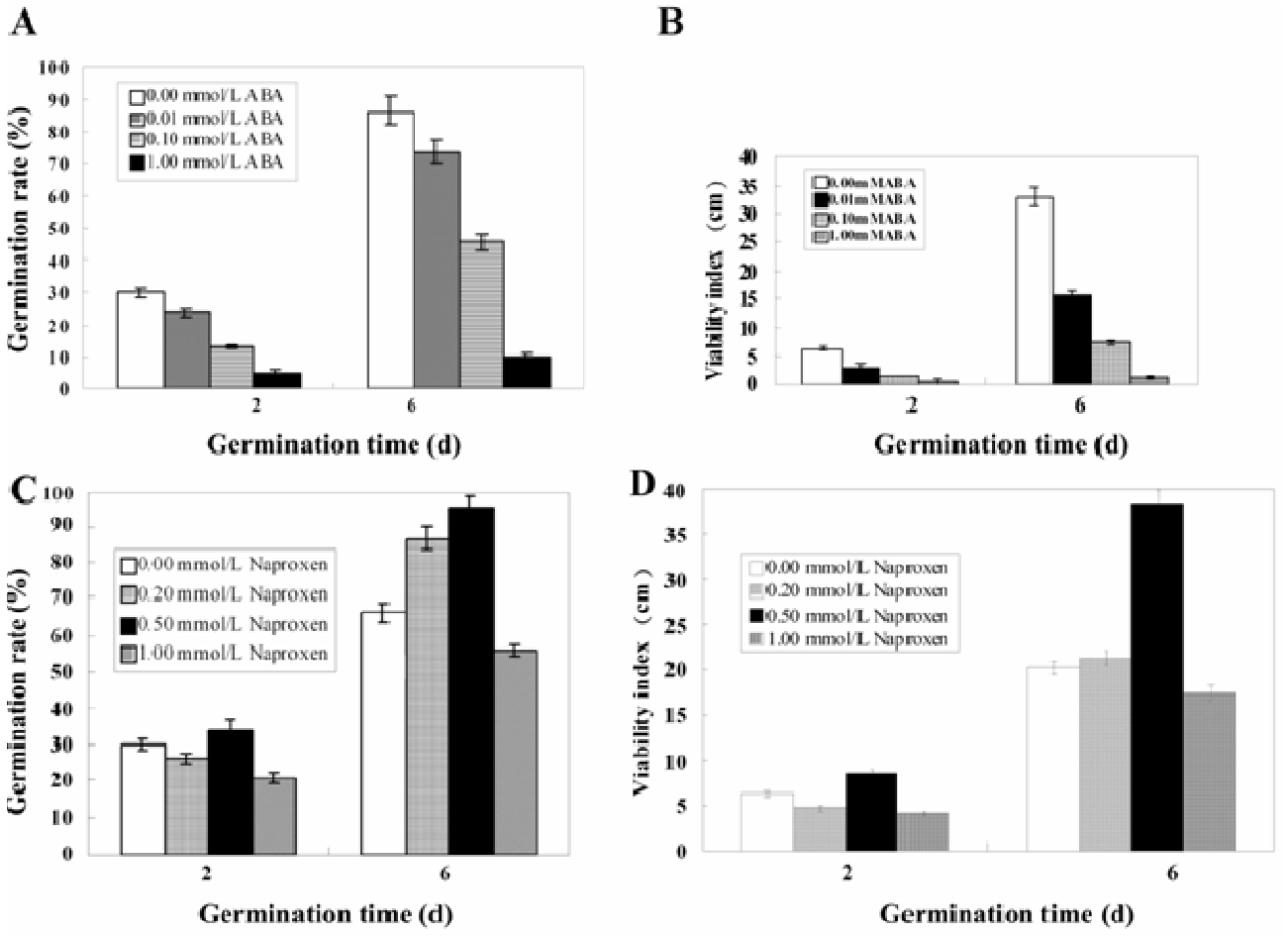

Figure 1. Effects of ABA and naproxen on germination rate and viability index of germinating peanut seeds. Imbibition of exogenous ABA by peanut seeds decreases the germination rate (A) and viability index (B) of germinating seeds in a dose-dependent manner; imbibition of 0.5 mmol/l naproxen by peanut seeds significantly increases the germination rate (C) and viability index (D) of germinating seeds. The germination assay was carried out at day 2 and 6 after the onset of imbibition using three independent seed batchs with three replicates in each. Error bars represent standard deviation (SD).

Morphogenetic effects of ABA and naproxen treatment on germinating seeds

To determine developmental effects of exogenous ABA and naproxen on peanut seeds, the embryos were examined by means of both light and scanning electron microscopy. In the vertical sections of control embryos, at day 0 of imbibition, the cells were full of cytoplasm and inclusions (Figure 3A); clear plumule apex growth could be observed at day 2 of germination (Figure 3B); at day 6 the plumule apex extruded notably, leaf primordium elongation increased markedly and cell inclusions decreased (Figure 3C). Observation of vertical sections of embryos of ABA or naproxen treated seeds revealed that both apical plumule growth and leaf primordium elongation were inhibited by the 0.1mmol/l ABA treatment (Figures 3D and E), whereas 0.5 mmol/l naproxen treatment promoted the plumule growth and leaf primordium elongation and also markedly decreased cell inclusions (Figures 3F and G). At day 6 in the 0.5 mmol/l naproxen treatment, plumule apex growth and leaf primordium elongation were slightly more than in control seeds, and were markedly more than those in seeds treated with ABA (Figures 3C, E and G). As shown by scanning electron microscopy (Figure 4), in the embryos of control seeds at day 6 of germination, axial buds grew rapidly, with a length of 3 - 4 mm (Figure 4A), and some lateral buds of 0.6 - 0.8 mm long (Figure 4B); compound leaves elongated from 2.5 - 3.3 mm in length (Figure 4C). In contrast, imbibition of 0.1 mmol/l ABA inhibited the apical meristem growth and postembryonic development significantly, resulting in much shorter axial buds of 1 -2 mm in length (Figure 4D) and lateral buds of 0.3 - 0.5 mm in length (Figure 4E), and also underdeveloped compound leaves of 0.4 - 0.8 mm long (data not shown). Imbibition of ABA also decreased the number of axial and lateral buds. In contrast, imbibition of 0.5 mmol/l naproxen led to increased number and length of axial and lateral buds,

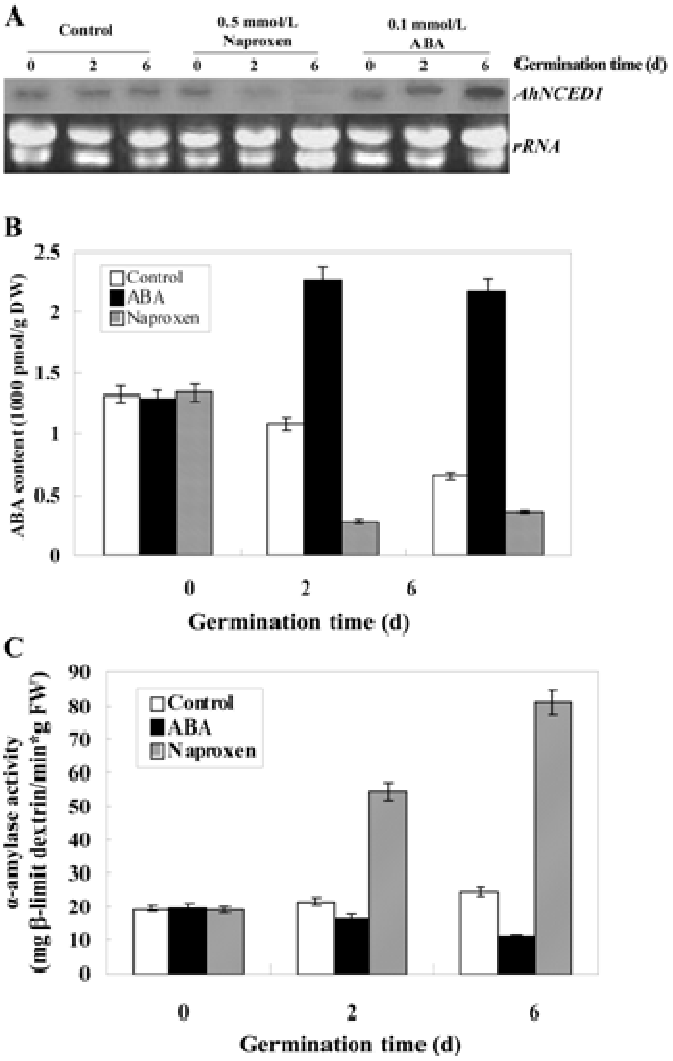

Figure 2. Effects of ABA and naproxen on the levels of *AhNCED1* gene transcript (A), ABA (B) and the α-amylase activity (C) in peanut seeds during germination. Total RNA and ABA were prepared separately from peanut seeds treated with 0.1 mmol/l ABA, 0.5 mmol/l naproxen or water as a control at day 0, 2 and 6 of germination. RNA gel blot analysis of the expression pattern of *AhNCED1* gene was performed as described in materials and methods. The ABA level and the α-amylase activity in peanut seeds treated with 0.1 mmol/l ABA, 0.5 mmol/l naproxen or water as a control were determined in triplicate at day 0, 2 and 6 after the start of imbibition. Error bars represent SD.

however there was no significant effect on the length of compound leaves (Figures 4F, G and H).

DISCUSSION

Seeds do not germinate in unfavourable conditions even after the breaking of dormancy. Phytohormones, ABA and GA, are well known to be involved in normal germination control (Tamura et al., 2006) - for example, de novo ABA biosynthesis is required for thermoinhibition of lettuce seeds germination (Yoshioka et al., 1998). In germinating peanut seeds there is a close relationship between the increase of vigor index and net loss of endogenous ABA content (Lin and Fu, 1996), as was also observed in this study (Figures 1 and 2). Exogenous ABA application results in a decrease of germination rate and α-amylase activity. An increase of ABA content and sodium tungstate treatment promotes seed germination, increases α-amylase activity, and decreases the endogenous ABA level in germinating peanut seeds (Guo et al., 2008). However, advanced molecular and morphogenetic investigation of ABA-mediated inhibition of seed germination in higher plants is still incomplete, especially concerning the possibility that inhibition involves a feedback regulation of ABA biosynthesis. This can partly be achieved by examining the effects of exogenous ABA and naproxen application on the levels of *NCED* gene transcript and endogenous ABA in peanut. In the present study, we demonstrated that such ABA treatment effected a significant increase in the levels of *AhNCED1* gene transcript (Figure 2A) and endogenous ABA (Figure 2B), and led to a marked decrease of α-amylase activity (Figure 2C), germination rate (Figure 1A) and viability index (Figure 1B) of germinating seeds. This was associated morphogenetically with inhibited plumule apex growth and leaf primordium elongation (Figures 3D and E), decreased number and length of axial and lateral buds and shorter length of compound leaves (Figure 4D and E) during seed germination.

Naproxen, the potent ABA biosynthesis inhibitor specifically targeting to *NCED*, is known to decrease endogenous ABA levels in higher plants (Han et al., 2004; Wan and Li, 2006). In the present study, we found that naproxen treatment decreased the levels of endogenous ABA (Figure 2B) and *AhNCED1* gene transcript (Figure 2A) and concomitantly increased α-amylase activity (Figure 2C), germination rate (Figure 1C) and viability index (Figure 1D) of germinating seeds. Morphogenetically, this is correlated with plumule apex growth increase and leaf primordium elongation (Figures 3F and G) as well as increased number and length of axial and lateral buds (Figures 4F and G), however without significant change in the length of compound leaves (Figure 4H). The ABA-mediated inhibition but naproxen-mediated promotion of seed germination suggests an involvement of ABA biosynthesis in the germination programme of peanut seeds.

Recent genetic and genomic analyses have revealed the molecular basis of the pathway and the genes/enzymes involved in ABA biosynthesis and catabolism. ABA is mainly inactivated through hydroxylation and conjugation (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). Among these pathways, the ABA 8'-hydroxylation pathway is the regulatory step in a variety of physiological processes. In *Arabidopsis*, *CYP707A1* to *CYP707A4* encode ABA 8'-hydroxylase (Kushiro et al., 2004; Saito et al., 2004). Gene expression and reverse-genetic analyses indicates that *CYP707A2* has a major

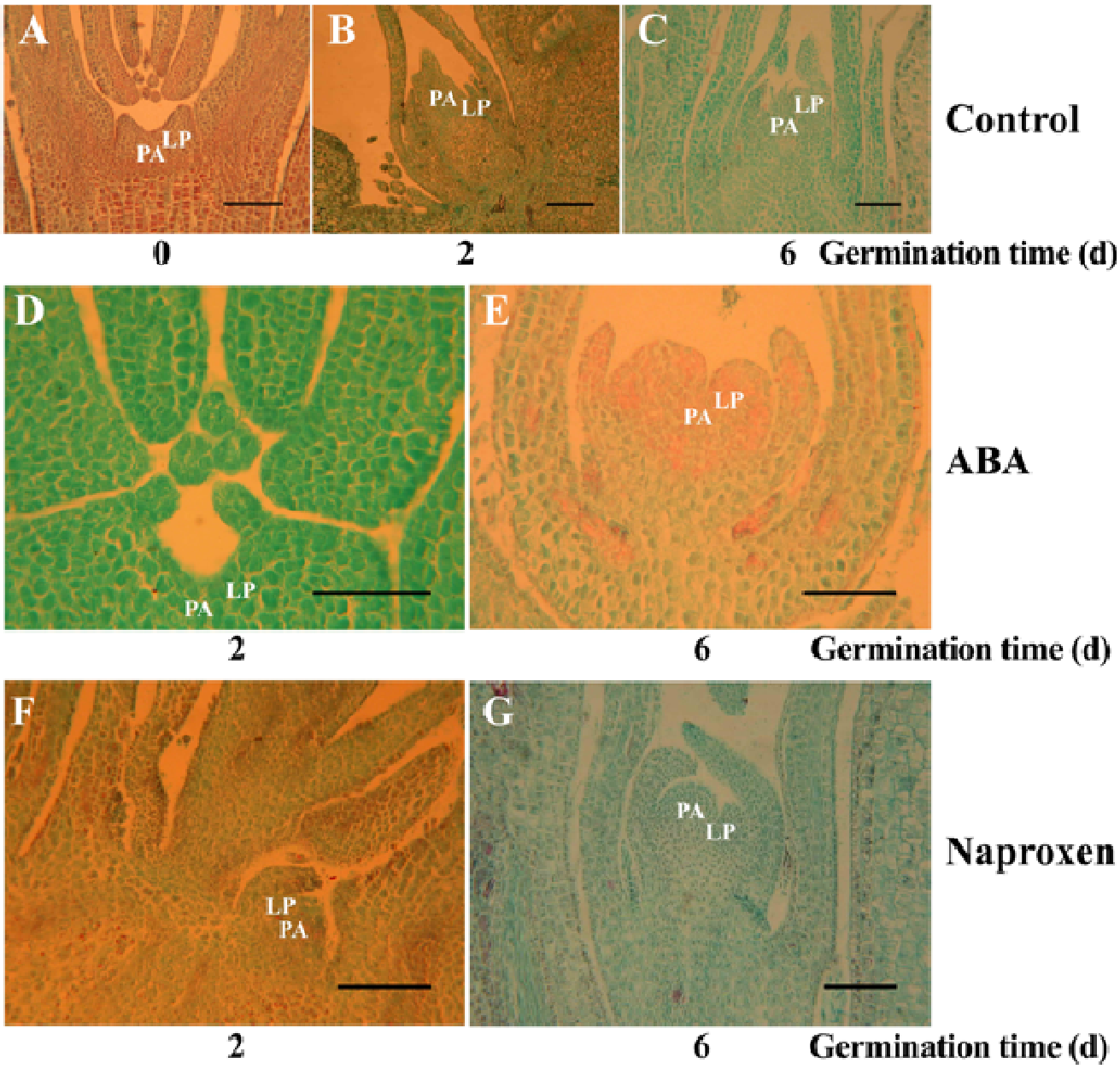

Figure 3. Histomorphology of the embryos of peanut seeds treated with water as control (A, B, C), 0.1 mmol/l ABA (D, E) or 0.5 mmol/l naproxen (F, G) at day 2 and 6 after the onset of imbibition during germination. The plumule apex growth and leaf primordium elongation were inhibited by imbibition of ABA (D, E), and promoted by imbibition of naproxen (F, G). PA = plumule apex; LP = leaf primordium. Scale bars = 200 μm. The reproducibility of this experimental result was confirmed by three independent experiments using independent samples prepared at different times.

role in the rapid decrease in ABA content in the first 6 to 12h of imbibition and implies that *CYP707A1* to *CYP707A3* are involved in seed germination (Kushiro et al., 2004; Okamoto et al., 2006). In our study, the endogenous ABA level in control peanut seeds decreased during germination as shown in Figure 2B and this decrease could have promoted germination and seedling development.

ABA has long been thought to negatively regulate ABA accumulation by activating its degradation (Cutler and Krochko, 1999; Qin and Zeevaart, 2002). Current indications are that the possible regulation of *NCED* gene expression by ABA has an important bearing on how ABA auto-regulates its own biosynthesis. Xiong et al. (2002) and Cheng et al. (2002) reported that *AtNCED3* gene could be induced by ABA in *Arabidopsis* ecotype Landsberg background. Additionally, *AtNCED3* gene transcripts under drought and salt stress treatments were significantly reduced in the ABA-deficient mutants *los5* and *los6* when compared with those in wild-type seedlings, demonstrating that ABA is required for full

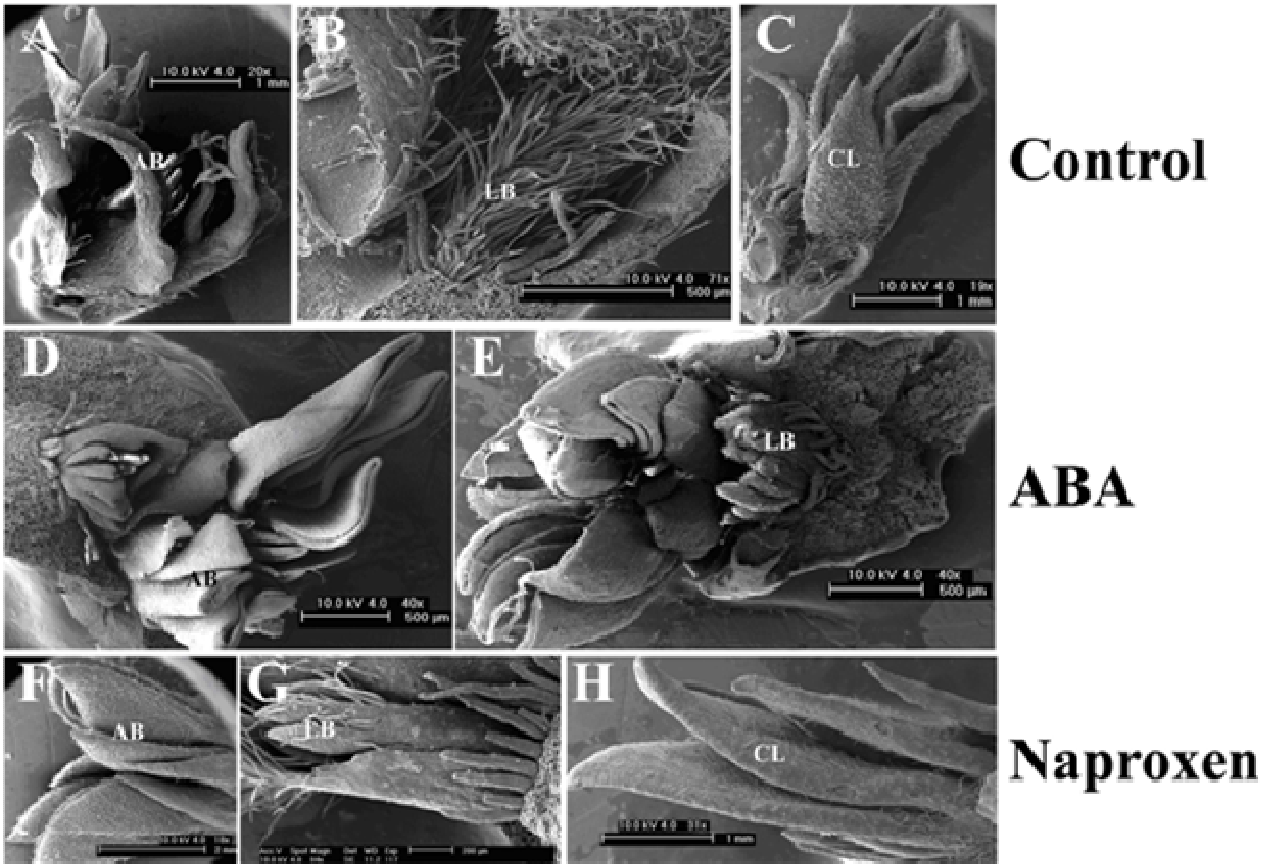

Figure 4. Scanning electron microscopy of the embryos of peanut seeds treated with water as a control (A, B, C), 0.1 mmol/l ABA (D, E) or 0.5 mmol/l naproxen (F, G, H) at day 6 after the start of imbibition during germination. Imbibition of ABA decreases the number and length of axial and lateral buds (D, E) and the length of compound leaves; imbibition of naproxen results in increased number and length of axial and lateral buds (F, G), but no significant change in the length of compound leaves (H). AB = axial bud; LB = lateral bud; CL = compound leaf. This experiment was repeated twice using independent seed batchs.

activation of *AtNCED3* by water stress (Xiong et al., 2002). Barrero et al. (2006) provided further evidence that strong induction of *AtNCED3* gene by ABA occurred through both ABA-dependent and ABA-independent pathways in *Arabidopsis*. We recently showed that the expression of *AhNCED1* gene in peanut plants was up-regulated by exogenous ABA treatment (Wan and Li, 2006; Guo et al., 2009). In the present study, imbibition of exogenous ABA by peanut seeds led to a significant increase in the levels of *AhNCED1* gene transcript (Figure 2A) and ABA (Figure 2B), and conversely imbibition of naproxen significantly decreased the levels of endogenous ABA (Figure 2B) and *AhNCED1* gene transcript (Figure 2A). Taken together, these observations in peanut imply a possible positive feedback regulation of ABA biosynthesis in higher plants.

α-Amylase is one of the important hydrolases which play a key role in seed germination and seedling development (Svensson et al., 1987). Embryonic α-amylase activity rises gradually during germination and this enzyme is regulated by plant hormones, such as ABA, as well as environmental factors (Sidenius et al., 1995). Curtis et al. (2004) showed that water stress reduced α-amylase activity and inhibited seed germination and seedling morphogenesis. Our study showed that α-amylase activity in control peanut seeds increased with germination time (Figure 2C), but strongly decreased in seeds treated with 0.1 mmol/l ABA; conversely, α-amylase increased in seeds treated with 0.5 mmol/l naproxen (Figure 2C). This increasing α-amylase activity would be nutritiously favourable to germination and seedling development.

In conclusion, we demonstrated that imbibition of exogenous ABA by peanut seeds resulted in a significant increase in the levels of both *AhNCED1* gene transcript and endogenous ABA, and this led to a marked decrease of α-amylase activity, germination rate and viability index of germinating seeds, morphogenetically resulting in depression of seed germination. Imbibition of naproxen by peanut seeds, on the other hand, significantly decreased the levels of endogenous ABA and *AhNCED1* gene transcript, and markedly increased α-amylase

activity, germination rate and viability index of germinating seeds, morphogenetically improving seed germination. These observations suggest the involvement of a positive feedback regulation of ABA biosynthesis in ABA-mediated inhibition of seed germination in peanut.

ACKNOWLEDGEMENTS

This work was jointly supported by the grants from National Natural Science Foundation of China (approved Nos. 30800077 granted to X. Wan and 30771297 granted to L. Li), Guangdong Natural Science Foundation (grant Nos. 06025049 and 06301202) and Higher Education Natural Science Research Project of Guangdong Province, China (grant No. 06Z009).

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