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RESEARCH PAPER

H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in *Arabidopsis* seed dormancy and germination

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Abstract

H_2O_2 is known as a signal molecule in plant cells, but its role in the regulation of aqbscisic acid (ABA) and gibberellic acid (GA) metabolism and hormonal balance is not yet clear. In this study it was found that H_2O_2 affected the regulation of ABA catabolism and GA biosynthesis during seed imbibition and thus exerted control over seed dormancy and germination. As seen by quantitative RT-PCR (QRT-PCR), H_2O_2 up-regulated ABA catabolism genes (e.g. *CYP707A* genes), resulting in a decreased ABA content during imbibition. This action required the participation of nitric oxide (NO), another signal molecule. At the same time, H_2O_2 also up-regulated GA biosynthesis, as shown by QRT-PCR. When an ABA catabolism mutant, *cyp707a2*, and an overexpressing plant, *CYP707A2-OE*, were tested, ABA content was negatively correlated with GA biosynthesis. Exogenously applied GA was able to over-ride the inhibition of germination at low concentrations of ABA, but had no obvious effect when ABA concentrations were high. It is concluded that H_2O_2 mediates the up-regulation of ABA catabolism, probably through an NO signal, and also promotes GA biosynthesis. High concentrations of ABA inhibit GA biosynthesis but a balance of these two hormones can jointly control the dormancy and germination of *Arabidopsis* seeds.

Key words: ABA, ABA catabolism, *Arabidopsis*, GA, GA biosynthesis, hydrogen peroxide (H_2O_2), nitric oxide (NO), seed dormancy.

Introduction

Seed germination is a complex process. Germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis (Bewley and Black, 1994; Holdsworth *et al.*, 2008). Seeds of most angiosperms are dormant at maturity, and the dormancy must be lost before germination can occur (Bewley, 1997). Seed dormancy has been defined by Finch-Savage and Leubner-Metzger as the incapacity of a viable seed to germinate under favourable conditions (Finch-Savage and Leubner-Metzger, 2006). Many factors are involved in seed dormancy regulation, including some plant hormones, such as abscisic acid (ABA), gibberellic acid (GA), and ethylene (Bewley, 1997; Zhou *et al.*, 1998; Ghassemian *et al.*, 2000; Nakajima *et al.*, 2006; Carrera *et al.*, 2008; Holdsworth *et al.*, 2008), some environmental factors, such as light intensity and low temperatures (Holdsworth *et al.*, 2008), and several signalling molecules, such as nitric oxide (NO) and some reactive oxygen species (ROS) (Batak *et al.*, 2002; Bethke *et al.*, 2004, 2006; Sarath *et al.*, 2007). However, the mechanisms of dormancy holding and breaking remain unclear because it is unknown how these factors are inter-related. The mechanisms of ABA catabolism and GA biosynthesis regulation are of particular interest.

H_2O_2 acts as a signalling molecule, participating in a series of processes including plant development, stress responses, and programmed cell death (Pei *et al.*, 2000; Bethke and Jones, 2001; Apel and Hirt, 2004; Foyer and Noctor, 2005). In plants, H_2O_2 is generated in chloroplasts, mitochondria, and peroxisomes (Mittler *et al.*, 2004). Plasma membrane NAD(P)H oxidase is reported to be the pivotal enzyme involved in H_2O_2 generation (Kauss and Jeblick, 1995,

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1996; Mur *et al.*, 1996; Shirasu *et al.*, 1997). The effect of H_2O_2 on plant development, stress responses, and programmed cell death has been thoroughly investigated (Pei *et al.*, 2000; Bethke and Jones, 2001; Apel and Hirt, 2004; Foyer and Noctor, 2005). The effect of H_2O_2 on seed germination has also been researched by some investigators. Fontaine *et al.* (1994) indicated that thioredoxin reduction by NADPH produced via the oxidative pentose phosphate pathway allows the mobilization of storage proteins of cereals, leading to germination. H_2O_2 is also regarded as having a function as a promoter of seed germination by oxidizing germination inhibitors in *Zinnia elegans* seeds (Ogawa and Iwabuchi, 2001). The sources of H_2O_2 during seed germination are not clear.

Bailly *et al.* (2008) indicated that in the dry state enzymes are probably not active and in this case ROS probably originate from non-enzymatic reactions such as lipid peroxidation or Amadori and Maillard reactions, and in hydrated seeds can be produced during the catabolism of lipids (glyoxysomes) and purines (peroxisomes), respiratory activity (mitochondria), electron transfer in photosystems (chloroplasts), or through the activity of NADPH oxidase (plasma membrane), amine oxidase, and peroxidase (cell wall) or cytochrome P450 (cytosol). They also indicated that accumulated H_2O_2 during imbibition is essential for seed dormancy breaking

ABA plays an important role in a number of physiological processes such as seed maturation, growth, and developmental regulation, seed dormancy, and adaptive responses to environmental stresses (Zeevaart and Creelman, 1988; Hoffmann-Benning and Kende, 1992; Kuwabara *et al.*, 2003; Nambara and Marion-Poll, 2005). In addition, ABA has been shown to be an important positive regulator in both the induction of dormancy during seed maturation and the maintenance of the dormant state in imbibed seeds (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003). ABA-deficient mutants in *Arabidopsis*, such as *aba1*, *aba2*, and *aao3*, show the absence of primary dormancy in mature seeds (Leon-Kloosterziel *et al.*, 1996; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003). Some ABA-insensitive mutants such as *abi1*, *abi2*, and *abi3* also lack or have decreased primary dormancy in mature seeds (Raz *et al.*, 2001; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Kushiro *et al.*, 2004; Nambara and Marion-Poll, 2005), whereas overexpression of some ABA biosynthesis genes increases seed ABA content and enhances seed dormancy or delays germination (Finkelstein *et al.*, 2002; Kushiro *et al.*, 2004; Nambara and Marion-Poll, 2005; Holdsworth *et al.*, 2008).

Some investigations have shown that ABA catabolism also plays a major role in seed dormancy maintenance and dormancy break. Seeds of the mutant *cyp707a2*, lacking the key enzyme in ABA catabolism, (+)-abscisic acid 8'-hydroxylase, accumulate much more ABA and show stronger dormancy during imbibition than the wild type (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Okamoto *et al.*, 2006). Earlier results (Liu *et al.*, 2009) also indicated that *CYP707A2* plays a major role in ABA catabolism during imbibition and regulates seed dormancy.

GA is a major plant hormone in a number of physiological processes, such as seed germination, stem elongation, leaf expansion, flowering, and seed development (Davies, 1993; Ogawa *et al.*, 2003; Yamauchi *et al.*, 2004). Together with ABA, GA is also involved in seed dormancy and germination control (Ogawa *et al.*, 2003) and is found to promote seed germination in many species (Koornneef and van der Veen, 1980; White *et al.*, 2000; Yamauchi *et al.*, 2004). Inhibitors of GA biosynthesis, such as paclobutrazol (PAC) and uniconazole, reduce seed germination in *Arabidopsis* (Jacobsen and Olszewski, 1993; Leon-Kloosterziel *et al.*, 1996; Toh *et al.*, 2008). Several GA-deficient mutants, such as *ga1-3* and *ga2-1*, have also delayed seed germination (Koornneef and van der Veen, 1980). It is proposed that GA plays two major roles in stimulating germination in *Arabidopsis*. The first role is in inducing radicle protrusion apparently by weakening the tissue that surrounds the embryo. The second role is in increasing the growth potential of the embryo, as indicated by the reduced growth rate of GA-deficient embryos (Groot and Karssen, 1987; Ogawa *et al.*, 2003).

The roles of ABA and GA in seed germination control have been indicated to be antagonistic by some investigators (Razem *et al.*, 2006; Weiss and Ori, 2007; Toh *et al.*, 2008). For example, GA induces transcription of α-amylase in the aleurone layer of cereal seeds that is significantly suppressed by ABA (Rogers and Rogers, 1992; Gómez-Cadenas *et al.*, 2001; Zentella *et al.*, 2002). ABA is also reported to inhibit seed germination by inhibiting GA biosynthesis directly under high temperature (Toh *et al.*, 2008). However, because of the complexity in their signalling pathways, the relationship of ABA and GA is not well understood in terms of their regulation. In this study, it was found that both GA and ABA are under the regulation of H_2O_2 in seed dormancy. Exogenous H_2O_2 increases ABA catabolism by enhancing the expression of *CYP707A* genes. At the same time, H_2O_2 enhances GA biosynthesis via enhancement of GA biosynthesis genes such as *GA3ox* and *GAw20ox* genes. The inhibition of seed germination by a low concentration of ABA is reversed by GA, but apparently GA cannot override the effect of high concentration of ABA. The present results also suggest that the H_2O_2-enhanced ABA catabolism requires the participation of NO, another small signalling molecule.

Materials and methods

Plant materials

The plants were grown in a growth chamber with a 16 h photoperiod at a photon flux density of ~200 μmol m^{-2} s^{-1} at a daytime temperature of 23 °C and a night-time temperature of 20 °C. In order to minimize the effect of seed maturation and storage conditions, plants of each genotype tested were grown in different sections of the same pot and seeds were harvested at the same time. Seeds were harvested in bulk 30 d after the petals appeared on the first flowers. These seeds maintained stronger

dormancy. Only freshly harvested seeds were used in the experiments. The rest of the seeds were stored at –80 °C, at which temperature dormancy can be maintained for more than a year (Millar *et al.*, 2006; Fujii *et al.*, 2007).

T-DNA insertion line

The seeds of *Arabidopsis thaliana cyp707a2* (SALK_083966) generated by the Salk Institute Genomic Analysis Laboratory (http://signal.sal.edu/) were obtained from the ABRC. The seeds were planted on agar plates containing kanamycin, and the kanamycin-resistant plants were transferred to soil. Seeds were harvested separately from individual plants. Subsequently, to confirm the mutant line as homozygous, PCR was performed with the genomic DNA of *cyp707a2* using gene-specific oligonucleotides (LP, AATCCCAAATATGCCTTAGGC; and RP, TATGTGGGGACTTTGATGGAC).

Chemical treatments

Sodium nitroprusside (SNP) was used as the NO donor to release NO steadily, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was used as the NO scavenger (Bright *et al.*, 2006). Diphenyliodonium (DPI) was used to inhibit NADPH oxidase to decrease production of H_2O_2 (Orozco-Cardenas *et al.*, 2001). Diniconazole and PAC were used to inhibit ABA catabolism and GA biosynthesis, respectively (Han *et al.*, 2004; Kitahata *et al.*, 2005; Toh *et al.*, 2008).

Germination assay

Fifty seeds were placed in 55 mm diameter Petri dishes with three Whatman No. 1 filter papers and 2.2 ml of sterile double-distilled water or treatment solutions. Plates were then placed in a 21 °C growth chamber under continuous light at 100 μM m^{-2} s^{-1} for 7 d. The seeds were regarded as germinated when the radicle emerged. Experiments were performed in quadruplicate for each treatment.

Determination of NO

NO was detected by the Nitric Oxide (total) Detection Kit (Assay Designs, USA). About 0.2 g of seeds were put into 1.5 ml tubes, then 200 μl of reaction buffer and 100 μl final dilution of NADH were added; 100 μl of water was added to a parallel tube as a control. Then 100 μl of nitrate reductase (NR) was added to the samples and 100 μl of reaction buffer was added to the control tubes. A 400 μl aliquot of reaction buffer without seeds, NADPH, and NR acted as a blank. The blank, control, and sample tubes were mixed well and incubated at 37 °C for 30 min. After incubation they were centrifuged at 3000 *g* for 1 min and 300 μl of supernatant was transformed to a new tube. A 100 μl aliquot of Griess reagent I was added to the control, sample, and blank, and after being well mixed, 100 μl of Griess reagent II was added. The tubes were mixed by shaking and then they were incubated at 25 °C for 10 min. The optical density (OD) of samples and controls was measured at 540 nm. The OD of each sample was labelled as ODs and that of each control as ODc. Then the average net OD was calculate and labelled as ODn. Each average ODn=average ODs–average ODc. Each ODn could be calculated from a standard curve. Sodium nitrate at 0–100 μM was used as a standard, and a standard curve was produced. The amount of NO released was equal to the amount of nitrate. The mechanism of this kit is transformation of NO to nitrite and its measurement.

Extraction and determination of ABA

For estimation of endogenous ABA levels of imbibed seeds, 0.2 g of seeds was homogenized in 1 ml of distilled water and then shaken at 4 °C overnight. The homogenates were centrifuged at 12 000 g for 10 min at 4 °C and the supernatant were directly used for ABA assay. ABA analysis was carried out using the radioimmumoassay (RIA) method as described by Quarrie *et al.* (1988). The 450 μl reaction mixture contained 200 μl of phosphate buffer (pH 6.0), 100 μl of diluted antibody (Mac 252) solution, 100 μl of $[^3H]$ABA (~8000 cpm) solution, and 50 μl of crude extract. The mixture was then incubated at 4 °C for 45 min and the bound radioactivity was measured in pellets precipitated with 50% saturated $(NH_4)_2SO_4^-$ with a liquid scintillation counter.

Determination of H_2O_2

An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to measure H_2O_2 production in 2-week-old plants. Leaves were frozen in N_2 and then ground. Then 500 μl of phosphate buffer (20 mM K_2HPO_4, pH 6.5) was added to 50 mg of ground frozen tissue. After centrifugation, 50 μl of the supernatant was incubated with 0.2 U ml^{-1} horseradish peroxidase and 100 μM Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine) at room temperature for 30 min in darkness. The fluorescence was quantified using FLUOStar Optima (excitation at 560 nm and emission at 590 nm) (Xing *et al.*, 2008).

QRT-PCR analysis

Total RNA was isolated from seeds or leaves by an RNeasy kit (Invitrogen). DNA impurities in the isolated RNA were digested before synthesizing the cDNA by adding DNase (Invitrogen) and incubation for 30 min at 37 °C. DNase was then inactivated by incubation for 10 min at 65 °C. Then 2 μg of RNA was reverse transcribed to cDNA with SuperScriptIII RTS First-Strand cDNA Synthesis Kit (Invitrogen). After that, the cDNA was diluted 10 times, and 4 μl of cDNA was used to carry out the quantitative RT-PCR (QRT-PCR). IQ™ SYBR Green Supermix (Bio-Rad) was used for the QRT-PCR. Actin2 acted as the intramural standard. The QRT-PCR was executed with iCycle (Bio-Rad). The primers that were used in QRT-PCR are as follows: *CYP707A1* (F, TTGGAAAGAGGAGACTAGAG; R, GTGAACCACAAAAGAGGAAC), *CYP707A2* (F, AAATGGAGTGCACTCATGTC; R, CCTTCTTCATCTCCAATCAC), *CYP707A3* (F, ATTCTTGTCCAGGCAATGAG; R, ATAGGCAATCCATTCTGAGG), *CYP707A4* (F, GAAAGGAATACAGTACAGTC; R, GGATTAGATTTGGCTAACTAC), *GA20ox1* (F, GCCTGTAAGAAGCACGGTTTCT; R, CTCGTGTATTCATGAGCGTCTGA), *GA20ox2* (F, CCCAAGGCTTTCGTTGTCAA; R, CCGCTCTATGCAAACAGCTCT), *GA20ox3* (F, TCGTGGACAACAAATGGCA; R, TGAAGGTGTCGCCTATGTTCAC), *GA3ox1* (F, TCCGAAGGTTTCACCATCACT; R, TCGCAGTAGTTGAGGTGATGTTG), *GA3ox2* (F, GTTTCACCGTTATTGGCTCTCC; R, TCACAGTATTTGAGGTGGTGGC), *RD29A* (F, TGCACCAGGCGTAACAGGTA; R, TAATCGGAAGACACGACAGGA), *RD29B* (F, GAGCATCCAAAGTGTTGAAGAAAGT; R, GGTCTTGCTCGTCATACTCATCAT), *XTH5* (F, CACGTCGATGGATGTGAAGCT; R, CTTTCTGATCCCACCAACGTTT), *EXP2* (F, CCTCCAAACTTTGCCTTAGCT; R, CGGCCAAGTCAAAGTGCTTAA), *NCED6* (F, TGAGAGACGAAGAGAAAGAC; R, GTTCCTTCAACTGATTCTCG), *NCED9* (F, GGAAAACGCCATGATCTCACA; R, AGGATCCGCCGTTTTAGGAT), *GA2ox2* (F, CCCTCAAATTTTCCGTGAGT; R, CAGCATTTTACTCAGAGTGTC), *CAT1* (F, ACACATACGTGTTTTGGTGTTGAGC; R, CACCCGAGTTTGTAGTGAAGAAAGG), *CAT2* (F, CTCCAAGCTCTCTTCTCATCAAACCAT; R, GGAGCTCGGAGAAAGTCAGCACAA), *CAT3* (F, GAGGGATATTCGTGGTTTTGCTGTC; R, TTTGTTTTCGGGTTAGGTTTCAACG), *AT1G19230* (F, TCACTTTTACTGGGTCACAAGGGAG; R, AACTCCATGTTTGGCATGGTTCA), *AT4G11230* (F, GGTACCGCAAAACGGTATGGATGT; R, AATCATCTCCAGGGGAAGAAGTAATAGA), *AT4G25090* (F, TTGGCAAAGAGTTTGGGTGATAGC; R,

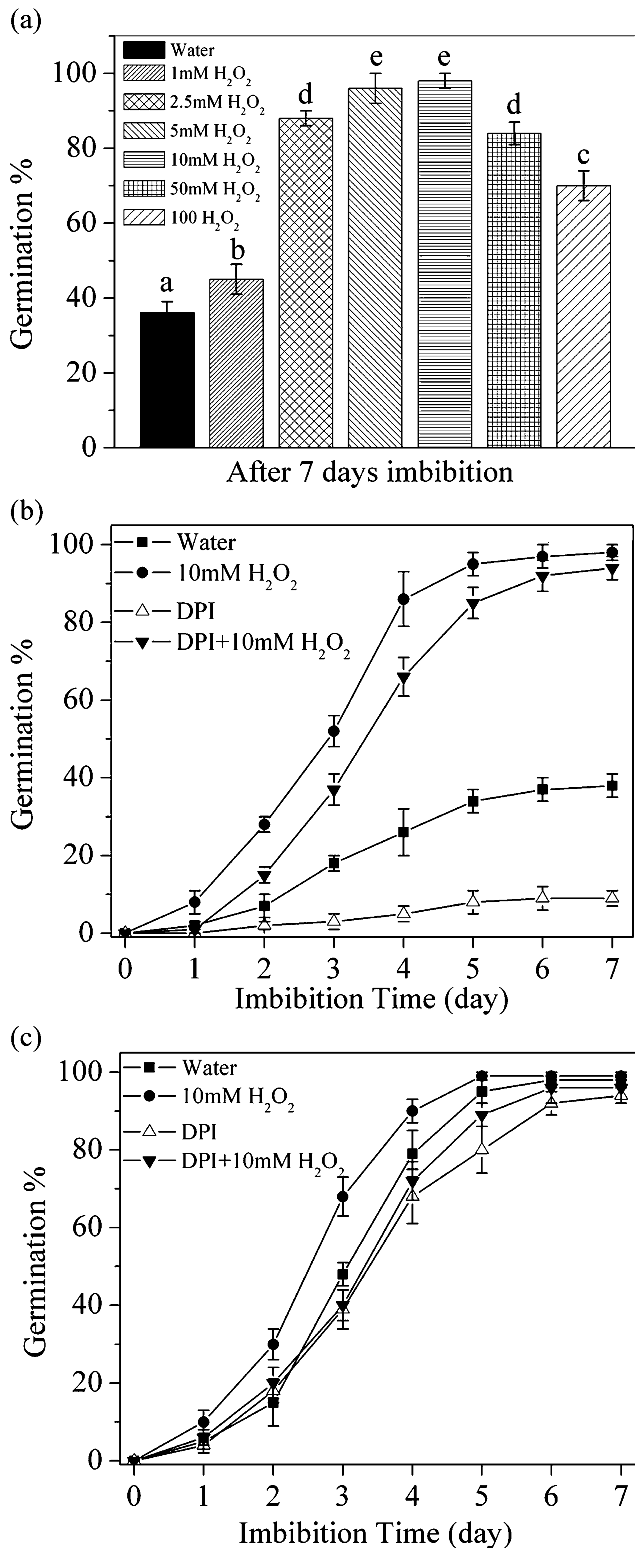

Fig. 1. The effect of H_2O_2 on seed germination and dormancy break in *Arabidopsis*. (a). Effect of different H_2O_2 concentrations on dormancy break of freshly harvested wild-type seed. Freshly harvested seeds were imbibed in water or different concentrations of H_2O_2 and the germination ratio was counted on the seventh day. (b) Effect of H_2O_2 and its production inhibitor DPI on freshly harvested seed dormancy and germination. (c) Effect of H_2O_2 and its production inhibitor DPI on non-dormant seed germination. Data represent the means±SE of four replicates, with 50 seeds each in a, b, and c. An ANOVA test followed by a rank test was performed. Different letters in (a) are used to indicate means that are significantly different (P <0.05).

GGTAACAGAATTAGGGCCATGTTTAGC), *Actin2* (F, TGTGCCAATCTACGAGGGT; R, GCTGGTCTTTGAGGTTTCC).

Generation of the CPY707A2-overexpressing line

Full-length *Arabidopsis CPY707A2* cDNA was obtained by using reverse transcription-PCR and cloned into the pENTR-TOPO cloning vector (Invitrogen) and sequenced. After the LR reaction, *CPY707A2* cDNA was inserted into the pGWB5 vector (a gift from Professor Liang, Yangzhou University) which had a 35S promoter; this vector was named pGWB5-*CPY707A2*. Transgenic *Arabidopsis* containing the cauliflower mosaic virus (CaMV) 35S promoter was generated using the floral dipping method (Clough and Bent, 1998) and transferred into Col-0 wild-type plants. Transformed plants were selected by growth on hygromycin-containing medium. Plants of the second generation after transformation were used for the experiments. The empty pGWB5 vector (the ccdb gene was substituted by a nonsense segment with a termination codon) which acted as control was also transferred into Col-0 wild-type plants.

Accession numbers

Sequence data from the article can be found in the GenBank data libraries or TIGR database (Arabidopsis thaliana Genome Project) under the following accession numbers: *CYP707A1*, At4g19230; *CYP707A2*, At2g29090; *CYP707A3*, At5g45340; *CYP707A4*, At3g19270; *GA20ox1*, At4g25420; *GA20ox2*, At5g51810; *GA20ox3*, At5g07200; *GA3ox1*, At1g15550; *GA3ox2*, At1g80340; *XTH5*, At5g13870; *EXP2*, At5g05290; *RD29A*, AT5g52310; *RD29B*, AT5g52300; *GA2ox2*, At1g30040; *NCED6*, At3g24220; *NCED9*, At1g78390; *CAT1*, At1g17680; *CAT2*, At4g35090; *CAT3*, At1g20620; and *Actin2*, At3g18780.

Results

H_2O_2 reduces dormancy of freshly harvested Arabidopsis seeds

After 7 d of imbibition in 5 mM H_2O_2, the dormancy of freshly harvested *Arabidopsis* seeds was broken. Much higher H_2O_2 concentrations, such as 100 mM, had less effect (Fig. 1a). The H_2O_2 effect was further confirmed by supplying DPI, a H_2O_2 scavenger, to reduce the level of H_2O_2 (Levine *et al.*, 1994; Alvarez *et al.*, 1998; Lee *et al.*, 1999). As shown in Fig. 1b, DPI enhanced seed dormancy significantly while exogenous H_2O_2 completely reversed the effect of DPI. When supplied to non-dormant seeds, H_2O_2 increased seed germination and DPI slightly slowed down the process (Fig. 1c).

H_2O_2 regulates genes involved in ABA catabolism and GA synthesis during imbibition

ABA and GA are known to regulate seed germination and dormancy but their signalling pathways have not yet been established. In the present experiments, H_2O_2 acted as a regulator of genes involved in both ABA and GA

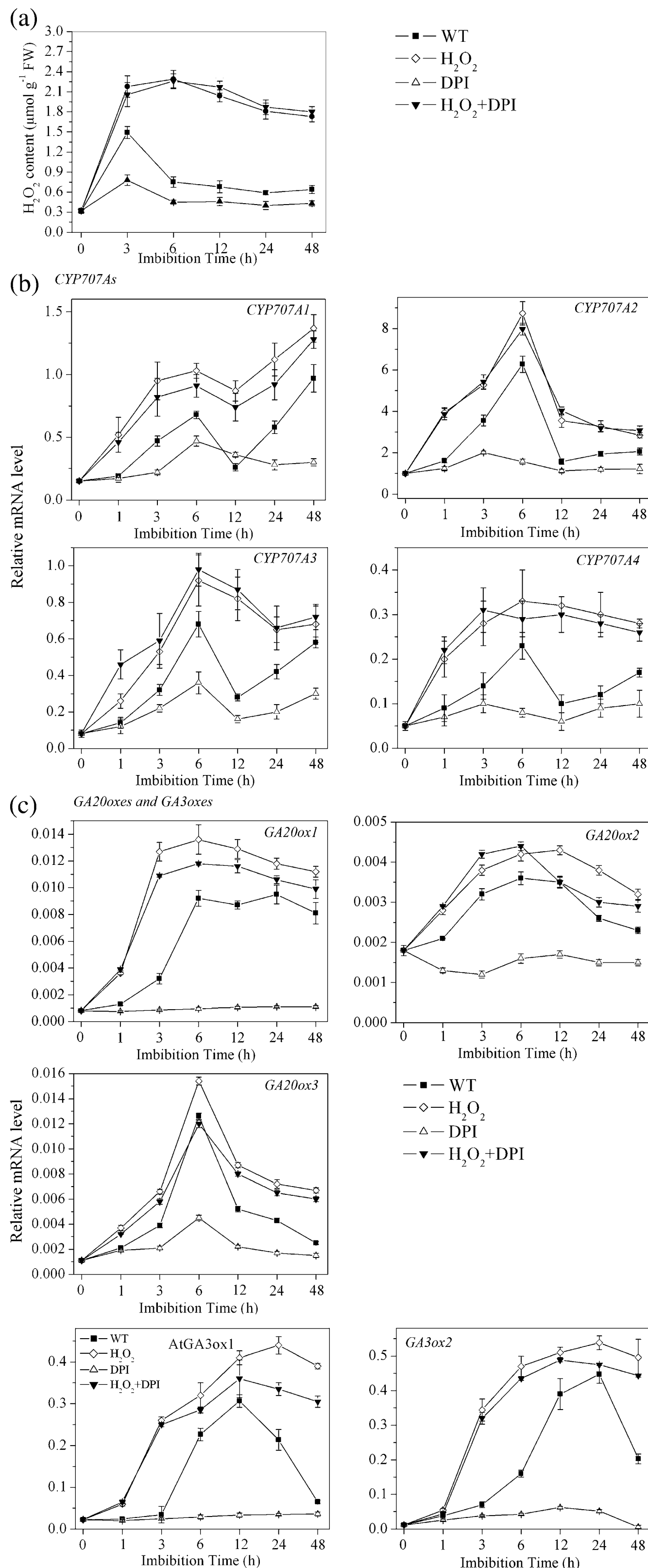

Fig. 2. Effect of H_2O_2 on the expressions of ABA catabolism and GA biosynthesis genes during imbibition. (a) Change of H_2O_2 content during imbibition under different treatments in the first 48 h of imbibition. (b) Change in the transcript levels of ABA catabolism genes in the first 48 h of imbibition. All four *CYP707A* genes were determined by QRT-PCR. (c) Change in the transcript levels of GA biosynthesis genes in the first 48 h of imbibition. All three *GA20ox* genes and two *GA3ox* genes were determined by QRT-PCR. H_2O_2 (10 mM) and DPI (10 μM) were used for these experiments. Values are the means±SE (n=4 for a and n=3 for b and c). An ANOVA test followed by a rank test was performed. Different letters are used to indicate means that are significantly different (P <0.05).

metabolism. As shown in Fig. 2a, the release of H_2O_2 from the imbibed seeds rapidly increased in the first few hours and reached a peak at 3 h, followed by a decrease after 6 h. DPI treatment decreased this H_2O_2 release significantly. When expression of ABA 8'-hydroxylase (*CYP707A1*, *CYP707A2*, *CYP707A3*, and *CYP707A4*), 9-*cis* epoxcartenoid dioxygenase (*NECD6* and *NCED9*), GA 20-oxidase (*GA20ox1*, *GA20ox2*, and *GA20ox3*), GA 3-oxidase (*GA3ox1* and *GA3ox2*), and GA 2-oxidase (*GA2ox2*) was investigated following different treatments during imbibition, the expression of four ABA catabolism genes (*CYP707A* genes) were induced within a few hours after imbibition of water, and then decreased after 6 h. When imbibed with H_2O_2, the transcription level of these four genes increased much more rapidly and was maintained at a high level during the entire imbibition period (Fig. 2a). When release of H_2O_2 was inhibited by DPI, there was no increase in transcription levels of these four genes compared with water. Further addition of exogenous H_2O_2 was able to reverse the inhibitory effect of DPI on transcription of the four genes (Fig. 2b). Transcription levels of the ABA biosynthetic genes *NCED6* and *NCED9* decreased in the water control within the first 6 h of imbibition and then increased. Exogenous H_2O_2 slightly enhanced the expression of these two genes (Supplementary Fig. S1 available at *JXB* online).

Expression of three *GA20ox* and two *GA3ox* genes was also significantly induced in the water control in the initial hours of imbibition. As shown in Fig. 2c, transcription levels of all three *GA20ox* genes increased rapidly during the first 6 h. Levels of *GA20ox1* remained high for the entire imbibition period, but *GA20ox2* and *GA20ox3* transcription levels decreased to a lower level after 6 h. Two *GA3ox* genes displayed a delayed initiation compared with the *GA20ox* genes. *GA3ox1* reached its maximum at 12 h and decreased thereafter, while *GA3ox2* peaked at 24 h. H_2O_2 enhanced the transcription of all five GA biosynthesis genes to a high level and these elevated levels were maintained throughout the remainder of the imbibition period. DPI significantly inhibited transcription of the five genes, but addition of exogenous H_2O_2 was able to reverse the DPI inhibition (Fig. 2c). The transcription level of the GA catabolic gene *GA2ox2* decreased during the first 6 h of imbibition and increased thereafter. Exogenous H_2O_2 enhanced this gene expression slightly (Supplementary Fig. S1 at *JXB* online).

H_2O_2 requires NO for regulation of genes involved in ABA catabolism and breaking of seed dormancy

As shown in Fig. 3a, both NO and H_2O_2 caused dormancy break in freshly harvested seeds. Treatment with DPI or

with the NO scavenger c-PTIO enhanced seed dormancy. Treatment with the NO donor SNP substantially reversed the inhibition caused by DPI, whereas exogenous H_2O_2 failed to reverse the inhibition caused by c-PTIO. The effect of H_2O_2 and SNP on seed dormancy break could be reversed by the ABA catabolism inhibitor diniconazole, which supposedly inhibits ABA 8'-hydroxylase activity (Fig. 3a).

Earlier results indicated that *CYP707A2* was much more abundant than the other *CPY707A* genes during germination (Saito *et al.*, 2004; Okamoto *et al.*, 2006), consistent with what was found in the present study (Fig. 3b). Both SNP and H_2O_2 enhanced *CYP707A* gene expression, especially that of *CYP707A2* (Fig. 3b). Treatment with c-PTIO or DPI decreased *CYP707A* gene expression. Treatment with SNP reversed the effect of DPI, while exogenous H_2O_2 treatment was not able to reverse the effect of c-PTIO (Fig. 3b).

The changes in ABA content reflected the expression of ABA catabolic genes. SNP and exogenous H_2O_2 treatments enhanced ABA catabolic gene expression, while scavengers or inhibitors decreased expression of these ABA genes (Fig. 3c). SNP reversed the inhibitory effects of DPI, but exogenous H_2O_2 was largely ineffective at reversing the effects of c-PTIO on the expression of ABA catabolic genes.

Expression of the ABA response genes *RD29A* and *RD29B* (Yamaguchi *et al.*, 2006; Fujii *et al.*, 2007) showed a similar response (Fig. 3d) to that shown for ABA catabolic genes (Fig. 3c).

The apparent requirement for NO to elicit the H_2O_2-responsive expression of ABA catabolic genes and breaking of seed dormancy suggests that H_2O_2 may also regulate NO production. As shown in Fig. 4a, during imbibition, exogenous H_2O_2 increased NO production while DPI decreased it. However, SNP and its scavenger c-PTIO showed a slight effect on H_2O_2 production (Fig. 4b).

Some genes involved in H_2O_2 production and catabolism were also measured during imbibition. Transcription of all three H_2O_2 catabolic genes, *CAT1*, *CAT2*, and *CAT3*, decreased rapidly and significantly during the first 3 h of imbibition (Fig. 4c). Transcription of the three NADPH oxidase genes, which are involved in H_2O_2 production, showed no change in the first 6 h of imbibition (Fig. 4d). Accumulation of H_2O_2 at the first stage of imbibition was apparently related primarily to decreasing H_2O_2 catabolism.

The expression of GA biosynthesis genes is enhanced by H_2O_2

Treatment with PAC, which inhibited GA biosynthesis (Kitahata *et al.*, 2005; Toh *et al.*, 2008), enhanced seed dormancy, and the enhancement of germination by H_2O_2 and NO could be reversed by PAC (Fig. 5a). When

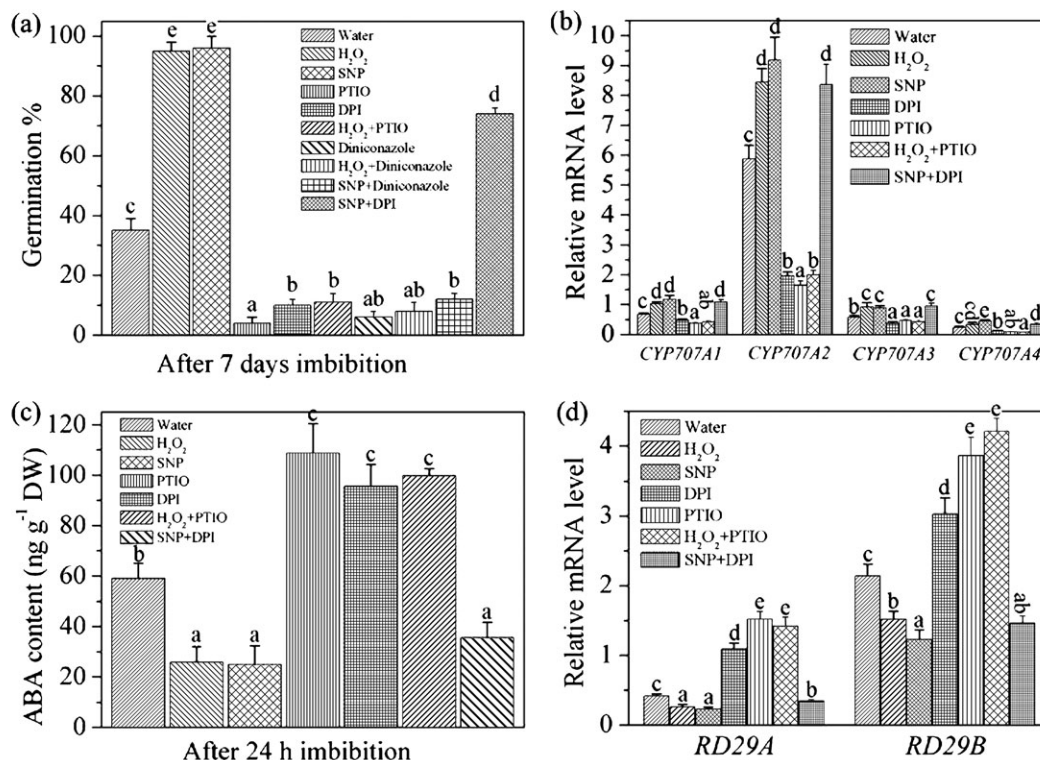

Fig. 3. The function of NO for H_2O_2-regulated ABA catabolism. (a) Effect of H_2O_2, NO, and their production inhibitor DPI or scavenger c-PTIO, or the ABA catabolism inhibitor diniconazole on freshly harvested seed dormancy and germination; the germination ratio was counted on the seventh day. (b) Change in the transcript levels of ABA catabolism genes under different treatments at 6 h imbibition. (c) Change in ABA content after 24 h imbibition. ABA contents were measured by RIA. (d) Change in the transcript levels of the ABA-regulated genes *RD29A* and *RD29B* under different treatments at 24 h imbibition. H_2O_2 (10 mM), DPI (10 μM), SNP (200 μM), c-PTIO (200 μM), and diniconazole (10 μM) were used for these experiments. Values are the means±SE (n=3 for a–d). An ANOVA test followed by a rank test was performed. Different letters are used to indicate means that are significantly different (P <0.05).

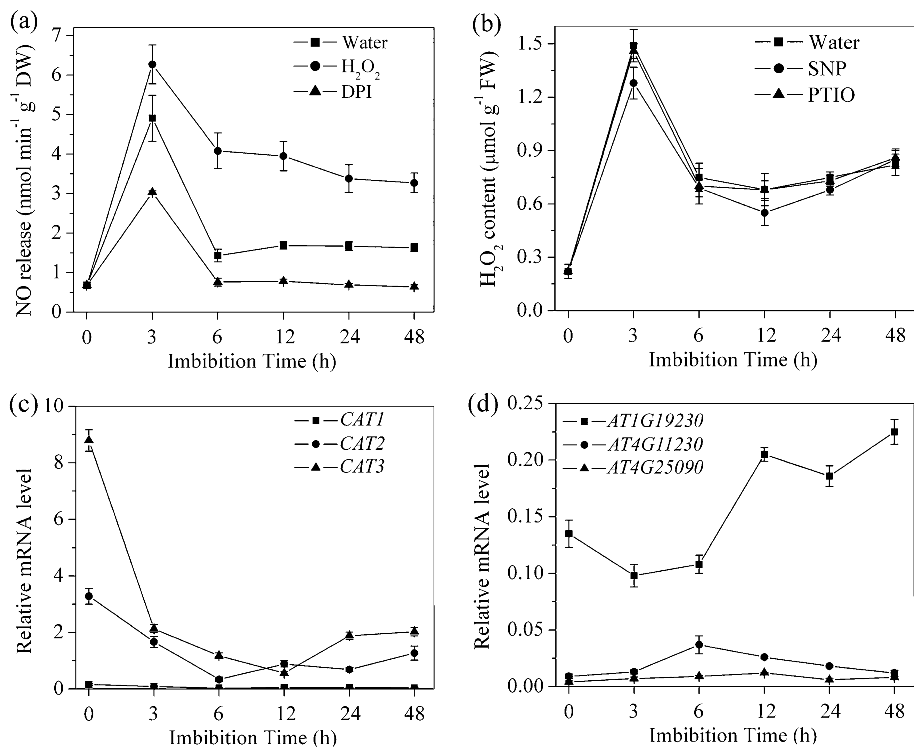

Fig. 4. Effect of H_2O_2 and NO on each other's production and the transcription of *CAT* and NADPH genes during imbibition. (a) Changes in NO release during imbibition with water, H_2O_2 (10 mM), and its production inhibitor DPI (10 μM). (b) Changes in H_2O_2 release during imbibition with water, SNP (200 μM), and its scavenger c-PTIO (200 μM). (c) The transcript levels of *CAT* H_2O_2 catabolism genes during imbibition. (d). The transcript levels of H_2O_2 NADPH production genes during imbibition. Values are means±SE (n=4 for a and b, and n=3 for c and d). An ANOVA test followed by a rank test was performed. Different letters are used to indicate means that are significantly different (P <0.05).

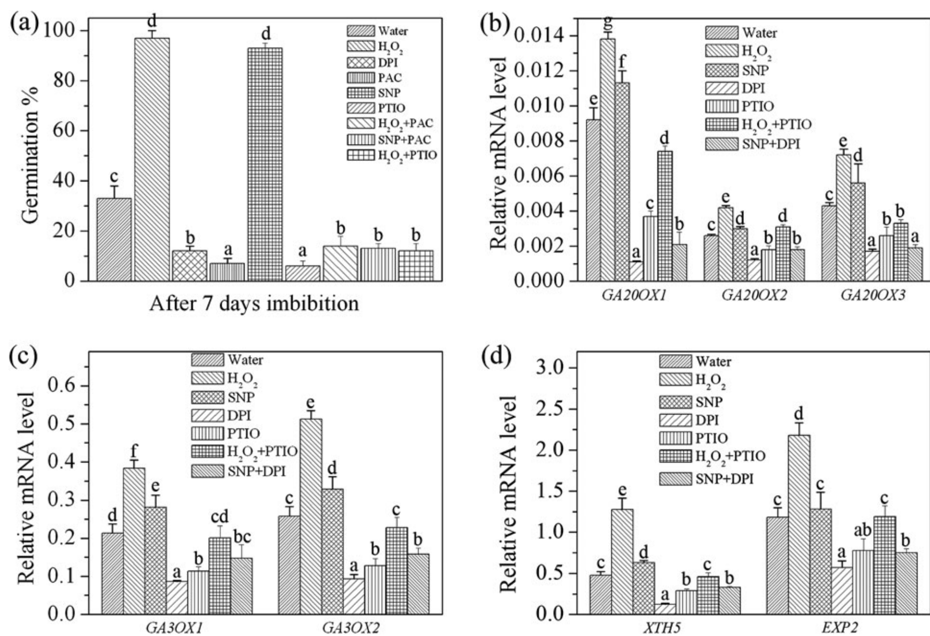

Fig. 5. The function of NO in H_2O_2-regulated GA biosynthesis. (a) Effects of H_2O_2, NO, and their production inhibitor DPI or scavenger c-PTIO, or the GA biosynthesis inhibitor PAC on the dormancy and germination of freshly harvested seed; the germination ratio was counted on the seventh day. (b) Changes in the transcript levels of *GA20ox* GA biosynthesis genes under different treatments at 24 h imbibition. (c) Changes in the transcript levels of *GA3ox* GA biosynthesis genes under different treatments at 24 h imbibition. (d) Changes in the transcript levels of GA-regulated genes *XTH5* and *EXP2* under different treatments at 24 h imbibition. H_2O_2 (10 mM), DPI (10 μM), SNP (200 μM), c-PTIO (200 μM), and PAC (10 μM) were used for these experiments. Values are the means±SE (n=3 for a–d). An ANOVA test followed by a rank test was performed. Different letters are used to indicate means that are significantly different (P <0.05).

expression of GA biosynthesis genes was investigated after 24 h imbibition, the transcription of the *GA20ox* and *GA3ox* genes was significantly up-regulated by exogenous H_2O_2, slightly up-regulated by SNP, and down-regulated by DPI and c-PTIO (Fig. 5b, c). The down-regulation by c-PTIO was reversed significantly by addition of exogenous H_2O_2, but this enhanced value was still low compared with seeds imbibed in H_2O_2 (Fig. 5b, c). The inhibition by DPI was increased slightly by addition of SNP (Fig. 5b, c).

Expression of two GA-regulated genes, *XTH5* and *EXP2*, was also measured after 24 h imbibition under different treatments (Rose *et al.*, 2002; Yamauchi *et al.*, 2004). Both *XTH3* and *EXP2* transcription levels were increased significantly by H_2O_2 and slightly by SNP, but were decreased by DPI and c-PTIO. Exogenous H_2O_2 reversed the effect of c-PTIO while SNP caused only a minimal reversal of the DPI effect (Fig. 5d).

H_2O_2 enhances and ABA suppresses the expression of GA biosynthesis genes

Treatment with H_2O_2 enhanced expression of GA biosynthesis genes during imbibition. It was therefore hypothesized that GA biosynthesis is up-regulated by H_2O_2 and suppressed by ABA. To examine this possibility, the ABA catabolism mutant *cyp707a2* and its overexpression line *CYP707A2-OE* were used. As shown in Fig. 6a, the *cyp707a2* mutant showed an absence of *CYP707A2* gene expression while *CYP707A2-OE* had a high level of *CYP707A2* gene expression. Freshly harvested *cyp707a2* seeds showed strong dormancy, while *CYP707A2-OE* seeds had a much weaker dormancy response compared with wild-type seeds (Fig. 6b). After 24 h imbibition, *cyp707a2* seeds retained a high ABA content whereas ABA levels in *CYP707A2-OE* seeds were much lower (Fig. 6c)

H_2O_2 and GA (10 μM) clearly enhanced germination of freshly harvested *cyp707a2* seed, while NO did not (Fig. 7a). *CYP707A2-OE* germination was inhibited completely by treatment with 0.5 μM exogenous ABA (Fig. 7a). QRT-PCR analysis demonstrated that the transcription of *GA3ox* genes was enhanced by exogenous H_2O_2 rather than by SNP after 24 h imbibition in *cyp707a2* (Fig. 7b). These results indicated that it was H_2O_2 rather than NO that exerts a regulatory effect on the expression of GA biosynthetic genes. QRT-PCR analysis also indicated that the transcription of *GA3ox* genes was high in *CYP707A2-OE* and was significantly decreased by treatment with 0.5 μM ABA (Fig. 7b). Expression of *GA3ox* genes was much lower in *cyp707a2* compared with the wild type and *CYP707A2-OE* (Fig. 7b, c). Thus, ABA treatment appeared to suppress expression of GA biosynthesis genes.

The empty pGWB5 vector showed no effect on expression of any gene (data not shown). The transcription levels of the GA-regulated genes *XTH5* and *EXP2* were similar to those of *GA3ox* genes. In the *cyp707a2* mutant, expression of these genes was enhanced by treatment with exogenous H_2O_2 but not by SNP after 24 h imbibition (Fig. 7c). Transcription of *XTH5* and *EXP2* was down-regulated by

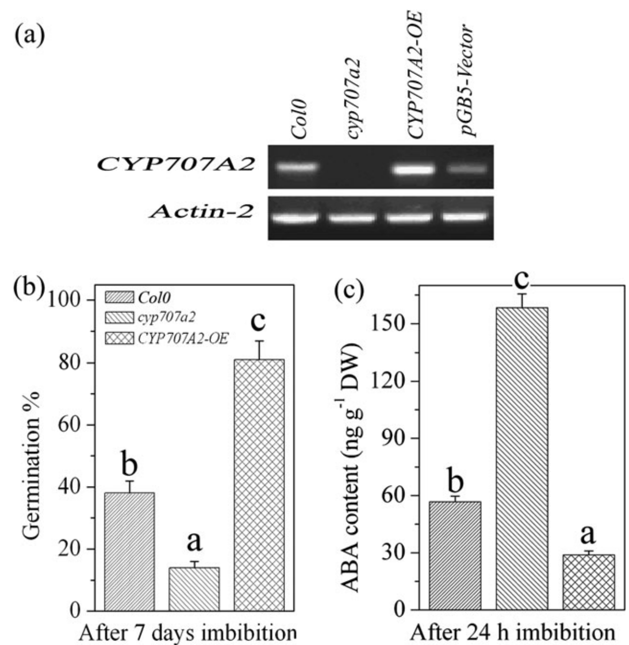

Fig. 6. The *CYP707A2* gene mediates seed dormancy and ABA catabolism. (a) Transcript levels of *CYP707A2* in the wild type, *cyp707a2*, the *CYP707A2* overexpression line, and pGB5 vector only in the wild type were analysed by RT-PCR. (b) The germination of the wild type, *cyp707a2*, and the *CYP707A2* overexpression line in freshly harvested seeds. The germination rates were recorded after 7 d of imbibition. (c) ABA contents of the wild type, *cyp707a2*, and the *CYP707A2* overexpression line after 24 h imbibition. Values are the means±SE (n=3 for b and c). An ANOVA test followed by a rank test was performed for b and c. Different letters are used to indicate means that are significantly different (P <0.05)

ABA in *CYP707A2-OE* after 24 h imbibition (Fig. 7c), suggesting that *XTH5* and *EXP2* transcription was much lower in *cyp707a2* compared with the wild type and *CYP707A2-OE* (Figs 5d, 7c). All of these results suggest that H_2O_2 enhances GA biosynthesis while ABA suppresses GA biosynthesis.

Discussion

It is well known that ABA and GA play important roles in seed dormancy and germination (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Ogawa *et al.*, 2003; Razem *et al.*, 2006; Weiss *et al.*, 2007; Toh *et al.*, 2008). Studies on numerous mutants have demonstrated that ABA catabolism and GA biosynthesis are required for seed germination (Koornneef and van der Veen, 1980; Groot and Karssen, 1987; Ogawa *et al.*, 2003; Kushiro *et al.*, 2004; Saito *et al.*, 2004; Okamoto *et al.*, 2006). The results from the present study may have clarified a signalling pathway for the mechanisms underlying these responses. A rapid NO-induced decrease in ABA is essential to break seed dormancy in *Arabidopsis*. Based on the present results, H_2O_2, acting as a signalling molecule, could regulate seed

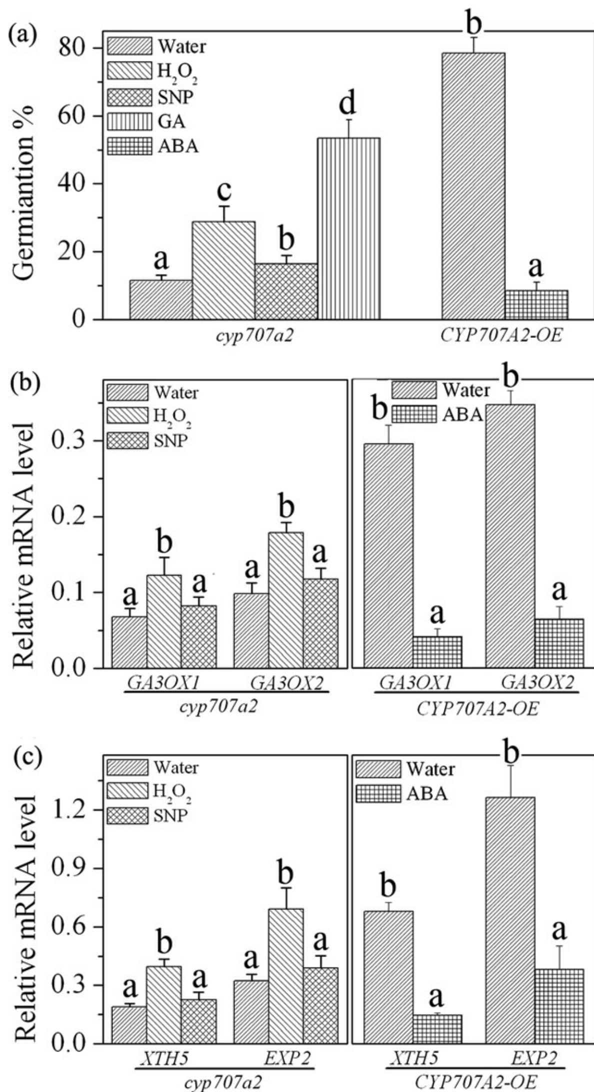

Fig. 7. H_2O_2 mediated GA biosynthesis and ABA suppressed GA biosynthesis. (a) The germination of *cyp707a2* and the *CYP707A2* overexpression line in freshly harvested seeds under different treatments. (b) Change in the transcript levels of *GA3ox* GA biosynthesis genes under different treatments in *cyp707a2* and the *CYP707A2* overexpression line after 24 h imbibition. (c) Change in the transcript levels of GA-regulated genes *XTH5* and *EXP2* under different treatments in *cyp707a2* and the *CYP707A2* overexpression line after 24 h imbibition. H_2O_2 (10 mM), SNP (200 μM), ABA (0.5 μM), and GA (10 μM) were used for these experiments. Values are the means±SE (n=3 for a–d). An ANOVA test followed by a rank test was performed. Different letters are used to indicate means that are significantly different (P <0.05)

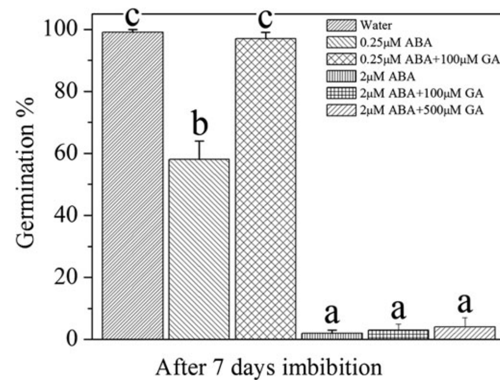

Fig. 8. The relationship of ABA and GA in seed germination. The germination of non-dormant seeds under different treatments. An ANOVA test followed by a rank test was performed. Different letters are used to indicate means that are significantly different (P <0.05).

dormancy by triggering both ABA catabolism and GA biosynthesis. The up-regulation of ABA catabolism by H_2O_2 would be carried out through NO. Concomitantly, as long as a high concentration of ABA exists, it inhibits the expression of GA biosynthesis genes so that a balance of these two hormones jointly controls the dormancy and germination of *Arabidopsis* seeds (Fig. 8).

As shown in Fig. 1, exogenous H_2O_2 decreased dormancy in freshly harvested seed. Inhibiting H_2O_2 production in turn enhanced seed dormancy. The action of H_2O_2 was associated with expression of genes related to ABA and GA biosynthesis and catabolism. Exogenous H_2O_2 treatment clearly increased the expression of *CYP707A*, *GA3ox*, and *GA20ox* (Fig. 2b, c) and decreased seed dormancy (Fig. 1b). Using DPI to inhibit H_2O_2 decreased the expression of *CYP707A*, *GA3ox* and *GA20ox* genes (Fig. 2b, c) and enhanced seed dormancy (Fig. 1b). It was also observed that the inhibition by DPI of the expression of these genes and of seed germination was completely reversed by exogenous H_2O_2.

When ABA catabolism and GA biosynthesis were directly inhibited by diniconazole and PAC, the enhancement of seed germination by H_2O_2 disappeared (Figs 2, 4). H_2O_2 showed a slight effect on expression of genes involved in ABA biosynthesis and GA catabolism compared with its effects on expression of genes involved in ABA catabolism and GA biosynthesis (Fig. 2 and Supplementary Fig. S1). These results indicated that the effect of H_2O_2 on seed dormancy break might be connected with transcription of genes involved in both ABA catabolism and GA biosynthesis.

A role for NO, another widespread signalling molecule, in breaking of dormancy was also apparent. Up-regulation of genes responsible for ABA catabolism by H_2O_2 required the participation of NO. The enhancement of seed germination by H_2O_2 treatment was significantly reversed by the NO scavenger c-PTIO. DPI inhibited H_2O_2 generation and enhanced seed dormancy, but these effects were substantially reversed by addition of the NO donor SNP (Fig. 3a). Use of c-PTIO as an NO scavenger reversed the effect of H_2O_2 on the expression of *CYP707A* genes and of ABA catabolic genes (Fig. 3b–d). Treatment with H_2O_2 also modulated NO release during imbibition (Fig. 4a, b), and the accumulation of H_2O_2 at the first stage of imbibition, primarily by decreasing H_2O_2 catabolism (Fig. 4c, d).

The regulation by H_2O_2 of expression of GA biosynthetic genes is different from its effects on genes involved in ABA catabolism. Although SNP, similarly to H_2O_2, increased

seed germination (Fig. 5a), SNP did not significantly increase transcription of GA biosynthetic genes when compared with H_2O_2 (Fig. 5b,c). SNP could not reverse the inhibition by DPI of transcription of GA biosynthesis genes, while exogenous H_2O_2 substantially restored the inhibition induced by c-PTIO (Fig. 5b, c). In particular, when the *cyp707a2* mutant was used to measure the effect of H_2O_2 and NO on GA biosynthesis, it was found that SNP had no effect on transcription of GA biosynthesis genes, while H_2O_2 increased this transcription (Fig. 7b, c), It was also found that the expression of *GA3ox* genes was much lower in *cyp707a2* than in the wild type imbibed with water or exogenous H_2O_2 (Figs 5c, 7b). The enhancement of seed germination induced by exogenous H_2O_2 and GA was also much lower in *cyp707a2* compared with the effects on the wild type (Figs 3a, 7a). As shown in Fig. 6c, *cyp707a2* had higher ABA levels compared with the wild type and *CYP707A2-OE*. The present results also support the conclusion that ABA down-regulated genes responsible for GA biosynthesis and ABA catabolism, both of which are functions that are necessary for breaking seed dormancy.

The transcription of GA biosynthesis genes was suppressed by exogenous ABA (Fig. 7b). From these results, it can be hypothesized that H_2O_2 could directly regulate GA biosynthesis and indirectly regulate ABA catabolism. The effect of SNP on GA biosynthesis may occur via regulation of ABA catabolism during imbibition. The results also suggest that GA reverses the inhibitory effect of a low concentration of ABA on seed germination but GA alone is insufficient to reverse germination inhibition when the ABA content is high (Fig. 8). Thus, ABA may regulate seed germination by two pathways, one that acts directly on some as yet uncharacterized factors and the second that acts through the regulation of GA biosynthesis. The data presented in Figs 3 and 5 indicate that even at low concentrations of ABA and under H_2O_2 and SNP treatments, the seeds failed to germinate if GA biosynthesis was inhibited by diniconozole or PAC. This indicates that both ABA catabolism and GA biosynthesis are absolutely necessary for seed dormancy break.

Figure 9 shows a hypothetical schematic model that could explain the results documented in the present paper. In this scheme, H_2O_2 may relieve dormancy of freshly harvested *Arabidopsis* seeds by two pathways. One pathway relies on enhancement of ABA catabolism and GA biosynthesis. The signal molecule NO does not regulate GA biosynthesis directly, but acts as an interim signalling molecule involved in H_2O_2 regulation of ABA catabolism. In the second pathway, ABA negatively regulates GA biosynthesis. In this way, both ABA and GA act in concert to regulate seed dormancy and germination.

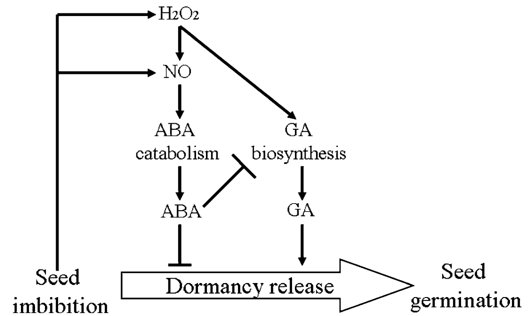

Fig. 9. Model showing how H_2O_2, NO, ABA, and GA regulate seed dormancy and germination. Seed imbibition leads to increases in H_2O_2 and NO. H_2O_2 up-regulates ABA catabolism through NO, and also GA biosynthesis. A high concentration of ABA also inhibits GA biosynthesis, but a balance of these two hormones jointly controls the dormancy and germination of *Arabidopsis* seeds.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Effects of H_2O_2 on the expression of ABA biosynthesis and GA catabolism genes during imbibition. The genes were analysed by QRT-PCR. H_2O_2 at 10 mM and DPI at 10 μM were used to manipulate H_2O_2 levels for these experiments. Values are the means with SE (n=4 for A and n=3 for B and C). (A) Changes in the transcript levels of *NCED6* in the first 48 h during imbibition. (B) Changes in the transcript levels of *NCED9* in the first 48 h during imbibition. (C) Changes in the transcript levels of *GA2ox2* in the first 48 h during imbibition.

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