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Research article

Reactive oxygen species induced by cold stratification promote germination of *Hedysarum scoparium* seeds

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ABSTRACT

Seed germination is comprehensively regulated by multiple intrinsic and extrinsic factors, and reactive oxygen species (ROS) are relatively new among these factors. However, the role and underlying mechanisms of ROS in germination regulation remain largely unknown. In this study, we initially found that cold stratification could promote germination and respiration of *Hedysarum scoparium* seeds, especially at low temperature. We then noted that a ROS environment change induced by hydrogen peroxide (H_2O_2) or methylviologen (MV) could similarly promote seed germination. On the other hand, the ROS scavenger N-acetyl-L-cysteine (NAC) suppressed germination of cold-stratified *H. scoparium* seeds, indicating a stimulatory role of ROS upon seed germination. An increased accumulation of O_2^- was detected in embryonic axes of cold-stratified seeds, and stratification-induced ROS generation as well as progressive accumulation of ROS during germination was further confirmed at the cellular level by confocal microscopy. Moreover, protein carbonylation in cold-stratified seeds was enhanced during germination, which was reversed by NAC treatment. Finally, the relationship between ROS and abscisic acid (ABA) or gibberellin (GA) in germination regulation was investigated. ABA treatment significantly inhibited germination and reduced the H_2O_2 content in both cold-stratified and non-cold-stratified seeds. Furthermore, we found that cold stratification mediates the down-regulation of the ABA content and increase of GA, suggesting an interaction between ROS and ABA/GA. These results in *H. scoparium* shed new light on the positive role of ROS and their cross-talk between plant hormones in seed germination.

1. Introduction

Seed germination is an intricate process that leads to elongation of the embryonic axes from a seed, allowing subsequent seedling emergence. Completion of germination requires activation of a complex regulatory system that is affected by intrinsic (i.e., embryo vigour) and extrinsic (i.e., environmental conditions, such as temperature, oxygen and water availability) factors. Recent studies have suggested that reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\cdot$) and singlet oxygen ($^1O_2$), are released and involved in seed germination (Schopfer et al., 2001). During seed germination, the active mitochondrion is likely one of the major sites to produce superoxide ($O_2^{\bullet-}$) and subsequently H_2O_2 (Møller, 2001). NADPH oxidases of the plasma membrane are another major source of superoxide radicals (Lamb and Dixon, 1997). It has been proposed that application of an NADPH-oxidase inhibitor leads to retarded radicle emergence, indicating a putative function for NADPH

Abbreviations: ABA, Abscisic acid; DCF, dichlorofluorescein; DCFH-DA, dichlorodihydrofluorescein diacetate; DIC, Differential interference contrast; DNPH, 2, 4-dinitrophenylhydrazone; DW, dry weight; FW, fresh weight; GA, Gibberellic acid; MDA, Malondialdehyde; HPLC, High Performance Liquid Chromatography; H_2O_2, hydrogen peroxide; LSM, laser scanning microscopy; MV, methylviologen; NAC, N-acetyl-L-cysteine; NBT, Nitroblue tetrazolium chloride; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SD, standard deviation.

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oxidases in germination (Sarath et al., 2007).

Although ROS have been widely considered to be detrimental to seeds, researchers are increasingly observing the positive role played by ROS in seed germination. Treatment with exogenous H_2O_2 or the ROS-generating compound methylviologen (MV) has been shown to induce the germination of dormant seeds in many species, such as barley (Fontaine et al., 1994), rice (Naredo et al., 1998), *Zinnia elegans* (Ogawa and Iwabuchi, 2001), apple (Bogatek et al., 2003) and sunflower (Oracz et al., 2007, 2009). Some evidence has also indicated that ROS generated in seeds may act as a signal in dormancy alleviation and germination initiation. In sunflower seeds, it was documented that there is a close correlation between dormancy alleviation and ROS accumulation, as well as protein carbonylation in embryonic axes (El-Maarouf-Bouteau et al., 2007; Oracz et al., 2007). The oxidation of reserve proteins could partially account for the mechanism of ROS in stimulating germination. Similar to sunflower embryos treated with hydrogen cyanide (HCN), H_2O_2 production and protein carbonylation were triggered and accompanied by dramatically stimulated germination (Oracz et al., 2009).

It is well known that the plant hormones abscisic acid (ABA) and gibberellic acid (GA) play significant roles in seed germination (Bewley, 1997). Studies with genetic mutants showed that GA releases dormancy and promotes germination, while ABA induces dormancy and inhibits germination (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). Therefore, the critical balance between GA and ABA contents seems to contrarily determine seed germination (Piskurewicz et al., 2008). Cross-talk occurs between ROS and plant hormones during seed germination. In a previous study of Arabidopsis seeds, it was found that the ABA contents were decreased with the upregulation of GA biosynthesis when H_2O_2 was applied and germination was stimulated (Liu et al., 2010). H_2O_2 can also alleviate seed dormancy by activating GA signalling and synthesis while simultaneously inhibiting its catabolism in Barley (Bahin et al., 2011). Recent studies further demonstrated the cross-talk of ROS with ethylene and ABA. Ethylene treatment provoked ROS generation in embryonic axes and conferred a beneficial effect on the germination of sunflower seeds, and MV suppressed the inhibitory effect of ABA (El-Maarouf-Bouteau et al., 2014). These findings suggest the importance of both ROS and their interaction with hormone signalling pathways. However, further efforts are required to explore the pivotal role of ROS in the regulation of germination.

Hedysarum scoparium is a shrub species that is characterized by fast growth and high drought resistance. Due to its economic and ecological value, it has been widely used for grassland restoration. The seeds of *H. scoparium* exhibit a limited germination rate at low temperatures. In one study, stratification was required to break dormancy or promote germination (Baskin et al., 2006; Chen et al., 2015; Imani et al., 2011) by accelerating the morphological development of the embryo, changing the levels of related hormones and increasing gene expression and nutrient accumulation. For instance, *Acer morrisonense* seeds germinated to 87% after being cold-stratified at 5 °C for 12 weeks, while the germination of non-stratified seeds was only 61%; the total ABA content of the cold-stratified seeds was only 28% of that in fresh *Myrica rubra* embryos (Chen et al., 2015).

The aims of this study are to investigate the role and underlying mechanisms of ROS in the germination of *H. scoparium*. Either cold stratification or ROS/ROS donor treatment dramatically promoted germination and respiration of *H. scoparium* seeds at 10 °C, accompanied by a progressive accumulation of ROS during imbibition. ROS enhanced the carbonylation of embryo proteins with seed imbibition. Either stratification or a ROS/ROS donor mediated the down-regulation of the ABA level and also increased the GA content, which was demonstrated by amylase activity. In return, ABA inhibited germination and reduced the ROS contents of seeds. All of these results suggest the benefit of ROS in the germination of *H. scoparium* seeds as well as a relationship between ROS and hormones.

2. Materials and methods

2.1. Plant material and germination test

Hedysarum scoparium seeds used in this study were harvested and provided by Ordos Forestry Desert Control Research Institute in Inner Mongolia in 2012. Germination assays were performed with seeds without pericarp in 9 cm Petri dishes (50 seeds per dish, three replicates) with a double layer of filter paper. Seeds were imbibed either with distilled water or with the indicated solutions, and the assays were carried out at the indicated temperature with 16 h of light and 8 h of dark daily. The germination test was conducted for seven or ten days as previously mentioned, and a seed was considered germinated when the radicle protruded though the seed coat (Ming et al., 2010). Seeds for cold stratification were mixed with sands and deposited in 4 °C for 10 days before imbibition (Chen et al., 2015).

2.2. Chemical treatments

Methylviologen (MV) was used as a reactive oxygen species (ROS) donor, and N-acetyl-L-cysteine (NAC) worked as an antioxidant. Treatment with MV was carried out by placing the seeds in MV solution in darkness for 3 h, followed by thoroughly rinsing three times with distilled water before the germination test. Treatment with H_2O_2, NAC and abscisic acid (ABA) was carried out by replacing distilled water in filter paper with H_2O_2, NAC or ABA solution to execute the germination test (Ishibashi et al., 2012; Maia et al., 2014).

2.3. Oxygen uptake measurements

The metabolic rates of the axes were estimated from the rate of oxygen consumption following the previously described methods (Walters et al., 2001). De-coated *H. scoparium* seeds were imbibed for 24 h or 48 h, and the oxygen they took up was measured manometrically using a biological oxygen meter (YAXIN-1151). Before measurements, a KOH-water solution (1: 4 w/w) was placed in the central well of Warburg flasks to absorb CO_2. Measurements were conducted from imbibed seeds at 25 °C over a 4 h period. At least five seeds were detected in each group. The oxygen uptake rates were calculated from linear regressions of pressure-time-course data ($R^2 > 0.90$).

2.4. In situ NBT staining and determination of O_2^- content

For NBT staining, embryos were separated from imbibed seeds and incubated in 10 mM Tris-HCl buffer (pH 7.4) containing 6 mM nitroblue tetrazolium chloride (NBT) in darkness at 20 °C for 20 min.

Intact embryos were washed with deionized water three times, and O_2^- was visualized as precipitates of dark blue insoluble formazan compounds (Beyer et al., 1987). The rinsed embryo was cut into transections to observe the location of O_2^- in the embryos.

For determination of the O_2^- content, seeds were quickly ground into powder in liquid nitrogen and incubated in potassium phosphate buffer (20 mM, pH 6.0) containing 0.5 mM XTT (Polyscience Europe, Eppelheim, Germany) in darkness at 20 °C (Schopfer et al., 2001). The absorbance of the supernatant at 470 nm was measured

with an Infinite M200 fluorescence microplate (Tecan, Germany). Reagent blanks (without tissue) and tissue blanks (without reagent) were run in parallel to correct for unspecific absorbance.

2.5. In situ localization of ROS by fluorescence

ROS production was visualized after staining with 5-(and-6)-chloromethyl-2,7-dichlorofluorescein diacetate (DCFH-DA) using confocal laser scanning microscopy (LSM) (Schopfer et al., 2001). DCFH-DA permeates cells and is hydrolysed by esterases to liberate dichlorofluorescein (DCF), which reacts with H_2O_2 or hydroperoxides to form a fluorescent DCF-derived compound. Excised axes were cut longitudinally and incubated in 20 mM potassium phosphate buffer (pH 6.0) containing 100 mM DCFH-DA for 15 min at 10 °C. Axes were rinsed for 15 min in a potassium phosphate buffer solution. Images were acquired (excitation, 488 nm; emission, 525 nm) (Leica, Wetzlar, Germany) with a Leica SP5 confocal microscope (Die et al., 2012; Wang et al., 2015).

2.6. Malondialdehyde measurements

The extent of lipid peroxidation was explored by measuring the malondialdehyde (MDA) content from 0.2 g of fresh weight (FW) embryonic axes according to a previous method (Heath and Packer, 1968) with slight modification. The results are expressed as $\mu mol \cdot g^{-1}$ FW of seeds and correspond to the means ± SD of measurements carried out with three extracts.

2.7. Immunoblotting of carbonyl groups

Total axes proteins were lysed at 4 °C in RIPA buffer (150 mM sodium chloride, 1.0% (v/v) Tergitol-type NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris, pH 8.0) with a protease inhibitor. The proteins, once separated by discontinuous SDS-polyacrylamide gel (12% separation gel, 4% stacking gel) electrophoresis (PAGE), were transferred to nitrocellulose membranes (Bio-Rad). The appearance of carbonyl groups in proteins was monitored by derivatization of protein extracts with 2, 4-dinitrophenylhydrazone (DNPH) and immunological detection of the DNP adducts with a monoclonal anti-DNP antibody (OxiSelect™ Protein Carbonyl Immunoblot Kit, CELL BIOLABS, INC.) followed by chemiluminescence (Tanase et al., 2016).

2.8. Endogenous ABA measurements

Endogenous ABA was analysed by High Performance Liquid Chromatography (HPLC). After imbibition, 0.2 g of seeds was frozen in liquid nitrogen and ground to a powder. Samples were extracted in 10 ml of 80% methanol (HPLC grade) at 4 °C overnight. Methanol was then removed by evaporation in a rotary evaporator. The remaining aqueous phase was extracted in ethyl acetate three times, and the solvent was then removed by a rotary evaporator. Then, the samples were dissolved in 3% methanol and 97% 0.1 M acetic acid and filtered through a 0.45-μm membrane. The fractions containing ABA were ready for HPLC analysis. The standard ABA (HPLC grade) was purchased from Sigma.

2.9. Determination of H_2O_2 content

Axes from seeds were ground to a powder in liquid nitrogen. The samples were reacted with reagents provided in the Hydrogen Peroxide Fluorescent Detection Kit (ARBOR ASSAYS, USA), and the fluorescent products were determined at 590 nm with a spectrophotometer. Each sample had three replicates.

2.10. Amylase activity assay

Frozen axes from seeds were homogenized, and soluble proteins were extracted. Amylase activity was measured by a spectrophotometric method described in previous literature (Ye et al., 2012).

2.11. Statistical analysis

All values are expressed as the mean ± standard deviation (S.D.) of no less than three replicates. The data were analysed with Duncan's multiple range test by SPSS software, and differences at $P < 0.05$ were considered significant.

3. Results

3.1. Cold stratification promotes germination of H. scoparium seeds

A previous study showed that moist cold stratification played a

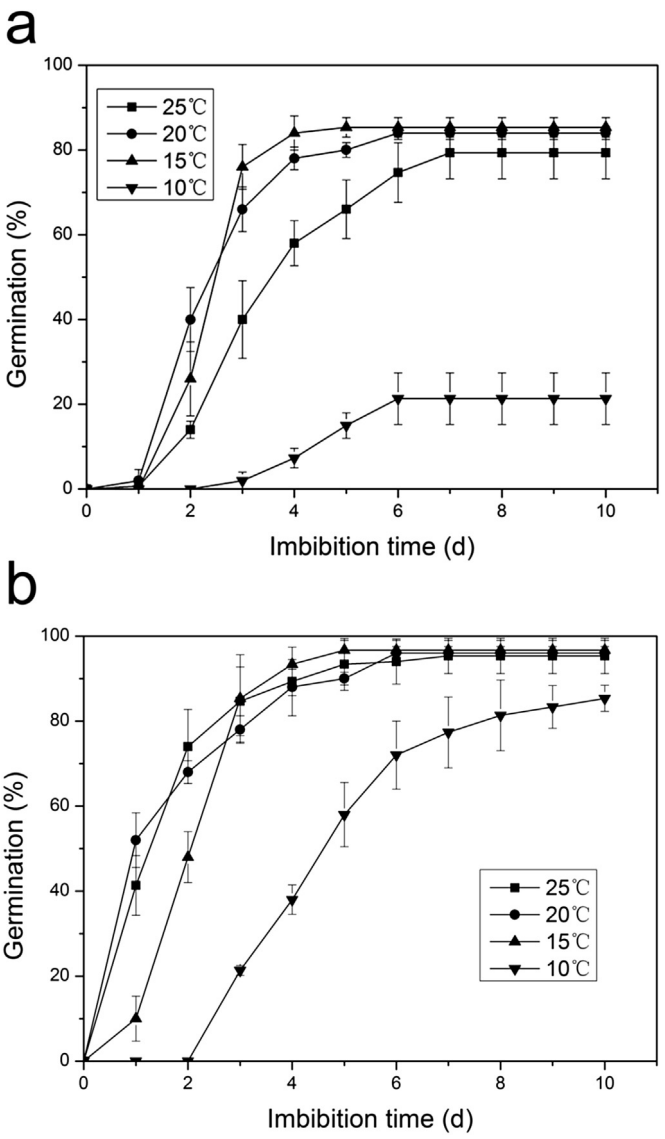

Fig. 1. Cold stratification promotes germination of *H. scoparium* seeds (naked) at various temperatures. (a) Non-cold stratified (Con) seeds germinated within 10 days at 10 °C, 15 °C, 20 °C and 25 °C. (b) Germination of seeds at 10 °C, 15 °C, 20 °C and 25 °C after cold stratification (Str) for ten days. Values are means ± standard deviation (SD) of three replicates.

vital role in breaking dormancy in various seeds. To determine whether cold stratification benefited the seed germination of *H. scoparium*, we first compared the germination behaviour of cold-stratified and non-cold-stratified, de-coated *H. scoparium* seeds. The results showed that at 15, 20 and 25 °C, more than 80% of non-cold-stratified seeds germinated after seven days of

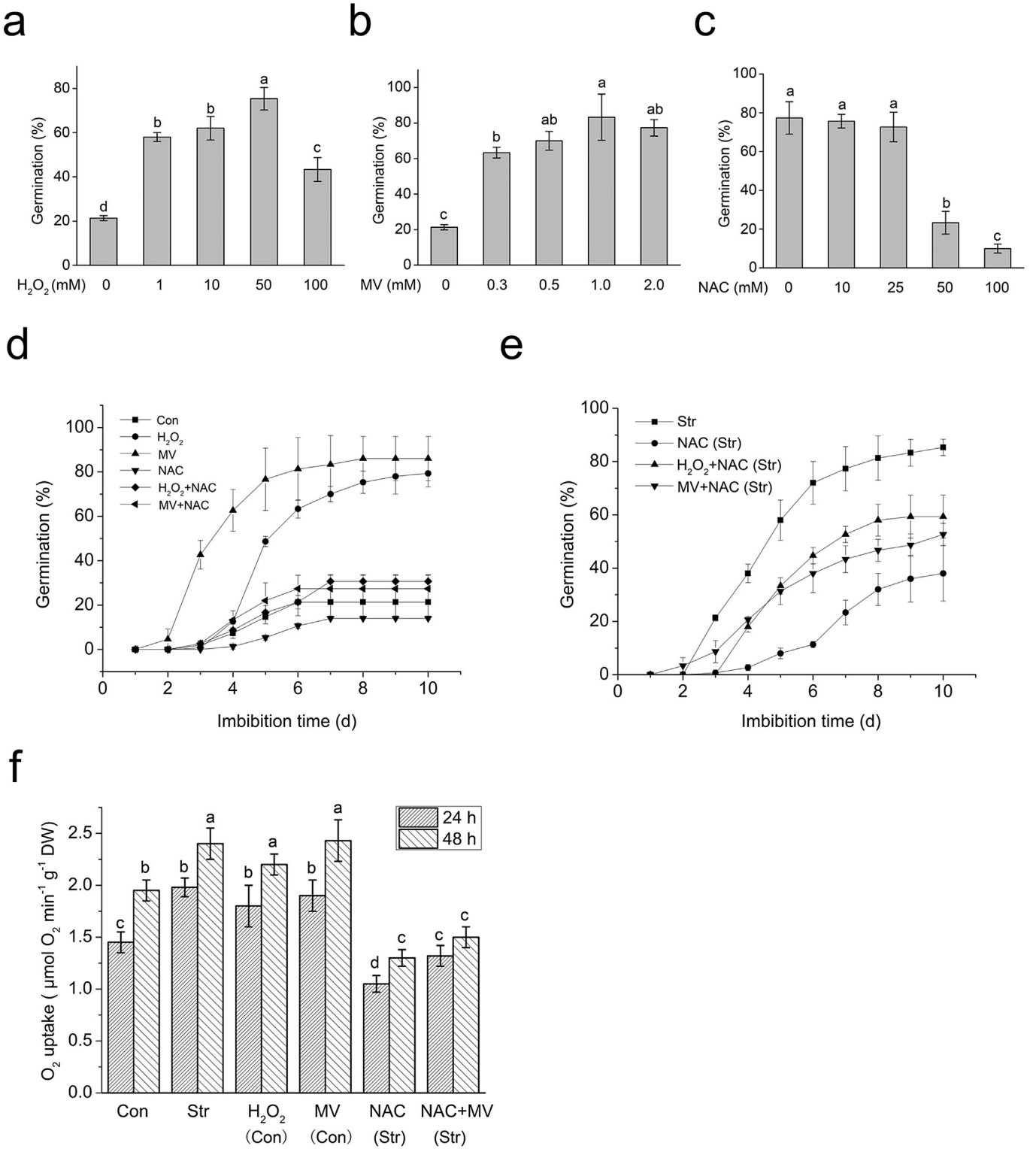

Fig. 2. ROS/ROS generating compounds promote seed germination and O_2 consumption as stratification. Germination of non-cold stratified (Con) seeds treated with indicated concentration of (a) hydrogen peroxide (H_2O_2) or (b) methylviologen (MV) at 10 °C after seven days of imbibition. (c) Germination of cold stratified (Str) seeds treated with indicated concentration of N-acetylcysteine (NAC) at 10 °C after seven days of imbibition. (d) Changes in germination of non-cold stratified seeds treated with 50 mM H_2O_2, 1 mM MV, 50 mM NAC, or together with both NAC and H_2O_2 or NAC and MV at 10 °C during ten-day imbibition. (e) Changes in germination of cold stratified seeds treated with 50 mM NAC or together with both NAC and H_2O_2 or NAC and MV at 10 °C during ten-day imbibition. (f) The effects of stratification, H_2O_2, MV and NAC treatment on O_2 consumption of whole seeds imbibed for 24 h or 48 h. DW, dry weight. Values are means ± SD of three replicates. Different superscript letters indicate significant difference at the $P < 0.05$ level.

imbibition; however, only 21% of seeds germinated by 10 days at 10 °C (Fig. 1a). Cold stratification treatment dramatically enhanced both the germination rate and final percentage of germination at all temperatures tested. In particular, 80% of cold-stratified seeds were able to germinate at 10 °C within 10 days (Fig. 1b), and stratified seeds germinated very quickly and evenly. Cold stratification clearly widened the temperature window of germination and promoted *H. scoparium* seed germination, especially at 10 °C. Consequently, we conducted the germination assay at 10 °C in the subsequent experiments to clarify the influence of cold stratification on *H. scoparium* seed germination.

3.2. ROS/ROS-generating compounds promote seed germination and respiration similar to stratification

A recent study reported that ROS generation is essential for germination of certain seeds (Bailly and El-Maarouf-Bouteau, 2008). To test whether ROS could promote *H. scoparium* seed germination, non-cold-stratified seeds were treated with H_2O_2 or MV. Germination of the control after seven-day imbibition was 21%, while 50 mM H_2O_2 or 1 mM MV treatment greatly enhanced it to 79% or 84%, respectively (Fig. 2a, b, 2d). Much higher concentrations, such as 100 mM H_2O_2 or 2 mM MV, had less of an effect. These results verified that ROS/ROS-generating compounds promoted seed germination with a similar effect as stratification, which raised a hypothesis that stratification might increase seed germination of *H. scoparium* by generating more ROS in the seeds. This was supported by the results that an antioxidant NAC could suppress germination of *H. scoparium* seeds, especially cold-stratified ones (Fig. 2c, d, 2e). Germination of cold-stratified seeds was approximately 77% after seven days of imbibition, whereas that of stratified seeds treated with 50 mM NAC dropped to only 23% compared to that of non-cold-stratified seeds (Fig. 2c and e). In addition, germination of seeds treated with a solution containing both NAC and H_2O_2 or NAC and MV showed a significant reversal of the inhibitory effects caused by NAC alone (Fig. 2d and e). Taken together, ROS treatment on *H. scoparium* seeds can promote germination without the need for cold stratification treatment.

To further investigate the effect of stratification or ROS on seed vigour, the amount of oxygen consumed during germination was measured as an indicator of the respiration rate. After 24 h of imbibition, cold-stratified seeds significantly consumed more than 43% oxygen compared to the non-cold-stratified control (Fig. 2f). Similarly, more oxygen was consumed by H_2O_2 or MV-treated, non-cold-stratified seeds than seeds without oxidative agent treatment. On the other hand, ROS scavenger NAC treatment reduced the uptake of O_2 to three-quarters that of cold-stratified seeds. However, seeds treated with both MV and NAC showed a significant reversal of the inhibitory respiration caused by NAC alone. Moreover, the promotion effect of stratification or ROS on the respiration rate was similar when seeds were imbibed for 48 h, though with a higher absolute amount of oxygen consumption than that at 24 h (Fig. 2f).

3.3. Cold stratification increases accumulation of ROS during seed germination

To directly link stratification with ROS generation in seeds, we characterized ROS production during seed germination. The O_2^- contents were first determined in axes excised from cold-stratified and non-cold-stratified seeds. Compared with non-cold-stratified seeds, the level of O_2^- had almost doubled in axes from cold-stratified seeds after 24 h of imbibition at 10 °C. After 48 h of imbibition, the O_2^- level in cold-treated axes increased by approximately 150% compared with that in the control, whereas it dropped to a value comparable with the control in seeds treated with 50 mM NAC (Table 1). Cold stratification was also associated with a slight but significant increase in malondialdehyde (MDA) content after 24 h of imbibition, indicating the occurrence of lipid peroxidation (Table 1).

Table 1
Superoxide (O^{2-}) and malondialdehyde (MDA) contents of axes from control, cold stratified and chemical treated seeds.

	O_2^- (A_{470nm})		MDA(μmol·g^{-1} FW)	
	24 h	48 h	24 h	48 h
Con	0.16 ± 0.01^{c}	0.21 ± 0.01^{bc}	4.68 ± 0.15^{c}	6.18 ± 0.24^{b}
Str	0.30 ± 0.02^{ab}	0.53 ± 0.04^{a}	6.48 ± 0.36^{b}	7.24 ± 0.22^{ab}
NAC(Str)	0.33 ± 0.01^{ab}	0.26 ± 0.03^{b}	8.37 ± 0.64^{a}	6.34 ± 0.22^{b}

In this table, 24 h or 48 h indicates time of imbibition. Con, non-cold stratified seeds; Str, cold stratified seeds. Values are means ± SD of three replicates. Different superscript letters indicate significant difference at the $P < 0.05$ level.

In situ accumulation of O_2^- in excised axes is shown in Fig. 3. Staining of non-cold-stratified axes by NBT was very slight except for the tip axes and was predominantly located in the outer layers of the axes. By contrast, the axes of cold-stratified seeds were homogeneously dark stained, as seen from the outside (Fig. 3a). Transverse sections through the axes confirmed the presence of formazan deposits inside the axes of stratified seeds, indicating wide-scale initiation of cellular ROS production (Fig. 3b).

ROS were also visualized in axes by fluorescence using DCFH-DA, which produces fluorescent spots within the cells at the sites of ROS formation (Fig. 4). The axes of non-cold-stratified seeds imbibed for 24 h showed a weak fluorescence signal in the outer layers of the axes, while axes imbibed for 48 h showed a slightly stronger fluorescence signal, suggesting that ROS specifically

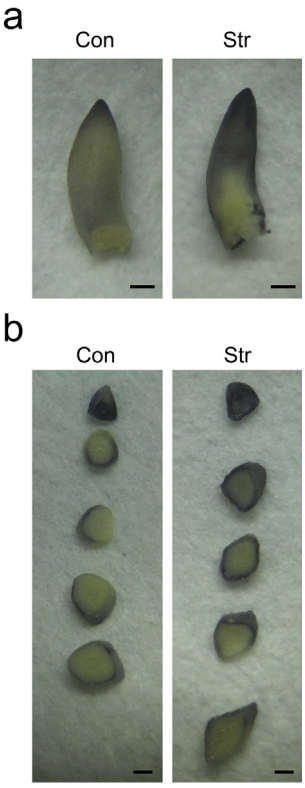

Fig. 3. Cold Stratification increases *in situ* accumulation of O_2^- during seed germination. Localization of O_2^- in (a) the whole seed embryonic axes and (b) hand-cut transections of Con and Str axes were visualized with NBT staining. Scale bars: 1 mm.

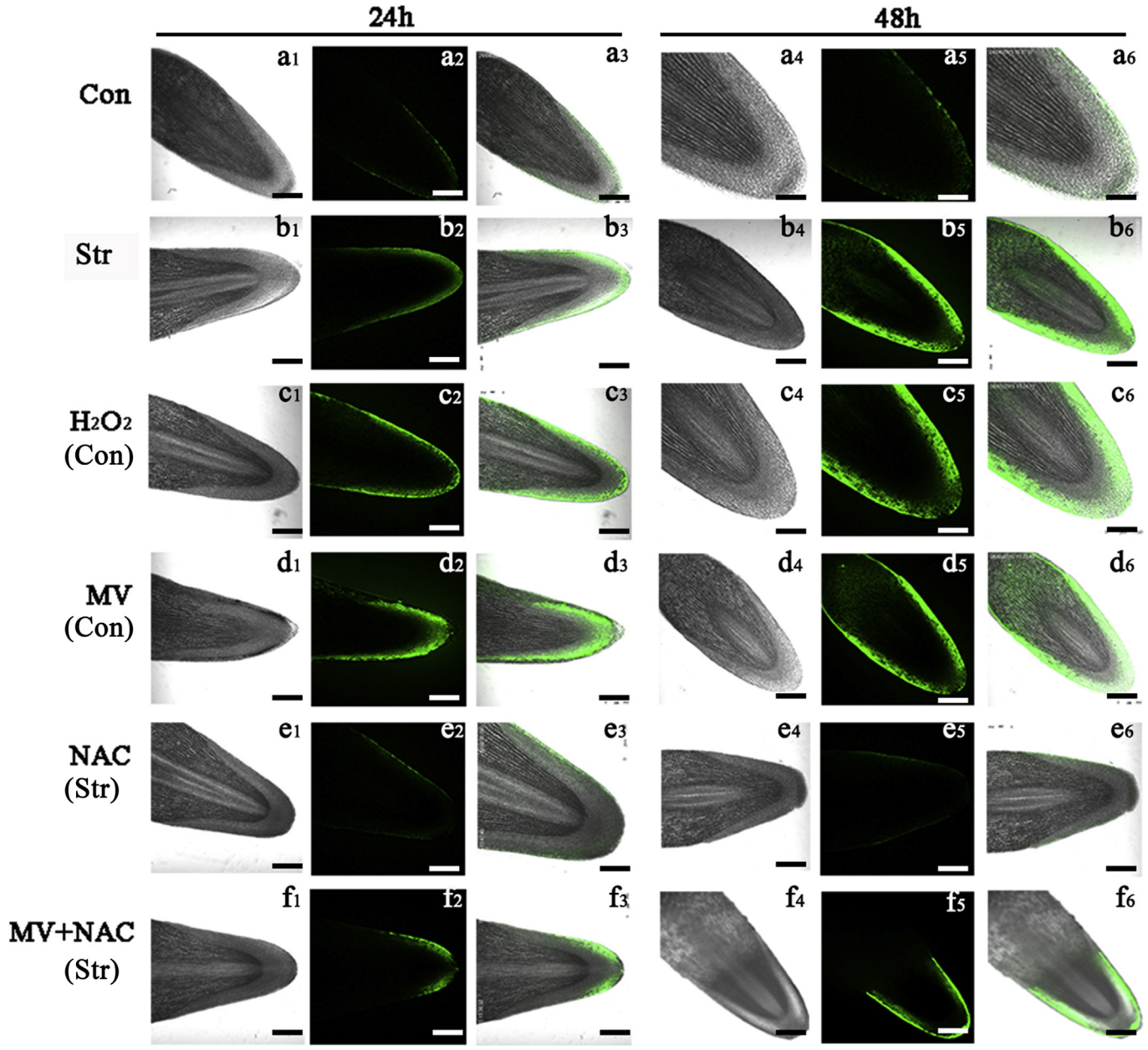

Fig. 4. Cold Stratification increases *in situ* accumulation of ROS during seed germination. Non-cold stratified (Con) and cold stratified (Str) seeds were imbibed for 24 h or 48 h. Axes were separated, longitudinally sectioned and loaded with dichlorodihydrofluorescein diacetate (DCFH-DA). *In situ* localization of ROS production was observed by confocal laser scanning microscopy (LSM). (a_1-f_1, a_4-f_4) Differential interference contrast (DIC) of DCFH-DA stained sections. (a_2-f_2, a_5-f_5) Fluorescence microscopy images of DCFH-DA stained sections. (a_3-f_3, a_6-f_6) Merged pictures of DIC and corresponding fluorescence images. Scale bars: 100 μm.

accumulate as imbibition proceeds (Fig. 4). By contrast, cold-stratified seeds imbibed for 24 h had a much more pronounced fluorescence, localized close to the radicle tip of the embryonic axes. After 48 h of imbibition, a much stronger signal appeared in the outer layers of axes and dense ROS were also deposited in the cells inside the axes. NAC treatment resulted in a marked reduction in fluorescence in cold-stratified axes, confirming the specificity of ROS induced by cold stratification. To further confirm the cold stratification-induced changes in ROS accumulation, non-cold-stratified seed axes were treated with H_2O_2 or MV. Both chemicals were associated with a marked increase in fluorescence signal, with similar intensity and location as that in stratified seeds. Our results indicated that ROS specifically accumulated as imbibition proceeded and that cold stratification could significantly increase ROS in the axes of *H. scoparium* seeds. Furthermore, ROS treatment of non-cold-stratified seeds increased the accumulation of O_2^- in axes and was thus likely to play a positive role in controlling seed germination.

3.4. Cold stratification enhances protein carbonylation during seed germination

We demonstrated that ROS specifically accumulated in axes during seed germination. To determine whether ROS production during seed germination could be associated with protein oxidation (carbonylation), one-dimensional PAGE of seed protein extracts was performed, and the presence of carbonyl groups was detected by western blotting using the DNPH immunoassay (Fig. 5).

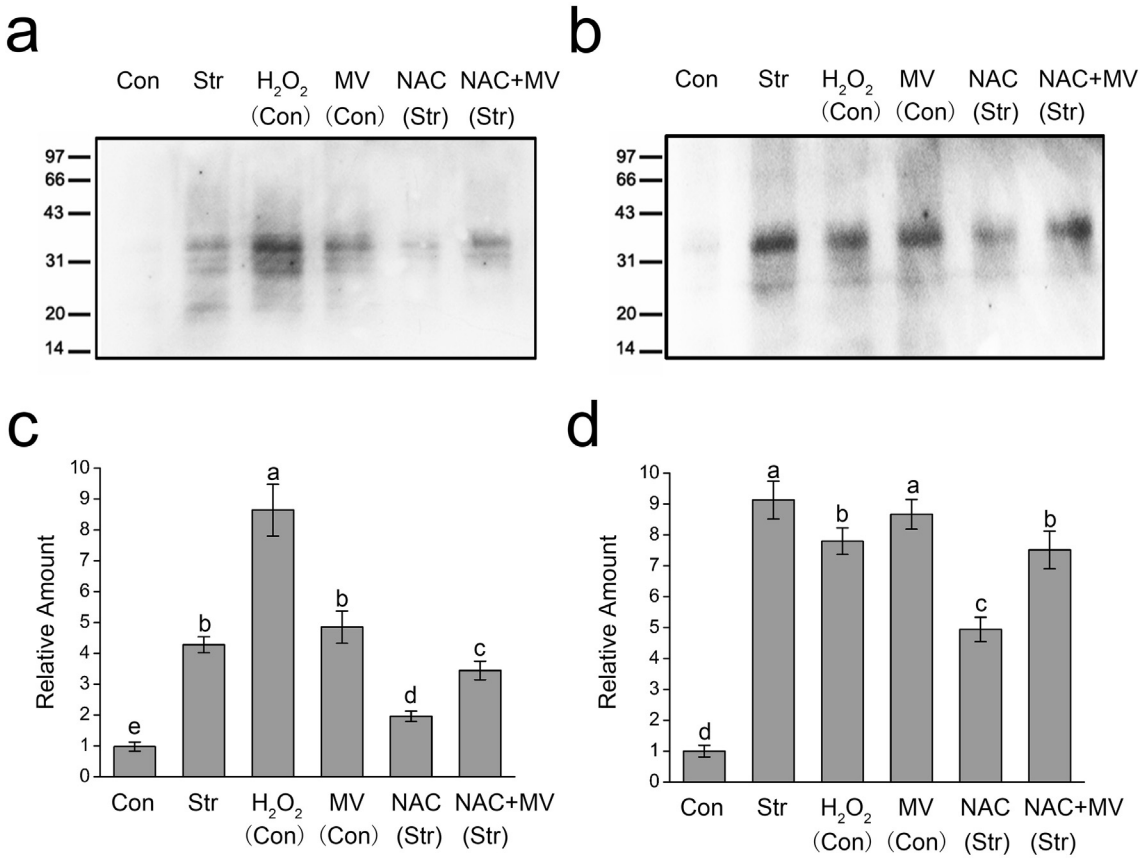

Fig. 5. Cold stratification enhances protein carbonylation during seed germination. One-dimensional PAGE of carbonylation modified proteins from axes of Con, Str or the chemical treated seeds imbibed for 24 h (a) or 48 h (b). Relative amount of each band in (a) and (b) was quantified by Image J, normalized to "Con" and presented as means ± SD in (c) and (d), respectively. Con, non-cold stratified seeds; Str, cold stratified seeds. Different superscript letters indicate significant difference at the $P < 0.05$ level.

Protein oxidation patterns from non-cold-stratified, cold-stratified and ROS-treated embryonic axes are shown in Fig. 5. The soluble proteins from non-cold-stratified axes displayed one major faint carbonylated band between 31 and 43 KD. By contrast, cold-stratified axes had a much higher signal of protein carbonylation, and a number of new carbonylated bands were detected. Moreover, the extent of protein carbonylation was much higher in soluble proteins from H_2O_2- or MV-treated, non-cold-stratified seed axes. On the other hand, ROS scavenger NAC treatment reduced the extent of protein carbonylation. However, seeds treated with MV and NAC had a relatively higher level of carbonylation than NAC-treated seeds. These results were consistent at both 24 h and 48 h of imbibition before seeds started to germinate. Given that cold stratification treatment and ROS/ROS generating compounds increased protein oxidation at the early stage of germination, we inferred that *H. scoparium* seeds probably benefited from enhanced protein carbonylation during seed germination.

3.5. ABA inhibits germination and reduces ROS content in seeds

We demonstrated that ROS specifically accumulated during seed germination and played an important role in cold stratification-mediated germination promotion in axes. To ascertain if there was a causative link between ROS metabolism and seed germination, we determined the endogenous ABA levels of imbibed seeds. Before imbibition, non-cold-stratified seeds contained 37.35 ng of ABA per gram of fresh weight (FW), while cold-stratified seeds had only half the level of ABA (17.45 ng/g FW). Then, the ABA contents decreased significantly after the onset of imbibitions in both groups. However, the ABA level in cold-stratified seeds was always lower than that in non-cold-stratified seeds, occupying only 60% and 27.6% of that in non-cold-stratified seeds at 24 h and 48 h of imbibition, respectively (Fig. 7a). To further reveal the actions of ABA on seed germination, we treated cold-stratified seeds with ABA. Seed germination was effectively suppressed by ABA in a concentration dependent manner (Fig. 6a). These results indicated that a relatively low ABA level in cold-stratified seeds is favourable for germination.

According to our results, ROS treatment of seeds promotes germination, while ABA treatment can suppress this process. We therefore investigated whether ABA worked as an antagonist of ROS by detecting the H_2O_2 content in germinating seeds. Notably, the H_2O_2 content increased during seed imbibition in cold-stratified seeds. ABA treatment significantly reduced the H_2O_2 content in both cold-stratified and non-cold-stratified seeds (Fig. 6b), suggesting that ABA can suppress ROS production during seed germination.

3.6. ROS regulate ABA and GA contents during seed germination

To study the possible effect of ROS on ABA and GA metabolism, we determined the ABA and GA contents under H_2O_2, MV and NAC treatments. Treatment of non-cold-stratified seeds with H_2O_2 or MV resulted in a decrease of 20% of the ABA content in embryonic axes after 24 h of imbibition. NAC treatment of cold-stratified seeds consistently led to a significant increase in the ABA content (Fig. 7a). After 48 h of imbibition, MV treatment significantly decreased the ABA levels (Fig. 7a), seeming to further release the inhibitive ability

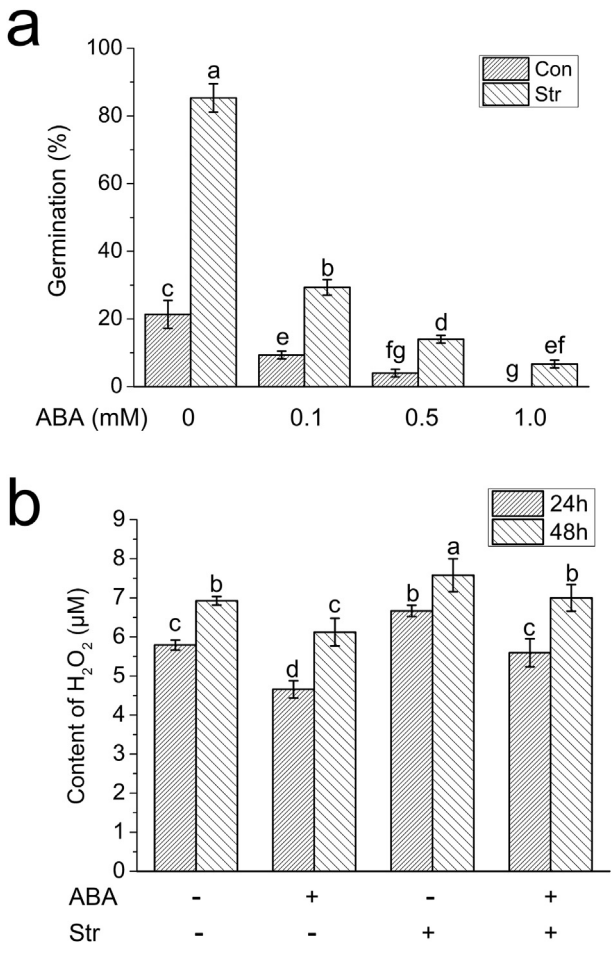

Fig. 6. ABA inhibits germination and reduces H_2O_2 content in seeds. (a) Indicated concentration of exogenous ABA inhibits germination of the Con and Str seeds. (b) Exogenous ABA (0.5 mM) reduces H_2O_2 content of axes from Con and Str seeds imbibed for 24 h or 48 h. Con, non-cold stratified seeds; Str, cold stratified seeds. Values are means ± SD of three replicates. Different superscript letters indicate significant difference at the $P < 0.05$ level.

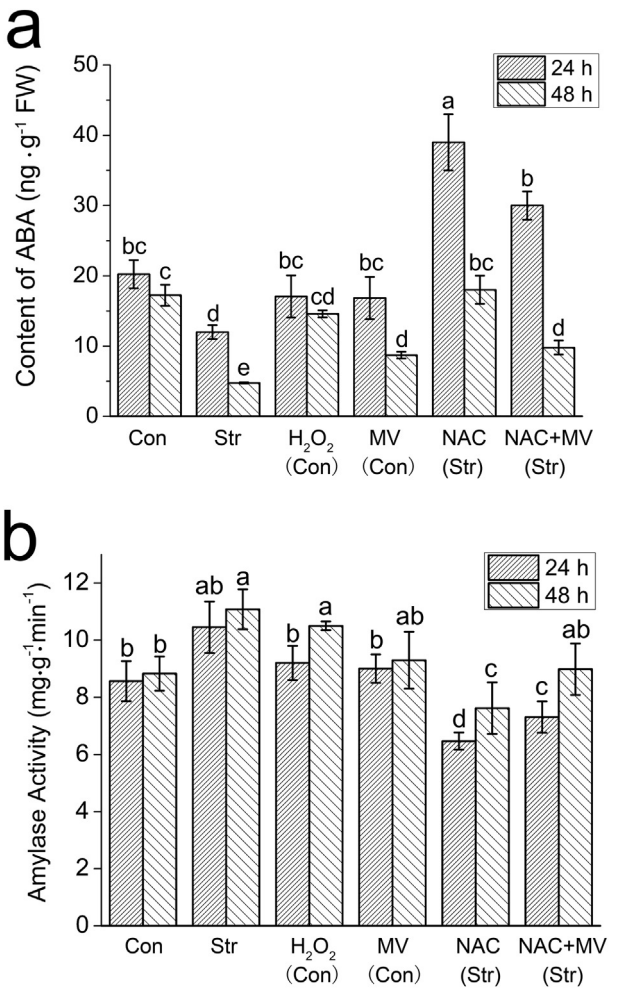

Fig. 7. The effect of ROS on ABA and GA contents during seed germination. (a) Contents of ABA in Con, Str and the chemical treated seeds imbibed for 24 h or 48 h. (b) Amylase activity, an indicator of GA accumulation, was measured in Con, Str and the chemical treated seeds imbibed for 24 h or 48 h. Con, non-cold stratified seeds; Str, cold stratified seeds; FW, fresh weight. Values are means ± SD of three replicates. Different superscript letters indicate significant difference at the $P < 0.05$ level.

of ABA on germination.

The antagonism between ABA and GA plays a key role in the release of seed dormancy. Amylase, which is indicative of GA accumulation during seed germination, is significantly induced by GA (Ye et al., 2012). We indirectly determined the GA content under different treatments. Fig. 7b shows the GA content, as indicated by amylase activity, during imbibition at 10 °C. In stratified seed axes, amylase activity showed a 22.4% increase at 24 h and a 25% increase at 48 h of imbibition compared with its activity in non-cold-stratified seeds. After 48 h of imbibition, it increased by 19.3% in cold-stratified embryos treated with H_2O_2 and slightly rose by MV treatment. NAC treatment of non-cold-stratified seeds consistently led to a significant decrease in amylase activity. On the other hand, in non-cold-stratified seeds treated with MV and NAC together, the inhibition of NAC treatment alone was released (Fig. 7b). Overall, the data presented above suggest that ROS could repress the ABA content and increase the GA content during seed germination.

4. Discussion

During seed imbibition, embryonic axes and surrounding tissues begin to hydrate and then initiate extension growth, leading to emergence of the radicle. Seed germination depends on both internal and external conditions, among which temperature, water and oxygen are the most important external factors. Cold stratification of seeds at low (0–10 °C) temperatures is frequently used to break dormancy to improve seed germination. Seeds of *H. scoparium* are often considered to have shallow dormancy. After cold stratification, germination of *H. scoparium* seeds reached 80–90% at 10 °C (Fig. 1), widening the temperature window for germination. ABA and GA act as the main regulators during seed germination, but recent studies have also implicated ROS. By applying a simple, suboptimal condition, i.e., germination at 10 °C, we were able to study the role of ROS in seed germination in *H. scoparium*.

ROS can act as a key component in cell wall loosening, testa and endosperm weakening, as well as protein oxidation in seed germination (Oracz et al., 2007; Schopfer et al., 2001; Schweikert et al., 2000). Methylviologen (MV) is a ROS-generating compound that can be reduced with or without light (less rapidly) (Slooten et al., 1995). In germinating seeds, MV probably enters in the mitochondria to produce ROS through the electron transport chain. It may gain electrons from reductants to form the cation radical $MV^{\bullet+}$ and then react with oxygen to yield superoxide ($O_2^{\bullet-}$) (Calderbank and Slade, 1976). By treating seeds with an increasing concentration

of H_2O_2 or MV, germination was first elevated in a dose-dependent manner and then decreased at a higher concentration (Fig. 2). This suggested that the ROS level is likely to play a vital role in seed germination. This result was consistent with the "oxidative window" model that regulated ROS generation, i.e., a restricted ROS level in a critical range is required for seed germination (Bailly and El-Maarouf-Bouteau, 2008). Treatment with 50 mM H_2O_2 or 1 mM MV could raise germination to higher than 80% by simply mimicking the effect of ten-day cold stratification. Moreover, germination of cold-stratified seeds dropped to only 23% with the 50 mM NAC treatment, equivalent to that of non-cold-stratified seeds. These results showed that ROS treatment could promote *H. scoparium* seed germination independent of cold stratification treatment. Thus, a causal link between ROS production and germination is likely to exist. In addition, in case of equivalent germination, the following biochemical data were somewhat comparable among seeds receiving different treatments.

Germination is clearly associated with an accumulation of superoxide anions and hydrogen peroxide in the embryonic axes (Table 1, Figs. 3 and 4). Fluorescence imaging showed that cold stratification dramatically promoted ROS production. ROS mainly concentrated in the outer layer of the embryonic axes and also deposited in cells inside the axes as dense dots. DCFH-DA staining of embryonic axes also demonstrated that ROS specifically accumulated as imbibition proceeded, which is in agreement with previous reports that ROS can act as cell messengers (Bailly, 2004). Because germination began after 48 h of imbibition at 10 °C (Fig. 1), it was inferred that enough ROS had accumulated by then to initiate germination. These molecules could therefore act as a signal to allow dormancy release and to favour subsequent seed germination.

Protein carbonylation is a widely accepted indicator for protein oxidation. In plants, it appears in several physiological stages during the life cycle (Johansson et al., 2004) and even confers benefits on seed dormancy alleviation (Oracz et al., 2007), as opposed to being a deleterious marker of ageing in animals. In our system, both accumulated ROS and lipid peroxidation product MDA (Burcham and Kuhan, 1996) were likely to introduce carbonyl groups into proteins, leading to enhanced protein carbonylation in cold-stratified *H. scoparium* seeds (Fig. 5). Among a number of carbonylated bands, the major bands appeared between 31 and 43 KD (Fig. 5), which may correspond to some oxidized storage proteins (such as oxidized 12S-globulins subunits) (Job et al., 2005). Interestingly, the major bands were very condensed after 48 h of imbibition (Fig. 5b, d). Considering that oxidized proteins are susceptible to proteolytic attack (Dunlop et al., 2002), these carbonylated storage proteins seem to prepare for mobilization during germination.

It is well known that ABA plays a significant role in seed germination inhibition. The results in barley and Arabidopsis demonstrated that the ABA content in seeds should be lowered to break dormancy (Millar et al., 2006). Additionally, during phase || of water uptake in rice, a higher concentration of ABA completely arrested germination (Ye et al., 2012). In this study, ABA was present at a relatively high level in dry seeds and decreased rapidly after the onset of imbibition. However, the ABA content is always lower in cold-stratified seeds than control seeds during imbibition (Fig. 7a), in agreement with higher germination in cold-stratified seeds. As shown in Fig. 6a, ABA effectively inhibited seed germination in both non-cold-stratified and cold-stratified seeds. These results demonstrated that stratification, or the resulting ROS production, could significantly reduce the ABA level but could not change the sensitivity of seeds to ABA in promoting germination.

Along with the increasing emergence of new regulators (such as ROS) in germination, the relationship between ROS and ABA/GA is also receiving more attention. Unlike in leaves, where ABA increased ROS production and induced oxidative stress (Jiang and Zhang, 2001), ABA was able to reduce the amount of ROS in imbibed rice seeds, especially in the embryo (Ye et al., 2012). In *H. scoparium* seeds, the H_2O_2 content was also reduced in response to ABA (Fig. 6b). In return, treatment of non-cold-stratified seeds with H_2O_2 or MV resulted in a decrease in the ABA content in embryonic axes, which was reversed by NAC treatment. These results suggested a complex counteractive modulation of the ROS and ABA levels in *H. scoparium* seeds, possibly realized by multiple mediators. We also noticed that stratification was more effective at changing the ABA/GA content than H_2O_2 or MV treatment, suggesting an additional effect of unknown factors with ROS. The above results indicated that ROS may have multiple functions; their enhanced levels are essential for dormancy breaking and seed germination in *H. scoparium*, and probably in other seeds.

In sum, our results allow us to propose a ROS regulation mechanism for germination. This mechanism involves a change in protein oxidation resulting from an accumulation of ROS during *H. scoparium* seed germination. Furthermore, we showed that ROS/ROS donor could repress the ABA content and increase GA activity during seed germination, which is consistent with the general model accounting for seed germination regulation. Overall, we suggest that ROS could induce protein oxidation and modulate the ABA/GA balance, thus serving as important regulators of seed germination in *H. scoparium*.

Author contributions

X.F.W., H.X., L.Q.S. and Q.Y.L. designed the experiments; L.Q.S., and H.X. performed the experiments, analysed the data, and wrote the initial version of the manuscript; X.F.W., Q.Y.L. and H.W.P. revised the manuscript.

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