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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