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Reactive oxygen species mediate axis-cotyledon signaling to induce reserve mobilization during germination and seedling establishment in *Vigna radiata*

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ABSTRACT

Seeds represent an excellent opportunity to investigate the role of reactive oxygen species (ROS) in control of metabolism during germination and seedling establishment. Cotyledons, the storage organs in *Vigna*, do not display growth/cell division while the embryonic axis shows rapid growth and intense metabolic activity. The present study investigates the possibility of ROS generated during respiration in the axis serving as messengers guiding storage reserve mobilization from cotyledons at the pre-greening stage. Seeds were germinated in the presence of hydroxyurea to halt cell division in the S-phase and separately in Edaravone, a potent free radical scavenger. Both treatments caused a decrease in germination percentage, seedling growth and protein mobilization. In the growing axis, both treatments resulted in a decrease in hydrogen peroxide (H_2O_2), total ROS, MDA and protein carbonyls. The picture in cotyledons was quite different, owing to the physiological dissimilarities between the tissues. The status of redox as evident by GSH/GSSG ratios tended toward oxidizing in axis in comparison to the highly reducing environment found in cotyledons. This is construed as a tendency to maintain redox buffering on the oxidizing side in the axis, to facilitate the passage of ROS message. These results strongly indicate that suppression of cell division or scavenging of ROS adversely affects protein reserve mobilization. It is proposed that apart from H_2O_2 being a transportable signal, the final message perceived in cotyledons also comprises lipid peroxidation, protein carbonylation and alteration of redox status of the glutathione pool.

1. Introduction

Seeds represent an important stage in the life cycle of a plant. Temporally as well as developmentally, seed stands at the cusp of two generations. The new generation of the plant exists as a tiny embryo, surrounded by protective as well as storage tissue. Germination and seedling establishment constitute a brief, non-green, heterotrophic phase in the otherwise autotrophic life of the plant. Cell division ceases during the final stage of seed development while cotyledons develop and storage reserves of protein, starch and/or oil are deposited (Bewley and Black, 1994). Metabolic activity declines to make the seed quiescent through induction of abscissic acid induced primary dormancy, followed by desiccation. During this period of quiescence or primary dormancy, seed survival and viability is ensured by collection of cellular adaptive mechanisms (Bailly, 2004).

Seed germination marks the end of dormancy. It is a complex process triggered by imbibition of water under appropriate conditions of temperature. Initiation of cell division in the embryo causes embryonic axis elongation, seed coat rupture and radicle protrusion (Bewley and Black, 1994). Carbohydrate reserve mobilization is the first biochemical process to begin post-imbibition (PI), providing reduced sugars for initiation of respiration. The small amounts of starch and storage proteins in the quiescent embryo that fuel growth are quickly exhausted (Bewley and Black, 1994). Further growth depends on the supply of carbohydrates and amino acids from the cotyledons. Mobilization of proteins from storage vacuoles generates amino acid monomers for *de novo* protein synthesis

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to support the growth of the embryonic axis. Reserve mobilization begins in the cotyledons during early stages of germination (Tan-Wilson and Wilson, 2012) and continues through seedling establishment. Reserves deplete by the time greening occurs and autotrophy takes over. While respiration in the cotyledons occurs at a moderate pace, the respiratory rates in the growing axis are very high due to the high rates of cell division. One of the consequences of these high respiratory rates is the increased production of reactive oxygen species (ROS). In the absence of photosynthesis, mitochondria are the main source of ROS during germination and in the pre-greening stage of seedling establishment (Bailly, 2004). Cell division and expansion in the rapidly growing axis and storage reserve mobilization from the cotyledons occur within the same period. This leads to the question whether the production of ROS and reserve mobilization have a cause and effect relationship, or are independent programs? Axis and cotyledons exhibit very different rates of growth. Barring the instances where cotyledons undergo greening and conduct photosynthesis, they serve as stores of food reserve to fuel the rapid growth in the axis (Halliwell and Gutteridge, 2006).

Reactive oxygen species generation and the resulting oxidative changes are inevitable outcomes of respiratory electron transport. High rates of electron transport are frequently associated with a proportionate generation of ROS (Verma and Sharma, 2012). By extension, the growing embryonic axis would represent a major source of ROS. Most species of ROS possess a short half-life and are scavenged either by enzymatic and/or non-enzymatic systems (Verma and Sharma, 2010). Hydrogen peroxide (H_2O_2), a non-polar molecule possessing the longest half-life among all ROS, reacts relatively slowly and can easily diffuse through biological membranes (Verma and Sharma, 2012). It is therefore, an important spinoff of mitochondrial electron transport and can diffuse a considerable distance depending on the presence or absence of scavenging systems. Externally added H_2O_2 or its enhanced apoplastic production leads to enhanced rates of storage protein mobilization in *Vigna radiata*, indicating the importance of ROS in axis cotyledons communication (Puntarulo et al., 1988). Production of hydrogen peroxide has also been demonstrated early during the imbibition period in radish (Schopfer et al., 2001), buckwheat (Dunaevsky and Belozersky, 1993), maize (Hite et al., 1999), sunflower (Bailly et al., 2000), wheat (Caliskan and Cuming, 1998), pea (Wojtyla et al., 2006) and tomato seeds (Morohashi, 2002). Thus, production of ROS during seed germination appears to be associated with high germination capacity and vigorous seedling development. A vigorous seed is therefore expected to possess an antioxidant system that balances the detrimental effect of generated ROS against the production of ROS-induced oxidative changes. Further, such changes may be indicative of respiratory rates in the axis and by implication, of the requirement of reserves mobilized from the cotyledons.

This study investigates if a cause and effect relationship exists between axis growth and reserve mobilization during germination and seedling establishment. Two approaches have been chosen. First, reducing the rate of cell division in the axis by exposure of germinating seeds to hydroxyurea, a metal binding hydroxamic acid and second, chemically quenching the ROS produced during axis growth by exposing seeds to Edaravone, a potent antioxidant and a scavenger of free radicals. *In vivo*, hydroxyurea gets converted to a free radical nitroxide that eventually inactivates ribonucleotide reductase. DNA synthesis is inhibited as a consequence and expansion of the dNTP pool that normally occurs during G_1/S phase, is prevented (Koç et al., 2004). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; M Wt 174.20) quenches hydroxyl radical (•OH) and inhibits •OH-dependent and •OH-independent lipid peroxidation (Yoshida et al., 2006). It exerts inhibitory effects on both water-soluble and lipid-soluble peroxyl radical (LOO•) induced peroxidation systems (Pérez-González and Galano, 2012) It has no significant effect on superoxide anion radicals (Yoshida et al., 2006).

2. Materials and methods

All chemicals used were of analytical grade or better and were obtained from Sigma–Aldrich unless indicated otherwise.

2.1. Plant material and growth condition

Mungbean (*V. radiata* (L.) Wilczek var. PDM-139) seeds were procured from Indian Institute of Pulses Research, Kanpur, India. Dry seeds (5 g) were washed thoroughly, imbibed in 25 mL of deionized water for 4 h and subsequently transferred to polycarbonate boxes layered with moist filter papers. The temperature was maintained at 34 °C throughout. Seeds were also imbibed in the presence of 10 mM hydroxyurea and 250 μg mL^{-1} Edaravone separately. These concentrations were arrived at after testing a range of concentrations for both hydroxyurea and Edaravone (Cayman). The seeds were exposed to 100, 150, 200, 250, 300, 350 and 400 μg mL^{-1} Edaravone. At 300 μg mL^{-1} Edaravone, germination was severely affected while at concentrations lower than 250 μg mL^{-1} there was no significant change in germination and seedling growth. Seeds did not germinate at all, at 350 and 400 μg mL^{-1} Edaravone. For hydroxyurea treatment, seeds were exposed to 5, 7.5, 10, 12.5 and 15 mM concentrations of the chemical. At concentrations lower than 10 mM, there was an insignificant change in both germination and seedling growth. Higher concentrations of hydroxyurea impaired germination severely or inhibited it completely.

2.2. Seed germination and growth parameters

Percent germination and radicle length for individual seeds in each set were measured 24 h post-imbibition (PI). Subsequently, axes and cotyledons were separated after removing seed coats. Fresh weight (FW) of the cotyledons and the axes was determined separately. The same cotyledons and axes were subsequently dried in a forced draft oven at 60 °C and weighed periodically till three successive constant dry weight (DW) readings were obtained.

2.3. Estimation of total soluble protein and SDS-PAGE analysis of cotyledon protein composition

20 pairs of cotyledons (two cotyledons per seed) from control and treated seed sets were homogenized in 5.0 mL of 50 mM Tris–HCl buffer (pH 8.0). The homogenate was filtered by squeezing through four layers of muslin and centrifuged at 10,000 × *g* for 10 min. Bradford's dye binding assay (Bradford, 1976) was used to determine the total soluble protein content of the extract. Protein reserve mobilization in these extracts was analyzed by SDS-PAGE on a 12% polyacrylamide gel (King and Laemmli, 1971). 15 μL extract was loaded in each lane and the protein bands were visualized after electrophoresis by staining with Coomassie Brilliant Blue R-250.

2.4. Preparation of extracts for enzyme assays

2 g cotyledons and embryonic axes from each set of seeds were separately homogenized in ice-cold deionized water adjusted to pH 7.5 with 10 mM Na_2CO_3. The grinding mixture also contained 0.1 mM ethylene diamine tetra acetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone (PVP) and 0.5% (v/v) Triton X-100. Homogenization was carried out on ice, using a pre-chilled mortar and pestle to prepare 5 mL homogenate. This was filtered through four layers of muslin and centrifuged at 22,000 × *g* for 20 min at 4 °C. This extract was used for all estimations except for protein carbonyls