30 A Role for Reactive Oxygen Species in Endosperm Weakening

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Abstract

In many seeds, including the established model species *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.) and the emerging *Brassicaceae* model system garden cress (*Lepidium sativum* L.), the process of germination features testa rupture and endosperm rupture as two separate events. Endosperm rupture requires cell wall weakening in the endosperm layer. Puncture force measurements are a useful tool for quantifying this endosperm weakening. We have established such measurements for *L. sativum* and have shown that endosperm weakening takes place prior to endosperm rupture. Various mechanisms have been proposed to promote endosperm weakening. A novel mechanism proposed by us involves the cleavage of cell wall polymers in the endosperm by reactive oxygen species, or more specifically, by apoplastic hydroxyl radicals (·OH) formed when superoxide (O_2^-·) and hydrogen peroxide (H_2O_2) undergo a Fenton reaction in the presence of peroxidases.

Introduction

Seed germination and endosperm weakening

The process of seed germination ends when the radicle has protruded through all the seed-covering layers. In many seeds, including economically important species, the endosperm is a germination-limiting tissue barrier. For an endospermic seed to complete germination, the growth potential of the radicle must be high enough to overcome the tissue resistance of the endosperm (Bewley, 1997a). Factors that influence germination, e.g. plant hormones, can thus influence the resistance of the endosperm tissue by promoting or inhibiting endosperm weakening.

In larger seeds, changes in the solidity of the endosperm tissue can be directly quantified by puncture force measurements. This method has proved to be useful

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in model species like lettuce (*Lactuca sativa* L.) (Abeles, 1986; Sung, 1998) and tomato (*Lycopersicon esculentum* Mill.) (Groot and Karssen, 1987; Chen and Bradford, 2000; Toorop *et al.*, 2000), and has recently been used to characterize changes in the seed coat of coffee (*Coffea arabica* L.) (Da Silva *et al.*, 2004, 2005). In all these species, a decline in the endosperm puncture force precedes endosperm rupture.

Endosperm weakening requires cell wall weakening. This process involves cleavage of cell wall polymers or loosening of bonds between the polymers. Several molecular mechanisms have been proposed for endosperm weakening. Most prominent among them is the digestion of cell wall polysaccharides by hydrolytic enzymes. The main focus so far has been on β-1,4-mannanase (Bewley, 1997b) and β-1,3-glucanase (Leubner-Metzger, 2003). Chen and Bradford (2000) further proposed that expansins also play a role in the endosperm weakening process. It can be safely assumed that endosperm weakening is not caused by one single mechanism, but by interaction of a variety of mechanisms (see Leubner-Metzger *et al.*, Chapter 20, this volume).

Action of reactive oxygen species on cell walls

In addition to cell wall modifying proteins, the action of reactive oxygen species (ROS), such as H_2O_2, O_2^- and ·OH, on cell wall polymers has also been proposed to cause cell wall loosening during fruit softening (Brummell *et al.*, 2004) and elongation growth (Schopfer, 2001). The amount of hemicelluloses, mostly xyloglucan, in cell walls is strongly reduced during auxin-induced elongation growth (Bret-Harte and Talbott, 1993). Schopfer (2001) has postulated that this reduction in hemicelluloses and the corresponding cell wall loosening are caused by ·OH. These radicals are highly reactive and therefore extremely short-lived. As their range of action is limited by diffusion, they must be produced directly in the cell wall in order to cleave cell wall polymers.

The following mechanism has been proposed for the formation of ·OH (Fig. 30.1): NADH oxidases located in the plasma membrane catalyse the formation of apoplastic O_2^-· anions. O_2^-· is dismutated by the antioxidant enzyme superoxide dismutase (SOD), leading to the formation of H_2O_2 and molecular oxygen. Thus O_2^-· and H_2O_2 are both present in the apoplast. In the presence of bivalent cations (e.g. Fe^{2+} or Cu^{2+}), ·OH can be formed from H_2O_2 in the so-called Fenton reaction, and regeneration of these catalytic bivalent cations can be achieved by the oxidation of O_2^-· (Vianello and Macri, 1991). The generation of ·OH in the cell wall by a Fenton-type reaction can also take place in the presence of peroxidases, which are abundant in the plant cell wall (Chen and Schopfer, 1999), or in the presence of ascorbate and bivalent cations (Fry, 1998). ·OH are able to cleave hemicelluloses and have been shown to cause *in vitro* cleavage of cell wall polysaccharides (Fry, 1998; Schweikert *et al.*, 2002). ROS have been shown to play a major role in the elongation growth of maize (*Zea mays* L.) roots, maize coleoptiles and sunflower (*Helianthus annuus* L.) hypocotyls (Liskay *et al.*, 2004). We propose that this mechanism is also involved in endosperm cell wall loosening during endosperm weakening and radicle elongation.

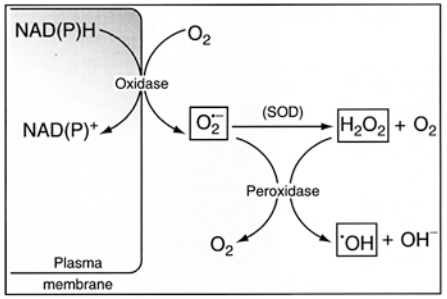

Fig. 30.1. Qualitative model for the generation of apoplastic hydroxyl radicals. NADH oxidases located in the plasma membrane catalyse the formation of apoplastic O_2^-· anions. O_2^-· is dismutated to H_2O_2 and molecular oxygen by superoxide dismutase (SOD). A Fenton-type reaction can take place in the presence of peroxidases, leading to the formation of ·OH. (From Schopfer *et al.*, 2001.)

Materials and Methods

Seeds and germination assays

Lepidium sativum L. 'Gartenkresse einfache' seeds (Juliwa, Heidelberg, Germany) were incubated in Petri dishes containing 6 ml 1/10 Murashige-Skoog medium without hormones or vitamins (Duchefa, Haarlem, The Netherlands) and two layers of filter paper. The Petri dishes were sealed with Parafilm, placed in a Sanyo Versatile Environmental Test Chamber MLR-350 (Sanyo, Loughborough, UK) and incubated at 18°C in continuous white light (8.35 µmol/m²·s). Germination was scored under a binocular microscope. Testa rupture was defined as a stage in which the whitish endosperm was visible through the cracked reddish-brown testa, and endosperm rupture as a stage in which the endosperm cap enveloping the radicle was no longer intact. For each data point, at least three replicates of 50 seeds were used in at least two independent experiments. If indicated, 10 µM (+/−*cis*–*trans*) abscisic acid (ABA; Sigma, Taufkirchen, Germany) and 10 mM H_2O_2 were added to the germination medium.

Puncture force measurements

Puncture force was measured using a custom-made machine (Fig. 30.2a and b). *L. sativum* seeds were cut in half, the embryo and remnants of testa stuck to the endosperm cap were carefully removed and the empty but intact endosperm cap placed in a seed-shaped mould. A metal probe (0.3 mm diameter) was slowly driven into the endosperm cap (2 mm/min) and the force it took to rupture the tissue was measured and registered as a peak on an attached recorder (Fig. 30.2c). These peaks were measured and the corresponding force was calculated. A calibration was performed using defined volumes of water. The method produces a certain background caused by friction between the metal probe and the endosperm. This background can be calculated by using the small peaks that result when endosperm tissues from already ruptured seeds are punctured. This background was subtracted from the individual values. At least 40 seeds from at least two independent experiments were used for each data point.

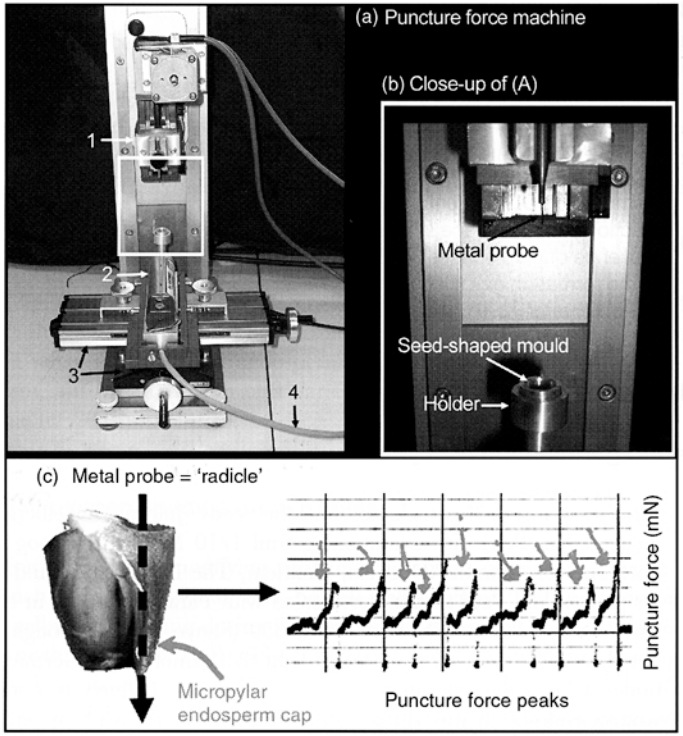

Fig. 30.2. Puncture force measurements. (a) and (b) The custom-made machine that was used to measure puncture force. Its main elements are labelled in the pictures (1 = slide for lowering the metal probe, 2 = strain gauge attached to beam, 3 = centering screws, 4 = connection to recorder). (c) A dissected *Lepidium* seed and a graph showing puncture force peaks. For the measurements, *Lepidium* seeds were cut in half and the embryo and remnants of testa stuck to the endosperm cap were removed, leaving the empty but intact endosperm cap into which the metal probe could be lowered.

Results and Discussion

Puncture force measurements – a useful method requiring a suitable seed system

The choice of the seed system for puncture force experiments strongly influences the experimental options offered by the method. It is essential that the seeds are large enough to be fixed properly and also leave enough space for the metal probe to be lowered on to the tissue with no or very little friction. This shows that the tiny seeds of the model plant *Arabidopsis thaliana* (L.) Heynh. are not suitable for puncture force measurements (see Leubner-Metzger *et al.*, Chapter 20, this volume).

In an optimal seed system for puncture force experiments, it should be possible to puncture the seed coat layers separately in order to assign the weakening to a

Table 30.1. Changes in puncture force during the germination of *Lepidium* seeds. Seeds were incubated at 18°C in continuous white light.

Treatment	Time (h)	Testa rupture (%)	Endosperm rupture (%)	Puncture force (mN)
(a) Control	8	86 ± 4	2 ± 1	38 ± 3[a]
(b) Control	18	99 ± 1	59 ± 1	19 ± 2[a]
(c) 10 µM ABA	18	99 ± 1	0	38 ± 2[a]
(d) 10 µM ABA	48	99 ± 1	0	45 ± 3[b]
(e) 10 Mm ABA + 10 mM H_2O_2	48	99 ± 1	41 ± 1	30 ± 2[b]

(a) and (b) control – puncture force declines over time before endosperm rupture takes place. (c) and (d) 10 µM ABA – ABA delays endosperm rupture and the decline in puncture force. (e) 10 µM ABA plus 10 mM H_2O_2 – the addition of H_2O_2 partially reverses the ABA-induced delay in endosperm rupture and weakening. Mean values ± standard error (SE) are presented.
[a]First seed batch.
[b]Second seed batch.

specific seed coat layer. When more than one covering layer or even the whole seed including the embryo is punctured, as has been done with lettuce (Abeles, 1986), it is hard to tell which layer of the seed is responsible for the observed changes in the puncture force.

Another relevant technical issue is the sensitivity of the measuring system in relation to the actual values measured. While most published results have been produced using commercially available texture-analysing machines, our custom-made machine proved sensitive enough to cope with the approximately three cell layers of the endosperm of *Lepidium* seeds. Our results range from ~40 mN to 20 mN (Table 30.1), while puncture forces measured for harder seed coats, like those for coffee or tomato, range from ~1400 mN to 600 mN (Da Silva *et al.*, 2004; Da Silva *et al.*, 2005) or from ~600 mN to 300 mN (Toorop *et al.*, 2000), respectively. In addition to differences in the sensitivity of the machines used, the size and shape of the metal probe has a significant impact on the absolute values measured. It may therefore be impossible to compare absolute puncture force values from different publications.

Another factor that might influence the choice of a suitable model system for endosperm weakening is the availability of molecular data and applicability of molecular and biochemical methods. Puncture force experiments alone, while delivering valuable results and giving clues to the mechanisms responsible for the observed processes, cannot be used to understand how these processes work. For most seeds on which puncture force measurements have been performed, especially for lettuce and tomato, molecular data and a large range of methods are available.

Lepidium sativum endosperm weakening occurs prior to endosperm rupture

While hardly any molecular data exists for *Lepidium*, it is closely related to *Arabidopsis*, and this offers the possibility of using the extensive molecular data available for the latter. With *Lepidium* seeds, a complete study of endosperm weakening might

be possible on different experimental levels, from puncture force measurements to transcriptome analysis using *Arabidopsis* microarray chips. While this has to be proven in our future experiments, it is already clear on the physiological level that *Lepidium* has distinct advantages over other established model systems (see Leubner-Metzger *et al.* Chapter 20, this volume): (i) *Lepidium* exhibits a two-step germination process with separate testa and endosperm rupture, which make it possible, at least after testa rupture, to manipulate the endosperm directly (Fig. 30.3); and (ii) ABA inhibits endosperm rupture, but does not inhibit testa rupture.

We measured the puncture force required at different stages during *Lepidium* seed germination (Table 30.1): (i) control after 8 h when most seeds have undergone testa rupture, but almost no endosperm rupture; (ii) control after 18 h when the majority of seeds have already progressed to endosperm rupture and the remaining seeds will do so within the next 2–4 h; (iii) ABA after 18 h; (iv) ABA after 48 h (the addition of 10 µM ABA delays endosperm rupture, thus none of the seeds has undergone endosperm rupture at 48 h); and (v) after 48 h in 10 µM ABA plus 10 mM H_2O_2, which partially reverts this delay in endosperm rupture.

Endosperm weakening is evident in *Lepidium* seeds prior to endosperm rupture (Table 30.1). ABA delays the onset of both endosperm weakening and endosperm rupture. Table 30.1 shows that the ABA-mediated delay of both endosperm weakening and endosperm rupture can be partially reverted by the addition of H_2O_2. Although 10 mM H_2O_2 did not cause visible oxidative stress responses, concentrations more than 50 mM H_2O_2 provoked visible oxidative stress symptoms in *Lepidium* seeds (i.e. a negative effect on germination and a bluish colour in the emerging radicles due to the presence of stress-induced anthocyanins).

There are no publications investigating the effect of ROS on endosperm weakening, although ROS have been postulated as a molecular mechanism for endosperm weakening (Leubner-Metzger, 2003; Bailly, 2004). Our results suggest that the H_2O_2-induced reversion of the ABA-mediated delay in endosperm rupture is, at least in part, caused by the promotion of endosperm weakening. This evidence supports our hypothesis that ROS are a novel molecular mechanism for endosperm weakening during seed germination.

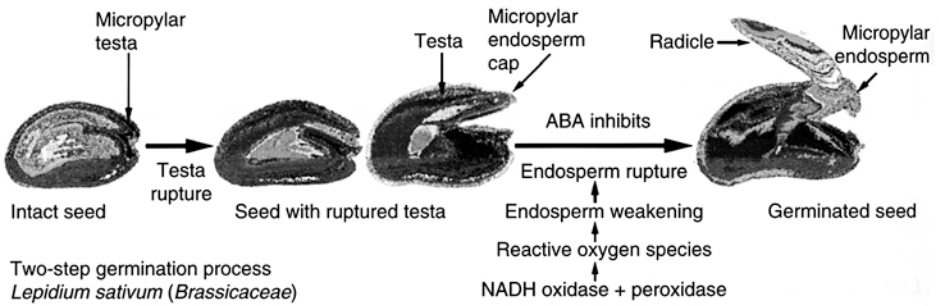

Fig. 30.3. *Lepidium* seed germination is a two-step process (i.e. testa rupture precedes endosperm rupture). ABA delays endosperm rupture, but not testa rupture. It is proposed that reactive oxygen species (ROS) produced in the cell wall play a role in endosperm weakening, which takes place before endosperm rupture.

Reactive oxygen species in seed germination

It is known that H_2O_2 promotes germination in various species. H_2O_2 can be used in high concentrations to promote germination of seeds with hard seed coats by scarification, it also has a germination-promoting effect in lower concentrations. Germination studies on *Zinnia elegans* Jacq. seed showed a dose-dependent promotion of germination by H_2O_2 (Ogawa and Iwabuchi, 2001). Inhibition of catalase in lettuce seeds led to higher concentrations of H_2O_2 in the seeds and to faster germination (Hendricks and Taylorson, 1975).

Our interpretation of this promotion is based on the model presented in Fig. 30.1: H_2O_2 reacts withE $O_2^{-}\cdot$ in the presence of cell wall peroxidases, leading to the formation of ·OH (Chen and Schopfer, 1999). These ·OH then act on the cell wall by causing polysaccharide cleavage resulting in endosperm weakening. This model and our hypothesis for endosperm weakening could also explain why tobacco (*Nicotiana tabacum* L.) seeds overexpressing a cell wall peroxidase germinate faster in the presence of osmotica than the corresponding wild type (Amaya *et al.*, 1999). Peroxidase activity increases in the micropylar endosperm of tomato seeds prior to endosperm rupture (i.e. during endosperm weakening) (Morohashi, 2002).

In addition to its effects on seeds, H_2O_2 promotes elongation growth requiring cell wall loosening in other plant parts. Overexpression of horseradish-peroxidase under the control of the CaMV-35S-promotor leads to faster elongation growth of zucchini (*Cucurbita pepo* L.) hypocotyls (Dunand *et al.*, 2003). In the roots of onion (*Allium cepa* L.), the highest peroxidase activity is found in elongating tissues (Cordoba-Pedregosa *et al.*, 2003). These tissues also show the highest concentrations of H_2O_2. However, H_2O_2 can also have an adverse effect (i.e. growth reduction). For example, addition of ABA to the growth medium leads to a higher activity of peroxidases, a higher concentration of H_2O_2 and reduced growth in the roots of rice (*Oryza sativa* L.) seedlings (Lin and Kao, 2001), and ROS production is essential for lignification and cross-linking of cell wall polymers in vascular tissue (Ogawa *et al.*, 1997).

We propose that cleavage of cell wall polymers by ·OH not only takes place in the endosperm, but also plays a role in radicle elongation. Here, cell walls have to be loosened in order to allow cell elongation, caused by water uptake, which takes place when the water potential in the embryo is lower than that of the surrounding medium.

When working with ROS, it is important to differentiate between ROS signalling effects (Laloi *et al.*, 2004) and direct action, such as the cleavage of cell wall polymers. The only way to prove the latter would be to experimentally show both the presence of ROS and the products of reactions caused by the ROS.

Acknowledgements

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