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Title
In vivo cell wall loosening by hydroxyl radicals during cress (Lepidium sativum L.) seed germination and elongation growth

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**Abstract**

Loosening of cell walls is an important developmental process in key stages of the plant life cycle, including seed germination, elongation growth and fruit ripening. Here we report direct *in vivo* evidence for hydroxyl radical (•OH)-mediated cell wall loosening during plant seed germination and seedling growth. We used electron paramagnetic resonance (EPR)-spectroscopy to show that •OH is generated in the cell wall during radicle elongation and weakening of the endosperm of cress (*Lepidium sativum* L., Brassicaceae) seeds. Endosperm weakening precedes radicle emergence, as demonstrated by direct biomechanical measurements. By ³H-fingerprinting we showed that wall polysaccharides are oxidised *in vivo* by the developmentally regulated action of apoplastic •OH in radicles and endosperm caps: the production and action of •OH increased during endosperm weakening and radicle elongation and were inhibited by the germination-inhibiting hormone abscisic acid. Both effects were reversed by gibberellin. Distinct and tissue-specific target sites of •OH attack on polysaccharides were evident. *In vivo* •OH attack on cell wall polysaccharides were not only evident in germinating seeds, but also in elongating maize (*Zea mays* L., Poaceae) seedling coleoptiles. We conclude that plant cell wall loosening by •OH is a controlled action of this type of reactive oxygen species.
**Introduction**

The plant cell protoplast is surrounded by the cell wall, a highly complex composite permeated by water and composed mainly of cellulose microfibrils embedded in a matrix of hemicellulosic and pectic polysaccharides, also containing proteins and phenolic compounds (Fry, 2000; Cosgrove, 2005; Knox, 2008). Inorganic ions and enzymes secreted into the plant cell walls, collectively called the apoplast, can be bound to specific wall components and contribute to the dynamic nature of this compartment. Plant cell growth is driven by water uptake and restricted by the cell wall: The structural properties and mechanical strength of the plant cell wall determine the shape and the rate and direction of growth of individual cells as well as the mechanical resistance of whole tissues (Cosgrove, 2005; Schopfer, 2006). Cell wall loosening is therefore an important process in all stages of plant development requiring elongation growth or tissue weakening. These include pollen tube elongation (Eckardt, 2005), root hair development (Foreman et al., 2003; Monshausen et al., 2007), fruit ripening (Brummell and Harpster, 2001; Fry et al., 2001), seedling elongation and seed germination (Finch-Savage and Leubner-Metzger, 2006; Müller et al., 2006), which is the focus of the present study.

In the mature seeds of most angiosperms the embryo is covered by two envelopes: the living endosperm and the dead testa. In order for seeds to complete germination successfully – germination being defined as the events between seed imbibition and radicle emergence – cell wall loosening is required for radicle elongation-growth driven by water-uptake and for weakening of the covering envelopes (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Nonogaki, 2006). A developmental switch from seed germination to seedling growth takes place after radicle emergence (Lopez-Molina et al., 2001). As these two different stages of plant growth are based on different developmental programmes, it is not known whether initial radicle elongation within the seed is driven by the same mechanisms as seedling elongation growth after radicle emergence.

Cell wall loosening requires structural changes in the wall, as load-bearing bonds must be broken. Known wall-modifying mechanisms in plants include enzymatic hydrolysis, transglycosylation and expansin action (Cosgrove, 2005). In seeds in particular, enzymatic hydrolysis of endosperm cell walls by endoglycanases such as β-1,3-glucanase
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(Leubner-Metzger, 2002) and β-1,4-mannanase (Nonogaki et al., 2000; Toorop et al., 2000; da Silva et al., 2004) has been shown to play a role during seed germination (for a detailed discussion and references see: Finch-Savage and Leubner-Metzger, 2006; Bewley, 1997). Expansins and xyloglucan endotransglucosylase/hydrolases are expressed in the endosperm cap of tomato seeds during germination (Chen et al., 2002), where they can contribute to endosperm weakening.

Hydroxyl radicals have been proposed as an additional plant cell wall loosening agent (Schopfer, 2001). These extremely reactive molecules can, if produced directly in the apoplast, attack cell wall polysaccharides and lead to breakage of load-bearing structures. While this process has been hypothesized to play a role in a variety of contexts, e.g. seed germination (Bailly, 2004) and seedling growth (Schopfer, 2001) it has so far only been shown directly in ripening pear fruits (Fry et al., 2001). Schopfer (2001) showed that extension can be induced in dead coleoptiles by exposing them to •OH, and that exposure to •OH accelerates growth of living seedlings. However, cell wall oxidation was not investigated in seedlings.

We investigated in vivo •OH production and oxidation of cell wall polysaccharides in defined tissues of germinating cress (Lepidium sativum L., Brassicaceae) seeds and maize (Zea mays L., Poaceae) seedlings. Germination and seedling elongation represent distinct key developmental processes that require wall loosening for elongation growth or tissue weakening. Production of reactive oxygen species (ROS), including •OH and superoxide (O2•−), has been reported in seeds and seedlings of various plant species during development (Bailly, 2004; Oracz et al., 2009) and the alleviation of dormancy (Oracz et al., 2007), but their role is not yet understood. Their known mode of action could be either indirect (cellular signaling, Oracz et al., 2009) or direct (e.g. scission of polymers), but the latter was often regarded as a 'negative role', causing toxicity and deterioration (Bailly, 2004; Winterbourn, 2008). Here we report direct in vivo evidence for a ‘positive’ developmental role and a novel direct action of apoplastic ROS during seed germination and seedling growth. Our approach is, to our knowledge, the first to combine direct biochemical and biophysical detection of ROS with an investigation of their in vivo action on the cell wall and alterations to biomechanical tissue properties.
Results and Discussion

Tissue Weakening during Seed Germination: Hydrogen Peroxide (H$_2$O$_2$) Inhibits and Hydroxyl Radical (•OH) Generation Promotes Weakening of the Endosperm Envelope

Seed germination of *Lepidium sativum* (garden cress, ‘cress’, Brassicaceae) comprises two sequential steps, testa and endosperm rupture, as does germination of the model plant *Arabidopsis thaliana* (‘Arabidopsis’, Brassicaceae) (Müller et al., 2006). Arabidopsis is a close relative of cress, and both species share a highly similar seed anatomy and germination physiology. As cress seeds are much larger than the tiny seeds of Arabidopsis, they are better suited to biochemical and biomechanical approaches at tissue- or organ-level.

Typically, cress embryos emerge from their covering layers by the elongating radicle penetrating the weakened endosperm cap, which covers the radicle after the testa has ruptured (Fig. 1A and B). Weakening of the cap, which consists of one to two cell layers, is required for typical germination and is inhibited by the germination-inhibiting hormone abscisic acid (ABA, Müller et al., 2006). Indirect evidence supports the view that cap weakening also occurs in Arabidopsis seeds and is regulated by hormones in the same manner (Finch-Savage and Leubner-Metzger, 2006; Bethke et al., 2007).

H$_2$O$_2$ treatment is known to stimulate germination of dormant seeds by releasing dormancy and by degradation of endogenous inhibitors such as ABA (Bailly, 2004). Our cress seed batch exhibits only a very shallow dormancy when fresh, and none in the after-ripened state which the seeds used in this study were in. For these non-dormant cress seeds, the addition of 10 mM H$_2$O$_2$ to the medium did not change the germination kinetics, but led to atypical germination in ca. 10 % of the seeds (Fig. 1D): The endosperm cap was torn off at its base instead of being penetrated by the radicle. Measurements of the tissue resistance of caps exposed to 10 mM H$_2$O$_2$ showed that cap weakening is inhibited (Fig. 1E). This might be caused by cell-wall tightening reactions which H$_2$O$_2$ is known to cause by cross-linking extraprotoplasmatic polymers in plants (Brisson et al., 1994; Schopfer, 1996; Encina and Fry, 2005) and animals (Wong et al., 2004). We cannot rule out cytotoxic effects of a 10-mM H$_2$O$_2$ treatment though we
observed that the seeds developed into normal-looking and healthy seedlings. It seems likely that the radicle, whose elongation was not influenced by 10 mM H\(_2\)O\(_2\) (data not shown), elongates as usual, while cap weakening fails to keep up, causing the atypical germination described above. This effect shows that the cap can act as a restraint to radicle elongation despite its thinness. These conclusions are in agreement with work on the thin lettuce endosperm for which chemical inhibition of weakening increases the percentage of seeds that exhibit either embryo expansion without protrusion (embryo buckling within the endosperm envelope) or atypical endosperm rupture (Pavlista and Haber, 1970).

While the addition of H\(_2\)O\(_2\) alone to the medium thus inhibited cap weakening, the generation of •OH in the cap cell walls via a Fenton reaction (Fe\(^{2+}\) + H\(_2\)O\(_2\) \rightarrow Fe^{3+} + \text{OH}^- + •OH) strongly accelerated it (Fig. 1E). We quantified this effect directly by puncture force measurements in which the tissue resistance of cress endosperm caps preincubated in ABA and then exposed to apoplastic •OH was determined. During typical germination, the force it took to rupture the endosperm tissue declined prior to endosperm rupture and radicle emergence from ca. 38 mN to ca. 20 mN (18 h CON). This cap weakening was inhibited by ABA (18 h ABA) and by H\(_2\)O\(_2\) (18 h H\(_2\)O\(_2\)). Incubation in ABA for 17 h followed by only 1 h exposure to apoplastic •OH led to a decline in tissue resistance: the puncture force was ca. 17 mN (Fig. 1E).

In caps that were incubated separately from radicles after dissection of the seeds, this decline was followed by local tissue dissolution and the formation of a hole at the tip of the cap where radicle emergence would usually occur (Fig. 1C). This developmentally regulated hole formation was inhibited by H\(_2\)O\(_2\) as well as by ABA in agreement with these substances’ influence on tissue resistance: after one day, four out of five caps incubated without H\(_2\)O\(_2\) and ABA had a hole, while none of the caps incubated in the presence of 10 mM H\(_2\)O\(_2\) or 10 µM ABA did. Taken together these results suggest a 'positive role' for •OH in cell wall loosening during cress seed germination, i.e. in the developmentally and hormonally controlled processes of hole formation and cap weakening required for seed germination.
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•OH and Superoxide (O₂⁻) are Produced In Vivo in the Apoplast during Cress Seed Germination

In order to have a cell wall loosening effect in vivo, •OH must be produced in the direct vicinity of wall polysaccharides: the radicals’ mobility range is extremely limited owing to their high reactivity and short lifespan (Fry et al., 2001; Schopfer, 2001). We used a spin trap which reacts with •OH forming a stable adduct to detect in vivo •OH production by electron paramagnetic resonance (EPR)-spectroscopy. This method is specific for apoplastic •OH, as Heyno et al. (2008) showed when they used the technique to detect the inhibitory influence of cadmium on •OH produced apoplastically at the plasma membrane independently of its stimulatory effect on intracellular •OH produced in mitochondria. It has been successfully used to detect •OH in Arabidopsis and cucumber seedling roots: In cucumber seedlings, but not in the small Arabidopsis seedlings, even localization to the growing zone was possible (Renew et al., 2005). We investigated in vivo apoplastic •OH production in cress seeds (Fig. 2), whose size made it possible to work with separate seed parts (Müller et al., 2006).

In vivo apoplastic •OH production in cress endosperm caps and radicles increased strongly between 8 h and 18 h (Fig. 2A). The 8 h time point is characterized by a still unweakened endosperm and non-elongating radicle (8 h CON), while at 18 h cap weakening has progressed and tissue resistance halved, and radicle elongation starts (18 h CON). ABA inhibited these physiological processes between 8 h and 18 h and inhibited •OH production in both tissues as well.

A tissue-specific ABA effect could be observed as the seeds progress to germination in the presence of ABA: Caps showed an increase of •OH production toward 72 h, while radicles showed an •OH production equal to that at 18 h ABA (Fig. 2A). Possible interpretations of this phenomenon are i) that cell wall loosening and thereby radicle elongation mechanisms differ in the presence and absence of ABA, and ii) that the increase in •OH production takes place during a very narrow time window, as it leads to immediate cell wall loosening and radicle growth driven by water uptake (Müller et al., 2006).

The reversion of the inhibitory ABA-effect on germination and endosperm weakening by its antagonist gibberellin (GA) (Müller et al., 2006) could also be observed at the level of
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*In vivo* apoplastic •OH production in radicles and endosperm caps (Fig. 2B). While we observe an increase in variance between the samples, which is possibly due to the fact that hormone interactions tend to vary strongly within a population, the overall effect was an obvious increase of •OH-production on reversion of the ABA-inhibition of germination with GA. These observations support our hypothesis that hormone-sensitive •OH-mediated effects in the cell wall contribute to endosperm weakening and radicle elongation.

We conclude that the increase in apoplastic •OH production might be a mechanism for endosperm cap weakening and radicle elongation during germination; and that the ABA-mediated inhibition of these processes might at least in part be caused by the ABA-inhibition of the apoplastic •OH production, which can be reversed by GA. ABA and GA are known for their antagonistic effects on the expression of cell-wall hydrolases in the endosperm cap during weakening just prior to endosperm rupture (Finch-Savage and Leubner-Metzger, 2006). Examples include β-1,3-glucanase in tobacco (Leubner-Metzger, 2002) and β-1,4-mannanase in tomato (Nonogaki et al., 2000; Toorop et al., 2000; da Silva et al., 2004) and coffee, where in addition an inhibitory effect of ABA on embryo growth potential has been demonstrated (da Silva et al., 2004).

Two hypotheses, which are not mutually exclusive, have been put forward to explain the source of •OH production in the cell wall: natural Fenton reactions dependent on a reductant (e.g. ascorbate), transition metal ions (e.g. copper) and a source of H₂O₂ (e.g. O₂ or O₂⁻) in the cell wall (Fry, 1998) or peroxidase-mediated Haber–Weiss-reactions (H₂O₂ + O₂⁻ → •OH + OH⁻ + O₂) (Schopfer, 2001). We found that superoxide (O₂⁻), a precursor of •OH according to either of the hypotheses and a product of the reaction of •OH with polysaccharides (Deeble et al., 1990), was produced in the apoplast of radicles and endosperm caps (Fig. 2C). In both seed parts, its production increased from 8 h to 18 h (CON) and was inhibited by ABA. With ABA we observed a delayed increase of O₂⁻-production in the endosperm cap, whose temporal pattern was highly similar to the ABA-regulation of the •OH-production. In the radicle, only a minor increase was observed, which is in accordance with the •OH-production pattern.

Figure 2D shows histochemical O₂⁻ detection, in which we observed that O₂⁻-production in embryos localized most strongly to the radicle – the part of the embryo that elongates
first and strongest and that comes into contact with the cap. While in 8 h-radicles the staining was exclusively localized to the very tip, by 18 h it had spread to additional adjacent parts of the elongation zone. The intensity of the staining, but not the spread of the localization was inhibited by ABA (data not shown). O$_2^•$ production by the 18 h endosperm cap occurred over its entire surface, while 8 h caps stained very weakly (Fig. 2D). The ROS production in the apoplast by any of the proposed mechanisms therefore appears to be spatially as well as temporally regulated.

In our system, different inhibitors of O$_2^•$ production had tissue-specific effects (Table I). O$_2^•$ production, which is required for the peroxidase-mediated mechanism of •OH production (Schopfer, 2001) and potentially contributes to the Fenton-mediated mechanism (since O$_2^•$ is rapidly dismutated to H$_2$O$_2$ and O$_2$), was more sensitive to inhibition by KCN in the endosperm cap than in the radicle, while diphenyleneiodonium chloride (DPI) led to inhibition in both seed parts. KCN is known to inhibit peroxidases, which can produce O$_2^•$ (Minibayeva et al., 2000), as well as ascorbate oxidase which is hypothesized to play a role in ROS-generation (Green and Fry, 2005) and other heme-containing enzymes, while DPI is an inhibitor of membrane-located NADPH oxidases and other flavoenzymes (Doussiere and Vignais, 1992). Our inhibitor results are in agreement with the hypothesis that NADPH oxidases, as well as apoplastic peroxidases or ascorbate oxidases, play a role in O$_2^•$ production in germinating cress seeds. Highly specific inhibitors of these different enzymes are not known, but the observed differences in the inhibition sensitivities between radicle and endosperm caps imply that the mechanisms differ qualitatively between the two seed tissues. It should be noted that differences in the permeability of the tissues might account for part of the difference between inhibitor effects.

The production of O$_2^•$ by NADPH oxidases has been linked to growth processes in various stages of plant, animal and fungal development: tobacco pollen tube elongation (Potocký et al., 2007), root hair tip growth in Arabidopsis (Foreman et al., 2003; Monshausen et al., 2007), ear development of mouse embryos (Kiss et al., 2006), fungal spore germination and appressorium formation in the rice pathogen Magnaporthe grisea (Egan et al., 2007), and to vegetative growth and ascospore germination of the fungus Podospora anserina (Malagnac et al., 2004). Liszkay et al.(2004) found that O$_2^•$ and
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•OH production are associated with maize root elongation and proposed that •OH causes cell wall loosening, but a direct in vivo action of these ROS on cell walls was not investigated. It has only recently begun to emerge that ROS play an important role in cell signaling throughout the kingdoms (Bailly, 2004; Laloi et al., 2004; D'Autréaux and Toledano, 2007). ROS signaling has recently also been investigated in the context of seed germination (Oracz et al., 2009). For the various modes of ROS action it is therefore important to carefully distinguish between signaling and direct mechanisms. We demonstrate in the next section that developmentally targeted in vivo •OH production in seeds and seedlings causes tissue-specific scission of cell wall polysaccharides in vivo.

•OH Loosens Cell Walls In Vivo and has Tissue-Specific and Hormonally Controlled Target Polysaccharides

Having established that apoplastic •OH is produced in vivo following a developmental pattern, we applied 3H-fingerprinting (Fry et al., 2001) to cell walls from the most informative sample comparisons (Fig. 3-5). This technique is the only accepted method that can demonstrate direct in vivo •OH action on cell wall polysaccharides. Such action can, depending on which atoms of the polysaccharide the •OH targets, (i) cause immediate chain scission, (ii) convert glycosidic bonds to unstable ester bonds, and (iii) introduce relatively stable oxo groups thus forming glycosulose residues (Miller and Fry, 2001). The fingerprinting method is based on 3H-labeling of the oxo groups, whose presence in polysaccharides (other than at the reducing terminus) is diagnostic of recent •OH attack (Fry et al., 2001, Miller and Fry, 2001). It has been shown that these 3H-fingerprints differ characteristically between unripe and ripe fruits, and that in vivo •OH attack increases during fruit ripening and may be an important mechanism of fruit cell wall loosening (Fry et al., 2001). •OH-attack on cell wall polymers potentially leads to the breakage of load-bearing polysaccharides and could thereby cause cell-wall loosening, but direct evidence that •OH attack on cell wall polysaccharides occurs in vivo and increases during seed germination and seedling growth was lacking. In germinating cress seeds, the onset of radicle elongation was associated with increased •OH attack on cell wall polysaccharides in the radicle that upon enzymic digestion yielded acidic as well as neutral [3H]labeled products (Figs. 3 and 5): We found an
increase in $[^{3}H]$labeling of the acidic product A1 and the two neutral products N1 and N2. This increase between 8 h CON and 18 h CON was ca. 1.5-fold for A1 and ca. 2-fold for N1 and N2. ABA completely inhibited this increase in \textit{in vivo} $\cdot$OH attack (Fig. 5A). Peaks N1 and N2 remain unidentified: they did not co-migrate exactly with any monosaccharide tested, and when eluted and re-run in a basic solvent, they migrated within the disaccharide zone (more slowly than the slowest monosaccharide, data not shown). They may therefore have been disaccharides containing unusual sugar residues not susceptible to Driselase digestion.

Our $[^{3}H]$-fingerprinting results for the corresponding endosperm caps (Figs. 4 and 5) differed qualitatively and quantitatively from those of the radicle: The most striking difference was the lack of a clearly defined $[^{3}H]$labeled acidic peak in the endosperm cap samples (Fig. 4A). The component that yields acidic product A1 is therefore either absent from cap cell wall polymers or not attacked by $\cdot$OH in the cap. In $[^{3}H]$labeled cap samples, the neutral peaks N1 and N2 were not detected, but two other neutral peaks (N3 and N4, Fig. 4B and 5B) with different migration patterns were evident. We identified the radioactive peak N3 in the endosperm cap samples as $[^{3}H]$arabinose by its exact co-migration with an arabinose internal marker during paper chromatography in several different solvents (Fig. 4C). That the product is $[^{3}H]$arabinose rather than $[^{3}H]$arabinitol indicates that we were detecting oxidized midchain or nonreducing-terminal sugar residues, not reducing-terminal arabinose moieties. A sugar residue that upon $\cdot$OH-attack forms an oxo derivative that is reducible by NaB$_3$H$_4$ to an $[^{3}H]$arabinose residue could originally have been either an arabinose residue or one of its epimers, e.g. a nonreducing-terminal xylopyranose residue (Miller and Fry, 2001). The unidentified neutral products present in our $[^{3}H]$-fingerprints (N1, N2, N4) may include rare epimeric monosaccharides (Miller and Fry, 2001) such as lyxose, or disaccharides resistant to enzymic digestion.

There was no increase in the \textit{in vivo} $\cdot$OH attack leading to $[^{3}H]$arabinose between 8 h CON and 18 h CON, but ABA decreased the \textit{in vivo} $\cdot$OH attack that leads to $[^{3}H]$arabinose (Fig. 5B). A small increase between 8 h CON and 18 h CON was evident for the \textit{in vivo} $\cdot$OH attack leading to N4. This increase was inhibited by ABA. Thus, seed germination is associated with developmentally regulated, qualitatively and quantitatively distinct patterns of \textit{in vivo} $\cdot$OH attack on cell wall polymers in radicle and cap tissues.
Our findings are in accordance with current knowledge summarized by Knox (2008) that plant cell wall polymers are extensively regulated developmentally and differ in structure and function between organs, tissues and taxa: For example, endosperm cell walls are known to contain more hemicellulose than somatic cell walls (Bewley, 1997). Cress radicles and caps would be expected to have distinct cell wall composition, potentially yielding different ‘fingerprints’ after •OH attack. In contrast to cell wall hydrolases, which tend to have high substrate specificity, •OH radicals can attack any polysaccharide (Fry, 1998), although not necessarily uniformly: for example, the fingerprint obtained from •OH-attacked xyloglucan contained 25× more [³H]xylose than [³H]glucose (Miller and Fry, 2001). Bethke et al. (2007) observed in seeds of Arabidopsis that endosperm cell walls become thinner during germination. The thinning is most obvious in the cap. Based on their physiological-microscopical experiments, the authors suggest that ROS is an attractive mechanism of cell wall loosening during germination. This hypothesis is in agreement with our direct biochemical evidence for a developmental role of in vivo •OH attack in cell wall loosening during germination.

In addition to seeds we investigated in vivo •OH attack on cell walls in maize seedling coleoptiles, a classical and well-characterized system for cell elongation. In vivo •OH production has been shown in this system (Schopfer, 2001), but a role for in vivo •OH attack of cell wall polysaccharides during elongation growth has never been demonstrated in this model system or in any other seedlings during elongation growth. We found a strong increase in in vivo •OH cell wall attack between slowly and rapidly elongating coleoptiles (Fig. 6). This trend is similar to the one we observed for non-elongating and elongating radicles.

Maize coleoptiles which had been induced to elongate by a red light pulse showed an over 5-fold increase in labeling of neutral compounds, but no acidic peak. The associated four neutral peaks differed qualitatively from the seed-derived peaks N1 to N3 as judged by their R_F-values, while N4 could be present. Previous ³H-fingerprinting work on maize coleoptile cell walls produced [³H]galactose (Fry, 1998), but no differences in ³H-fingerprints between auxin (20 µM indol acetic acid)-treated and control coleoptiles.

Endosperm cap weakening (this work) and fruit softening (Fry et al., 2001) are developmental processes that involve in vivo •OH cell wall attack and cell separation, but
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not cell elongation (Bewley, 1997). Radicle elongation during seed germination is a growth process involving cell elongation which likely includes an increase in in vivo •OH cell wall attack as well (Fig. 3). In addition, our results support a role of •OH in elongation growth of maize coleoptiles and complement the work by Schopfer (2001) with direct evidence for a corresponding mechanism. Taken together, our results suggest that in vivo •OH production during tissue weakening and elongation growth leads to •OH cell wall attack of tissue- and/or species-specific polysaccharide target sites.

Conclusion

We provide direct evidence that in vivo •OH production in the apoplast causes in vivo scission of specific cell wall polysaccharides in elongating maize coleoptiles as well as the radicles and endosperm caps of germinating cress seeds. This constitutes a novel mechanism for cell wall loosening during seed germination. The direct action of •OH on cell wall polysaccharides has tissue-specific target sites, is temporally, hormonally (GA-ABA antagonism) and developmentally regulated, and appears to be a mechanism of general importance as it is evident in diverse developmental processes during the plant lifecycle. Our findings shed new light on the role of ROS in plants and provide a novel interpretation frame for ROS production during seed germination.

In vivo •OH attack of cell wall polysaccharides appears to be a mechanism by which ROS mediate diverse developmental processes of plants. An intriguing issue of this mechanism is that its specificity is determined by the dynamic structural organization of the apoplast. Direct •OH attack on extraprotoplasmatic polymers would require a tight control of the amount and site of ROS production, the mechanism of which is still unclear at this point. However, the fact that we could detect only a small number of distinct peaks in the $^3$H-fingerprinting (Figs. 3, 4 and 5) strongly suggests that •OH attack of cell wall polysaccharides and therefore also its generation is not randomly distributed over all cell wall polysaccharides. This suggests that •OH is produced at specific sites, for example peroxidases, which can be preferentially associated with particular polysaccharides (Carpin et al., 2001), or at transition metal ions which are known to be complexed by specific cell wall polymers (Fry et al., 2002). The positive effects of a tightly controlled production of •OH may also play a role in other living systems, where developmental
processes require the loosening of extracellular matrices. As ROS have been detected in the context of growth or weakening in organisms from bacteria and fungi to plants and mammalian embryos (Gapper and Dolan, 2006), it seems likely that this mechanism can be found throughout the kingdoms.

Materials and Methods

Plant Material, Germination and Puncture-Force Measurements

For germination, cress seeds (*Lepidium sativum* L. ‘Gartenkresse einfache’, Juliwa, Heidelberg, Germany) were imbibed in petri dishes on two layers of filter paper with 6 mL 1/10 Murashige-Skoog salts in continuous white light (101.2 µmol m\(^{-2}\) s\(^{-1}\)) at 18° C as described (Müller et al., 2006). Where indicated, cis-S(+-)-ABA, GA\(_{4/7}\) or H\(_2\)O\(_2\) was added to the medium in the concentrations indicated. Tissue resistance was determined with the puncture force method as described (Müller et al., 2006). For •OH treatment, isolated intact caps were incubated in 1 mM FeSO\(_4\) in 10 mM phosphate buffer, pH 6.0, for 30 min and washed for 10 min in the buffer. Subsequently, to initiate the Fenton reaction, a freshly prepared mixture of H\(_2\)O\(_2\) and ascorbic acid was added to give a final concentration of 100 µM each. Maize (*Zea mays* L. 'Perceval', Asgrow, Bruchsal, Germany) seedlings were grown in plastic boxes on Vermiculite and deionized water at 25°C in darkness. Fast coleoptile growth was induced after 4 d by a 10 min red-light pulse (2.6 µmol m\(^{-2}\) s\(^{-1}\)), after which the seedlings were transferred back to darkness. Coleoptile segments for \(^3\)H-labeling were harvested 5 d after imbibition. These segments were taken 5 mm below the coleoptile tip and were 5 mm long.

Hydroxyl Radical (•OH) Detection by Electron Paramagnetic Resonance (EPR) Spectroscopy

Isolated radicles or endosperm caps (100) were incubated for 3 h in spin-trapping solution [50 mM 4-POBN (α-(4-pyridyl-1-oxide)-N-tert-butyl nitrone) containing 4% v/v ethanol] on a rotary shaker. EPR spectra were recorded for the incubation solution at room temperature in a flat cell with an ESR-300 X-band spectrometer from Bruker (Rheinstetten, Germany) at 9.7 GHz microwave frequency, 100 kHz modulation frequency, modulation amplitude 1 G and 63 mW microwave power as described (Renew}
et al., 2005). Representative spectra are shown in Supplemental Fig. S1. Signal size was calculated as signal to noise ratio.

**Superoxide (O$_2$$^•^-$) Detection**

O$_2$$^•^-$ production was measured by photometric determination of the reduction of XTT (3′-[1-phenylamino-carbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro] benzenesulfonic acid hydrate, Polysciences Inc., Eppelheim, Germany). Radicles or caps (100) were collected in 10 mM phosphate buffer, pH 6.5, on ice. Tissues were left for 20 min in order for wounding effects to subside (Roach et al., 2008). The reaction was started by adding 0.5 mM XTT followed by incubation on a rotary shaker at 300 rpm for 3 h. Absorption spectra of the incubation medium were measured. A$_{470}$ – A$_{650}$ (reference wavelength) was used to calculate XTT reduction. CuZn-superoxide dismutase (from bovine erythrocytes) was purchased from Sigma-Aldrich, Germany.

In addition, O$_2$$^•^-$ production was measured by photometric determination of the oxidation of epinephrine to adrenochrome. Radicles or caps (120) were collected in 10 mM phosphate buffer, pH 7.0, on ice. After the last dissection, tissues were left for 20 min in order for wounding effects to subside (Roach et al., 2008). The reaction was started by adding 1 mM epinephrine followed by incubation on a rotary shaker at 300 rpm for 3 h. A$_{480}$ was then measured in the incubation medium. Tissues that had been incubated for 18 h were used for inhibitor studies. Inhibitors were added at the indicated concentrations before the colorimetric reaction was started.

For the histochemical detection of O$_2$$^•^-$, cress seeds were dissected and the embryos and endosperm caps equilibrated for 10 min in 50 mM phosphate buffer, pH 6.0. Nitroblue tetrazolium chloride (NBT; 10 mM) was then added. When staining was visible, seed parts were removed from the staining solution, washed for 1 min in phosphate buffer and photographed.

**3H-Fingerprinting**

Fingerprinting of •OH-attacked polysaccharides was modified from (Fry et al., 2001). Radicles or endosperm caps or maize coleoptile tissue (100 mg fresh weight) were ground on ice in 1.5 ml buffered ethanol [ethanol:pyridine:acetic acid:water (75:2:2:19, v/v)] containing 10 mM sodium thiosulfate. Ethanol is an excellent scavenger of •OH, preventing any post-mortem action of •OH on polysaccharides; thiosulfate blocks the
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Fenton reaction, preventing further •OH-production (Fry, 1998). After washing in 75 % v/v ethanol, part of the suspension was used for dry weight determination and an equal part for ³H-labeling. For the latter portion, the suspension was centrifuged at 2300 g for 10 min and the pellet washed twice with 10 ml 75 % v/v ethanol. For saponification of pectin methyl-esters, the pellet was suspended in 200 µL of 0.2 M NaOH. After 5 min, 0.5 ml of labeling solution [1 M NH₃ containing either 1 mM NaB³H₄ at 1.95 MBq/µmol (for radicles and coleoptiles) or 5 mM NaB³H₄ at 0.39 MBq/µmol (for endosperm caps)] was added to each sample. Samples were left on a rotary shaker for 2 d. Excess NaB³H₄ was scavenged with 10 mg xylose at 20°C overnight, after which NH₃ was evaporated in a draft of air. The solution was then acidified with 100 µL acetic acid; polysaccharides were precipitated with 3.5 ml ethanol and washed three times with 75 % v/v ethanol. The ethanolic solution contained [³H]xylitol, indicating that a suitable excess of NaB³H₄ had been used (paper chromatography data, not shown). The dried pellets were digested in 200 µL 1 % (w/v) partially purified Driselase (Fry, 2000) in a volatile buffer [pyridine:acetic acid:water (1:1:98, v/v), pH 4.7] containing a volatile anti-microbial agent [0.5 % (w/v) chlorobutanol] for 5 d. Digestion was stopped with 35 µL 90 % v/v formic acid. Samples were briefly centrifuged and 40 µL of supernatant was run by high-voltage electrophoresis at pH 3.5 on Whatman 3MM paper (2.5 kV, 1 h) (Fry, 2000). Strips of the electrophoretogram were assayed for ³H by scintillation counting. Material that comigrated with marker glucose, that is the neutral fraction, was eluted from the paper with water (after removal of scintillation fluid by washing in toluene and drying) and re-run by paper chromatography on Whatman Number 1 in butanol:acetic acid:water (12:3:5, v/v). Markers were stained with AgNO₃ after removal of any scintillation fluid from the paper by washing in toluene.

Photographic Documentation

All photographs were taken with a Leica DCF480 digital camera (Bensheim, Germany) attached to a stereomicroscope (Leica Mz 12,5).

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Figure Legends

Figure 1. Developmental factors and ROS affect the weakening of the cress endosperm cap. (A) A mature cress seed consists of an embryo surrounded by two covering tissues: the testa and the endosperm. The cap covers the radicle tip. (B) Typical germination: Cress seeds germinate in two steps. After ca. 8 h, the brown testa ruptures, revealing the radicle still covered by the cap, which weakens until at ca. 18 h it is penetrated by the elongating radicle. CON (control) medium without additions. (C) In caps incubated after dissection at 18 h CON (‘isolated caps’), tissue weakening led to the formation of a hole at the place where the radicle would have emerged after 1-2 d. (D) Atypical germination after addition of H$_2$O$_2$ to the medium. (E) Effects of H$_2$O$_2$, ABA, and ABA followed by •OH (Fenton reaction initiated by 100 µM H$_2$O$_2$ and ascorbic acid in caps loaded with Fe$^{2+}$) on cap weakening quantified by puncture force measurements. In a control treatment with Fe$^{2+}$ and ascorbic acid, but without H$_2$O$_2$, the puncture force did not decline significantly (34.9 +/- 1.5 mN). Mean values +/- SE of at least three replicates of 25 caps are presented. (A and B) modified from (Müller et al., 2006).
**Figure 2.** *In vivo* detection of apoplastic •OH and O$_2$•$^-$ production in cress caps and radicles during seed germination. (A) Quantification of EPR signal sizes indicative of *in vivo* generated apoplastic •OH in caps and radicles dissected at the times indicated. Seeds were incubated in medium without (CON) or with 10 µM ABA (ABA) added. Note the different scales of the Y-axes for cap and radicle. For comparison to the germination response, the puncture force and endosperm rupture values are given below graph D. (B) ABA-GA antagonism: *In vivo* generated apoplastic •OH (EPR as in A) in caps and radicles treated with ABA or ABA+GA (5 µM ABA, 10 µM GA$_{4+7}$). Means of radicle samples (ABA vs. ABA + GA) differ significantly (P<0.05) as calculated by one way ANOVA followed by Tukey’s multiple comparison test (GraphPad Prism software). (C) Quantification of apoplastic O$_2$•$^-$ in intact caps and radicles by photometric determination of the reduction of XTT. (D) Histochemical staining of O$_2$•$^-$ production with NBT in embryos (10 min staining) and endosperm caps (15 min staining). (A-C) Mean values +/- SE of at least four replicates of 100 radicles and caps, respectively, are shown.
**Figure 3.** Detection of in vivo •OH attack on cress seed radicle cell walls by $^3$H-fingerprinting (Fry et al., 2001). (A) Representative $^3$H-fingerprints of acidic products from radicle samples. Signal intensity in the scintillation count is plotted against distance from the origin after high voltage paper electrophoresis (PE) at pH 3.5. Monosaccharide markers were run with the samples. (B) Representative $^3$H-fingerprints of neutral products from radicle samples. Neutral material (that which co-migrated with glucose during PE) was eluted and re-run by paper chromatography (PC) in an acidic solvent. Peaks N1 and N2 remain unidentified: they did not co-migrate exactly with any monosaccharide tested, and when eluted and re-run in a basic solvent, they migrated within the disaccharide zone (more slowly than the slowest monosaccharide, data not shown). They may therefore have been disaccharides containing unusual sugar residues not susceptible to Driselase digestion. Acidic markers: GalO = galactonic acid, GalA = galacturonic acid, GlcA = glucuronic acid. Neutral markers: Gal = galactose, Glc = glucose, Gal-ol = galactitol, Ara = arabinose, Xyl-ol = xylitol, Xyl = xylose, Fuc = fucose, Rib = ribose.
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**Figure 4.** Detection of in vivo •OH attack on cress seed endosperm cap cell walls by ³H-fingerprinting and identification of the neutral compound observed as peak N3 as arabinose. ³H-fingerprints of cap samples (A and B) differ quantitatively and qualitatively from those of radicle samples (see Fig. 3). (A) Representative electrophoretic ³H-fingerprints of ³H-labeled products from cap samples. Signal intensity in the scintillation count is plotted against distance from the origin after high-voltage paper electrophoresis (PE) at pH 3.5. No acidic peak was detected. (B) Representative chromatographic ³H-fingerprints of ³H-labeled products from cap samples. The samples were eluted from the fraction that comigrated with glucose during PE at pH 3.5 and re-run by paper chromatography (PC). (B and C) The neutral compound observed as peak N3 was identified as arabinose (Ara) by PC in different solvents with reference to internal and external markers (Fry, 2000). Peak N4 remains unidentified. Peak N3 comigrates with an external arabinose standard in butanol:acetic acid:water (12:3:5, v/v, ‘BAW’). (C) Peak N3 was eluted and re-run by PC in ethyl acetate:pyridine:water (8:2:1, v/v, ‘EPW’). Again, the peak comigrates with the external marker arabinose. The internal marker arabinose (arrow) comigrated with the EPW peak, as shown by AgNO₃-staining of the strips of chromatography paper after recovery from the scintillation fluid. For abbreviations of acidic and neutral markers see Fig. 3.
**Figure 5.** Quantification of peak areas indicative of •OH-attack on polysaccharides in the radicle (A) and the endosperm cap (B). While a distinct acidic peak (A1) was only present in the radicle, neutral peaks were detected in all samples, but differed qualitatively between radicles (N1, N2 – see Fig. 3) and caps (N3, N4 – see Fig. 4). N3 was identified as [3H]arabinose (see Fig. 4B and 4C). Areas under peaks were normalized by setting the value at 8 h to 100. The physiological state of the seeds at the time of dissection is indicated. Mean values +/- SE of at least four replicates (200 radicles and 1000 caps used for extraction, respectively) are presented.
**Figure 6.** Detection of *in vivo* •OH attack on maize seedling coleoptile cell walls by $^3$H-fingerprinting during elongation growth. (A) Representative $^3$H-fingerprint of labeled products from segments of slowly and rapidly elongating coleoptiles. Signal intensity in the scintillation count, that is $^3$H-labeling of former oxo groups, is plotted against distance from the origin after high-voltage paper electrophoresis (PE) at pH 3.5. Only the rapidly elongating coleoptiles showed an appreciable neutral peak (co-migrating with glucose), which was eluted and re-run by paper chromatography (PC). (B) Representative $^3$H-fingerprint of labeled neutral products from segments of rapidly elongating coleoptiles. The sample was eluted from the fraction that co-migrated with glucose during PE and re-run on PC. For abbreviations of acidic and neutral markers see Fig. 3.
**Table I.** Inhibitors of \( O_2^{\cdot-} \) production have tissue-specific effects on germinating cress seeds. \( O_2^{\cdot-} \)-production was measured in radicles and endosperm caps of cress seeds incubated for 18 h. Two photometric assays were used: oxidation of epinephrine and reduction of XTT. The addition of CuZn-superoxide dismutase (‘CuZn-SOD’, from bovine erythrocytes) led to an inhibition of around two-thirds of the total signal, indicating that this fraction is specifically caused by \( O_2^{\cdot-} \)-mediated reactions. As SOD can only reach the surface of the tissues, it is possible that the remaining fraction is (at least in part) also caused by \( O_2^{\cdot-} \), with the reaction taking place in regions inaccessible to SOD, but accessible to XTT and epinephrine.

The remaining third Mean values +/- SE at 18 h of at least three replicates (n = 120) are presented in comparison to the untreated control (CON). n.d. = not determined.

| Treatment                  | Radicle      | Endosperm cap          |
|----------------------------|--------------|------------------------|
|                            | Inhibition (%) | SE        | Inhibition (%) | SE     |
| Epinephrine method:        |              |           |              |        |
| CON                        | 0.0          | 0.0       | 0.0          | 0.0    |
| KCN (0.1 mM)               | 20.4 ± 12.6  | 60.5 ± 0.9 |
| KCN (1 mM)                 | 45.2 ± 6.3   | 57.2 ± 2.2 |
| DPI (15 µM)                | 5.2 ± 3.0    | 0.0 ± 4.2  |
| DPI (50 µM)                | 38.9 ± 16.0  | 34.8 ± 9.1 |
| CuZn-SOD (150 units mL\(^{-1}\)) | 60.1 ± 2.9  | 68.5 ± 2.2 |
| XTT method:                |              |           |              |        |
| CON                        | 0.0          | 0.0       | 0.0          | 0.0    |
| KCN (0.1 mM)               | 25.0 ± 7.5   | n.d.      |
| KCN (1 mM)                 | 33.1 ± 6.1   | n.d.      |
| DPI (50 µM)                | 26.4 ± 7.3   | n.d.      |
| CuZn-SOD (150 units mL\(^{-1}\)) | 60.3 ± 1.2  | 73.4 ± 1.9 |
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Figure 1. Developmental factors and ROS affect the weakening of the cress endosperm cap (cap). (A) A mature cress seed consists of an embryo surrounded by two covering tissues: the testa and the endosperm. The cap covers the radicle tip. (B) Typical germination: Cress seeds germinate in two steps. After ca. 8 h, the brown testa ruptures, revealing the radicle still covered by the cap, which weakens until at ca. 18 h it is penetrated by the elongating radicle. CON (control) medium without additions. (C) In caps incubated after dissection at 18 h CON ('isolated caps'), tissue weakening led to the formation of a hole at the place where the radicle would have emerged after 1-2 days. (D) Atypical germination after addition of H$_2$O$_2$ to the medium. (E) Effects of H$_2$O$_2$, ABA, and ABA followed by •OH (Fenton reaction initiated by 100 µM H$_2$O$_2$ and ascorbic acid in caps loaded with Fe$^{2+}$) on cap weakening quantified by puncture force measurements.

In a control treatment with Fe$^{2+}$ and ascorbic acid, the cap force did not decline significantly (34.9 +/- 1.5 mN). Mean values +/- SE of at least three replicates of 25 caps are presented. (A and B) modified from (Müller et al., 2006).
Figure 2. In vivo detection of apoplastic *OH and O₂*²⁻ production in cress caps and radicles during seed germination.

(A) Quantification of EPR signal sizes indicative of in vivo generated apoplastic *OH in caps and radicles dissected at the times indicated. Seeds were incubated in medium without (CON) or with 10 μM ABA (ABA) added. Note the different scales of the Y-axes for cap and radicle. For comparison to the germination response, the puncture force and endosperm rupture values are given below graph D. (B) ABA-GA antagonism: In vivo generated apoplastic *OH (EPR as in A) in caps and radicles treated with ABA or ABA+GA (5 μM ABA, 10 μM GA4+7). Means of radicle samples (ABA vs. ABA + GA) differ significantly (P<0.05) as calculated by one way ANOVA followed by Tukey’s post-hoc test. (C) Quantification of apoplastic O₂*²⁻ in intact caps and radicles by pho-oxidation of NBT. Endosperm rupture was scored. (D) Histochemical O₂*²⁻ detection with NBT in embryos and endosperm caps. (A-C) Mean values +/- SE of at least four replicates of 100 radicles and caps, respectively, are shown.
Figure 3. Detection of in vivo •OH attack on cress seed cell radicle cell walls by $^3$H-fingerprinting. (A) Representative $^3$H-fingerprints of acidic products from radicle samples. Signal intensity in the scintillation count is plotted against distance from the origin after high voltage paper electrophoresis (PE) at pH 3.5. Monosaccharide markers were run with the samples. (B) Representative $^3$H-fingerprints of neutral products from radicle samples. Neutral material (that which co-migrated with glucose during PE) was eluted and re-run by paper chromatography (PC) in an acidic solvent. Acidic markers: GalO = galactonic acid, GalA = galacturonic acid, GlcA = glucuronic acid. Neutral markers: Gal = galactose, Glc = glucose, Gal-ol = galactitol, Ara = arabinose, Xyl-ol = xylitol, Xyl = xylose, Fuc = fucose, Rib = ribose.
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Figure 6. Detection of in vivo •OH attack on maize seedling coleoptile cell walls by 3H-fingerprinting during elongation growth. (A) Representative 3H-fingerprint of labeled products from segments of slowly and rapidly elongating coleoptiles. Signal intensity in the scintillation count is plotted against distance from the origin after high-voltage paper electrophoresis (PE) at pH 3.5. Only the rapidly elongating coleoptiles showed an appreciable neutral peak (co-migrating with glucose), which was eluted and re-run by paper chromatography (PC). (B) Representative 3H-fingerprint of labeled neutral products from segments of rapidly elongating coleoptiles. For abbreviations of acidic and neutral markers see Fig. 3.