In Vivo Cell Wall Loosening by Hydroxyl Radicals during Cress Seed Germination and Elongation Growth1[W][OA]

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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 spectroscopy to show that OH is generated in the cell wall during radicle elongation and weakening of the endosperm of cress (Lepidium sativum; Brassicaceae) seeds. Endosperm weakening precedes radicle emergence, as demonstrated by direct biomechanical measurements. By 3H fingerprinting, we showed that wall polysaccharides are oxidized 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 evident not only in germinating seeds but also in elongating maize (Zea mays; Poaceae) seedling coleoptiles. We conclude that plant cell wall loosening by OH is a controlled action of this type of reactive oxygen species.

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, therefore, is 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 this 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, 1997b; 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 stages of plant growth are based on different develop-
opment programs, 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 expansion action (Cosgrove, 2005). In seeds in particular, enzymatic hydrolysis of endosperm cell walls by endoglucanases such as β-1,3-glucanase (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 Bewley, 1997a; Finch-Savage and Leubner-Metzger, 2006). Expansins and xyloglucan endotransglucosylase/hydrolases are expressed in the endosperm cap of tomato (Solanum lycopersicum) seeds during germination (Chen et al., 2002), where they can contribute to endosperm weakening.

Hydroxyl radicals (OH) 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, such as seed germination (Bailly, 2004) and seedling growth (Schopfer, 2001), it has so far only been shown directly in ripening pear (Pyrus communis) 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 the 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) seeds and maize (Zea mays) 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 Inhibits and OH Generation Promotes Weakening of the Endosperm Envelope

Seed germination of garden cress comprises two sequential steps, testa and endosperm rupture, as does germination of the model plant Arabidopsis (Arabidopsis thaliana; 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 the 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. 1, A 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).

Hydrogen peroxide (H2O2) 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 nondormant cress seeds, the addition of 10 mM H2O2 to the medium did not change the germination kinetics but led to atypical germination in approximately 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 H2O2 showed that cap weakening is inhibited (Fig. 1E). This might be caused by cell wall-tightening reactions that H2O2 is known to cause by cross-linking extraplasmatic polymers (Brisson et al., 1994; Schopfer, 1996; Encina and Fry, 2005). We cannot rule out cytotoxic effects of a 10 mM H2O2 treatment, although 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 H2O2 (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 (Lactuca sativa) 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$_2$ → Fe$^{3+}$ + 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 approximately 38 mN to approximately 20 mN (18h CON). This cap weakening was inhibited by ABA (18h ABA) and by H$_2$O$_2$ (18h H$_2$O$_2$). Incubation in ABA for 17 h followed by only 1 h of exposure to apoplastic OH led to a decline in tissue resistance: the puncture force was approximately 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 1 d, 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 mM 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).

‘OH and O$_2^-$ 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 life span (Fry et al., 2001; Schopfer, 2001). We used a spin trap that 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 (Cucumis sativus) 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 and 18 h (Fig. 2A). The 8-h time point is characterized by a still unweakened endosperm and nonelongating...
radicle (8h CON), while at 18 h, cap weakening has progressed, tissue resistance is halved, and radicle elongation starts (18h CON). ABA inhibited these physiological processes between 8 and 18 h and inhibited \( \cdot \text{OH} \) production in both tissues as well.

A tissue-specific ABA effect could be observed as the seeds progressed to germination in the presence of ABA: caps showed an increase of \( \cdot \text{OH} \) production toward 72 h, while radicles showed an \( \cdot \text{OH} \) production equal to that at 18 h of ABA (Fig. 2A). Possible interpretations of this phenomenon are (1) that cell wall loosening and thereby radicle elongation mechanisms differ in the presence and absence of ABA, and (2) that the increase in \( \cdot \text{OH} \) 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 in vivo apoplastic \( \cdot \text{OH} \) production in radicles and endosperm caps (Fig. 2B). While we observed 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 \( \cdot \text{OH} \) production on reversion of the ABA inhibition of germination with GA. These observations support our hypothesis that hormone-sensitive \( \cdot \text{OH} \)-mediated effects in the cell wall contribute to endosperm weakening and radicle elongation.

We conclude that the increase in apoplastic \( \cdot \text{OH} \) 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 \( \cdot \text{OH} \) 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 \( \beta \)-1,3-glucanase in tobacco (\textit{Nicotiana tabacum}; Leubner-Metzger, 2002) and \( \beta \)-1,4-mannanase in tomato (Nonogaki et al., 2000; Toorop et al., 2000; da Silva et al., 2004) and coffee (\textit{Coffea arabica}), 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 \( \cdot \text{OH} \) 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 \( \text{H}_2\text{O}_2 \) (e.g. \( \text{O}_2^- \) or \( \text{O}_2 \cdot \)) in the cell wall (Fry, 1998); and peroxidase-
mediated Haber-Weiss reactions (H$_2$O$_2$ + O$_2$ → ‘OH + OH$^-$ + O$_2$, Schopfer, 2001). We found that O$_2$^•−, a precursor of ‘OH according to either of the hypotheses and a product of the reaction of ‘OH with polysaccharides (Deebble et al., 1990), was produced in the apoplast of radicles and endosperm caps (Fig. 2C). In both seed parts, its production increased from 8 to 18 h (CON) and was inhibited by ABA. With ABA, we observed a delayed increase of O$_2$^•− production in the endosperm cap, whose temporal pattern was highly similar to the ABA regulation of ‘OH production. In the radicle, only a minor increase was observed, which is in accordance with the ‘OH production pattern.

Figure 2D shows histochemical O$_2$^•− detection, in which we observed that O$_2$^•− 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 cyanide (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 oxidase, 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 (Potocky 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 (Oryza sativa) pathogen Magnaporthe grisea (Egan et al., 2007), and vegetative growth and ascospore germination of the fungus Podospora anserina (Malagnac et al., 2004). Liszka et al. (2004) found that O$_2$^•− and ‘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; Laloï et al., 2004; D’Autreux 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, therefore, it is 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.

### Table I. Inhibitors of O$_2$^•− production have tissue-specific effects on germinating cress seeds

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radicle Inhibition (%)</th>
<th>Endosperm Cap Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>KCN (0.1 mM)</td>
<td>20.4</td>
<td>60.5</td>
</tr>
<tr>
<td>KCN (1 mM)</td>
<td>45.2</td>
<td>57.2</td>
</tr>
<tr>
<td>DPI (5 μM)</td>
<td>5.2</td>
<td>4.2</td>
</tr>
<tr>
<td>DPI (50 μM)</td>
<td>38.9</td>
<td>34.8</td>
</tr>
<tr>
<td>CuZn-SOD (150 units mL$^{-1}$)</td>
<td>60.1</td>
<td>68.5</td>
</tr>
<tr>
<td>XTT method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>KCN (0.1 mM)</td>
<td>25.0</td>
<td>7.5</td>
</tr>
<tr>
<td>KCN (1 mM)</td>
<td>33.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>DPI (50 μM)</td>
<td>26.4</td>
<td>7.3</td>
</tr>
<tr>
<td>CuZn-SOD (150 units mL$^{-1}$)</td>
<td>60.3</td>
<td>73.4</td>
</tr>
</tbody>
</table>

‘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 $^3$H fingerprinting (Fry et al., 2001) to cell walls from the
most informative sample comparisons (Figs. 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, (1) cause immediate chain scission, (2) convert glycosidic bonds to unstable ester bonds, and (3) 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 3H 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 approximately 1.5-fold for A1 and approximately 2-fold for N1 and N2. ABA completely inhibited this increase in in vivo OH attack (Fig. 5A).

Our 3H 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 3H-labeled acidic peak in the endosperm cap samples (Fig. 4A). The component that yields acidic product A1, therefore, is either absent from cap cell wall polymers or not attacked by OH in the cap. In 3H-labeled

![Figure 3](image_url)

**Figure 3.** Detection of in vivo OH attack on cress seed radicle cell walls by 3H fingerprinting (Fry et al., 2001). A, Representative 3H 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 3H fingerprints of neutral products from radicle samples. Neutral material (which comigrated with Glc during paper electrophoresis) was eluted and rerun by PC in an acidic solvent. Peaks N1 and N2 remain unidentified: they did not comigrate exactly with any monosaccharide tested, and when eluted and rerun in a basic solvent, they migrated within the disaccharide zone (more slowly than the slowest monosaccharide; data not shown). Therefore, they may have been disaccharides containing unusual sugar residues not susceptible to Driselase digestion. Markers are as follows: GalA, galacturonic acid; GalO, galactonic acid; Gal-ol, galactitol; GlcA, glucuronic acid; Xyl-ol, xylitol. CON, Control.

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 Ara. 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 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 Glc during paper electrophoresis at pH 3.5 and rerun by PC. B and C, The neutral compound observed as peak N3 was identified as 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 Ara standard in butanol:acetic acid:water (12:3:5 [v/v]). C, Peak N3 was eluted and rerun by PC in ethyl acetate:pyridine:water (EPW; 8:2:1 [v/v]). Again, the peak comigrates with the external marker Ara. The internal marker Ara (arrow) comigrates 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 Figure 3 legend. CON, Control.
cap samples, the neutral peaks N1 and N2 were not detected, but two other neutral peaks (N3 and N4; Figs. 4B and 5B) with different migration patterns were evident. We identified the radioactive peak N3 in the endosperm cap samples as [3H]Ara by its exact comigration with an Ara internal marker during paper chromatography (PC) in several different solvents (Fig. 4C). That the product is [3H]Ara rather than [3H]arabinitol indicates that we were detecting oxidized midchain or nonreducing terminal sugar residues, not reducing terminal Ara moieties. A sugar residue that upon OH attack forms an oxo derivative that is reducible by NaB₃H₄ to a [3H]Ara residue could originally have been either an Ara residue or one of its epimers (e.g. a nonreducing terminal xylopyranose residue; Miller and Fry, 2001). The unidentified neutral products present in our 3H fingerprints (N1, N2, and 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 in vivo OH attack leading to [3H]Ara between 8-h CON and 18-h CON, but ABA decreased the in vivo OH attack that leads to [3H]Ara (Fig. 5B). A small increase between 8-h CON and 18-h CON was evident for the in vivo 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 in vivo 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, 1997a). 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 times more [3H]Xyl than [3H]Glc (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

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 and N2; see Fig. 3) and caps (N3 and N4; see Fig. 4). N3 was identified as [3H]Ara (see Fig. 4, B and C). 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 1,000 caps used for extraction) are presented. CON, Control.

Müller et al.
one we observed for nonelongating and elongating radicles.

Maize coleoptiles that 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 RF values, while N4 could be present. Previous 3H fingerprinting work on maize coleoptile cell walls produced [3H]Gal (Fry, 1998) but no differences in 3H fingerprints between auxin (20 μM indole 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 \( \cdot \)OH cell wall attack and cell separation but not cell elongation (Bewley, 1997b). Radicle elongation during seed germination is a growth process involving cell elongation that likely includes an increase in in vivo \( \cdot \)OH cell wall attack as well (Fig. 3). In addition, our results support a role of \( \cdot \)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 \( \cdot \)OH production during tissue weakening and elongation growth leads to \( \cdot \)OH cell wall attack of tissue- and/or species-specific polysaccharide target sites.

CONCLUSION

We provide direct evidence that in vivo \( \cdot \)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 \( \cdot \)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 life cycle. 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 \( \cdot \)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 extraplastomembrane 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. 3–5) 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, such as peroxydases, that can be preferentially associated with particular polysaccharides (Carpin et al., 2001), or at transition metal ions that 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 'Gartenkresse einfache'; Juliwa) were imbibed in petri dishes on two layers of filter paper with 6 mL of one-tenth-strength Murashige and Skoog salts in continuous white light (101.2 μmol m\(^{-2}\) s\(^{-1}\)) at 18°C as described (Mueller et al., 2006). Where indicated, cis-[(S)-α-ABA, GA\(_{300}\), 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 (Mueller 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 'Perceval'; Asgrow) seedlings were grown in plastic boxes on vermiculite and deionized water at 25°C, on a rotary shaker at 300 rpm for 3 h. Absorption spectra of the incubation solution at room temperature in a flat cell with an ESR-300 X-band spectrometer from Bruker at 9.7-GHz microwave frequency, 100-kHz modulation frequency, 1 G modulation amplitude of 1 G, and 63-mW microwave power was used to calculate XTT reduction. Copper/zinc (CuZn)-superoxide dismutase (from bovine erythrocytes) was purchased from Sigma-Aldrich.

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\(_{380}\) – A\(_{450}\) (reference wavelength) was used to calculate XTT reduction.

\(^3\)H 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 of buffered ethanol (ethanol: pyridine:acetic acid-water, 12:3:5 [v/v]) containing 10 mM sodium thiosulfate. Ethanol is an excellent scavenger of OH, preventing any postmortem action of OH on polysaccharides; thiosulfate blocks the 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 \(^3\)H labeling. For the latter portion, the suspension was centrifuged at 2,300g for 10 min and the pellet was washed twice with 10 mL of 75% (v/v) ethanol. For saponification of pectin methyl esters, the pellet was suspended in 200 μL of 0.2 mM NaOH. After 5 min, 0.5 mL of labeling solution (1 mM NH\(_4\) containing either 1 mM NaB\(_4\)H\(_4\) at 1.95 MBq μmol\(^{-1}\) [for radicles and coleoptiles] or 5 mM NaB\(_4\)H\(_4\) at 0.39 MBq μmol\(^{-1}\) [for endosperm caps]) was added to each sample. Samples were left on a rotary shaker for 2 d. Excess NaB\(_4\)H\(_4\) was scavenged with 10 mg of Xyl at 20°C overnight, after which NH\(_4\) was evaporated in a draft of air. The solution was then acidified with 100 μL of acetic acid; polysaccharides were precipitated with 3.5 mL of ethanol and washed three times with 75% (v/v) ethanol. The ethanolic solution contained [\(^3\)H]ylitol, indicating that a suitable excess of NaB\(_4\)H\(_4\) had been used (PC data not shown). The dried pellets were digested in 200 μL of 1% (w/v) partially purified Dirlase (Fry, 2000) in a volatile buffer (pyridine:acetic acid:water, 1:1:98 [v/v/w]; pH 4.7) containing a volatile antimicrobial agent (0.5% [w/v] chlorobutanol) for 5 d. Digestion was stopped with 35 μL of 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 \(^3\)H by scintillation counting. Material that comigrated with marker Glc (i.e. the neutral fraction) was collected in 10 mM phosphate buffer, pH 7.0, on ice. Nitroblue tetrazolium chloride (10 μM) was then added. When staining was visible, seed parts were removed from the staining solution, washed for 1 min in phosphate buffer, and photographed.

Photographic Documentation

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

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure S1. Representative EPR spectra for radicle samples.

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