Published in final edited form as:

Plant Sci. 2014 March ; 0: 120–126. doi:10.1016/j.plantsci.2013.12.008.

Controlled free radical attack in the apoplast: A hypothesis for roles of O, N and S species in regulatory and polysaccharide cleavage events during rapid abscission by *Azolla*

Michael F. Cohen^{a,*}, Sushma Gurung^b, Jon M. Fukuto^c, and Hideo Yamasaki^b

Jon M. Fukuto: fukuto@sonoma.edu; Hideo Yamasaki: yamasaki@sci.u-ryukyu.ac.jp

^aDepartment of Biology, Sonoma State University, 1801 E. Cotati Ave., Rohnert Park, CA 94928, USA

^bFaculty of Science, University of the Ryukyus, Nishihara 903-0213, Japan

^cDepartment of Chemistry, Sonoma State University, 1801 E. Cotati Ave., Rohnert Park, CA 94928, USA

Abstract

Shedding of organs by abscission is a key terminal step in plant development and stress responses. Cell wall (CW) loosening at the abscission zone can occur through a combination chain breakage of apoplastic polysaccharides and tension release of cellulose microfibrils. Two distinctly regulated abscission cleavage events are amenable to study in small water ferns of the genus *Azolla*; one is a rapid abscission induced by environmental stimuli such as heat or chemicals, and the other is an ethylene-induced process occurring more slowly through the action of hydrolytic enzymes. Although free radicals are suggested to be involved in the induction of rapid root abscission, its mechanism is not fully understood. The apoplast contains peroxidases, metal-binding proteins and phenolic compounds that potentially generate free radicals from H₂O₂ to cleave polysaccharides in the CW and middle lamella. Effects of various thiol-reactive agents implicate the action of apoplastic peroxidases having accessible cysteine thiols in rapid abscission. The Ca²⁺ dependency of rapid abscission may reflect the stabilization Ca²⁺ confers to peroxidase structure and binding to pectin. To spur further investigation, we present a hypothetical model for small signaling molecules H₂O₂ and NO and their derivatives in regulating, via modification of putative protein thiols, free radical attack of apoplastic polysaccharides.

Keywords

ascorbate; hydrogen peroxide; hydroxyl radicals; non-enzymatic scission; cell wall; rapid abscission

1. Introduction

Plant organs such as leaves, flowers, fruits and roots detach from the plant in response to developmental programming, hormonal signaling or various stresses. Within the separation

© 2013 Elsevier Ireland Ltd. All rights reserved.

*Corresponding author. Tel. +1 707 664 3413 cohenm@sonoma.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

layer of an abscission zone polysaccharide cleavage and cell expansion occur, leading to detachment of the organ. The matrix of hemicellulose and pectin must be disrupted and cellulose microfibrils loosened in the apoplast [the extracellular space encompassing the cell wall (CW) and middle lamella]. This can proceed by relaxation of polysaccharide strand tension by expansin proteins [1] combined with CW hydration [2] and strand breakage, either through the action of hydrolytic enzymes such as pectinases and cellulases [3], or by free radical-mediated reactions [4]. Although an array of CW-hydrolyzing enzymes has been characterized, much remains to be learned about the means by which plants achieve controlled production of free radicals in the apoplast. In this review we discuss free radical-mediated CW loosening in a variety of contexts and possible mechanisms for regulation and localized generation of free radicals in the apoplast, with focus on the model plant *Azolla*.

2. Two modes of abscission in *Azolla*

Small floating water ferns of the genus *Azolla* are distributed worldwide in still or slow-moving bodies of water. Individual fronds are typically not much greater than a centimeter in diameter and have several roots that tend to become entangled, resulting in mat formation (Fig 1). The plants are particularly amenable to experimentation owing to their fast (ca. three-day) generation time and since they can be readily exposed to chemical treatments simply by supplementing their medium. Upon induction abscission zones, occurring both at the base of branches and at the attachment point between the root and frond, undergo CW loosening and cell expansion similar to that observed of other plants (Fig. 1). *Azolla* plants appear to be unique in having two distinct types of abscission responses that differ in timing, in the extracellular polymers targeted for degradation, and in the mechanism of regulation.

2.1 Ethylene-induced abscission

Abscission in plants typically occurs in response to ethylene. In *Azolla*, like other plants, the ethylene response is inhibited by cycloheximide, indicating the need for protein synthesis and subsequent export of protein components into the apoplast [5]. Ethylene-induced abscission in the small floating fern *Azolla filiculoides*, also similar to some other plants, is accompanied by an increase in cellulase and polygalacturonase activity along with a marked loss of primary CW structural integrity as observed by transmission electron immunomicroscopy [5]. A role for endogenously generated ethylene in root and frond abscission of *Azolla* plants has yet to be investigated but would presumably be involved in detachment of old roots and developmental control of frond size. The 6–8 h time lag for abscission to occur following exposure of *A. filiculoides* plants to ethylene is within the range of response times typically observed for flower and leaf abscission in other plants [5]; detachment of *Geranium* petals is the fastest documented abscission response to ethylene, occurring within 2.25 hours of treatment [6]. For a comprehensive review of ethylene-sensitive abscission we refer the reader to a recent review article [3].

2.2 The rapid abscission phenomenon

In addition to ethylene-induced abscission, *Azolla* plants exhibit the fastest abscission responses known in nature, separating branches and dropping roots within minutes in response to various chemical exposures [7, 8] and heat [9]. This frees the fronds from root entanglements, allowing them to escape to potentially better conditions.

Aside from its speed, the nature of the CW disruption is another key distinction between rapid abscission and ethylene-responsive in *Azolla*. Cell separation in *A. filiculoides* during rapid abscission coincides most notably with dissolution of pectin in the middle lamella rather than the primary CW dissolution seen during ethylene-induced abscission [10, 11]. In this sense the rapid abscission phenomenon is similar the *Impatiens* model for leaf

abscission, in which degradation of pectin in the middle lamella is associated with cell separation (see [10, 12] and refs. therein). Subsequent expansion of cells may be due to activity of expansins that loosen cellulose microfibrils [1, 2]. Cell separation during rapid abscission occurs under neutral to slightly alkaline conditions, unlike ethylene-sensitive abscission of *Phaseolus* leaf petioles, which is favored by low pH [10]. A role for preformed proteins in rapid abscission is implied in the lack of effect by actinomycin D or cycloheximide on the response [8]. Substantial declines in cell separation observed after application of the protease papain point to an apoplastic location for at least some essential proteins in the rapid abscission process [11]. Rapid abscission is not accompanied by any discernible increase in cellulase and polygalacturonase activity [5] and CW-degrading enzymes exogenously supplied to manually detached roots only partially incite the cell separation event, findings that are consistent with the hypothesis that breakage of CW polysaccharides in rapid abscission occurs via controlled free radical attack rather than hydrolytic enzymes [10]. In this review we introduce a hypothetical model for the mechanism of rapid abscission in *Azolla* that draws upon our understanding of free radical chemistry and CW loosening processes in other plant systems.

3. A role for free radicals in cell wall loosening

3.1 Techniques for studying free radical attack of apoplastic polysaccharides

^3H -fingerprinting has been applied in a variety of plant species to demonstrate free radical-mediated cleavage of polysaccharides during processes that involve CW loosening, including cress seed germination, cell elongation [4] and fruit ripening [13]. Free radical attack can, depending on the atom of the polysaccharide targeted, convert glycosidic bonds to unstable ester bonds or result in formation of relatively stable carbonyl groups in addition to causing chain scission. Tissues are treated in the ^3H -fingerprinting method with NaB_3H_4 to reduce carbonyl groups, leaving the reduced carbons bound to ^3H . The tissue is then enzymatically digested and the products analyzed. Exposure of CW polysaccharides to hydroxyl radicals ($\cdot\text{OH}$) *in vitro* results in patterns of degradation products similar to those seen in ripening pear fruit [13]. $\cdot\text{OH}$ are produced in the apoplasts of elongating radicle cells and of endosperm cells undergoing cap weakening, as ascertained by electron paramagnetic resonance (EPR) using and $\cdot\text{OH}$ spin traps to complement ^3H -fingerprinting results [4]. Similarly, spin traps form adducts with H_2O_2 -derived $\cdot\text{OH}$ in isolated pea root CW [14].

One difficulty in utilizing biochemical analysis to address roles for free radicals in abscission zone CW loosening lies in obtaining sufficient cell biomass that is free of uninvolved boundary cells. This is especially difficult for *Azolla* abscission zones which measure only about 300 μm in diameter. Synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy can be applied to investigate chemical changes in abscised root tips. It allows for non-invasive high resolution visualization of chemical bonds and functional groups *in situ* [15, 16]. The thin root diameter of *Azolla* is actually an advantage for spectromicroscopy since it permits penetration of the infrared light beam. After treatment with an inducer of rapid abscission, abscised ends of dropped roots have FTIR profile signatures that are diagnostic of $\cdot\text{OH}$ attack, which are distinct from those of other root regions and of abscission zones in untreated still-attached roots that do not show evidence of $\cdot\text{OH}$ attack (G. Birarda, unpublished results). The presence of a reaction “fingerprint” for $\cdot\text{OH}$ does not immediately indicate that abscission directly involves $\cdot\text{OH}$ -mediated oxidative damage/bond cleavage. Existing experimental evidence cannot rule out the possibility that the plant tissue in the vicinity of the cleavage site simply undergoes oxidative damage after abscission and, therefore, is merely a marker for abscission as opposed to a direct cause of abscission. Recent advancements in FTIR spectromicroscopy should allow for real-time monitoring of abscission to investigate whether the oxidative changes precede or follow the separation event.

3.2 Possible mechanisms of free radical generation and action

Up to this point, evidence for the idea that $\cdot\text{OH}$ -mediated oxidative damage/cleavage of plant tissue is involved in the mechanism of abscission has been presented and discussed. However, it is worth noting that $\cdot\text{OH}$ is extremely reactive, short-lived (10^{-9} s) in biological systems and acts at a short distance [17]. Moreover, conditions for its generation are very specific. $\cdot\text{OH}$ (or its reactive equivalent, *vide infra*) can be generated in primarily two ways. In biological systems: i) the Fenton reaction and ii) via the decomposition of peroxynitric acid (ONOOH). The Fenton reaction involves the reduction of H_2O_2 by a reduced metal [in biological systems either ferrous (Fe^{II}) or cuprous (Cu^{I}) ion (Reaction 1)]. A reductant is needed to reduce the oxidized metals back to their initial reduced states to make this system catalytic in the metal (Reaction 2).

Due to the high reactivity of $\cdot\text{OH}$, it is proposed that it never dissociates from the metal in the Fenton reaction but instead forms a metal bound species that is highly oxidizing (reviewed in [17]). Considering that the $\cdot\text{OH}$ produced via a Fenton reaction is so highly-reactive, for all intents and purposes it cannot travel beyond its site of generation (due to rapid reaction with its first chemical encounter) or remains associated with the metal responsible for its generation.

The specificity of these oxidations is based purely on the location of the redox metal. That is, if the Fenton reaction is responsible for the formation of the oxidant (either $\cdot\text{OH}$ or the metal-bound oxidant), then the oxidation chemistry will occur specifically where the metal is located. This implies that any physiological mechanism requiring “ $\cdot\text{OH}$ -like” reactivity, must have some means of regulating the location and timing of metal (or metalloprotein) binding. The presence and regulation of H_2O_2 as well as an appropriate reductant are necessary for the Fenton reaction to occur. A burst of H_2O_2 production coinciding with abscission [18] presumably provides substrate for generation of $\cdot\text{OH}$ with either ascorbate, superoxide [4, 13, 19] or dihydroxyphenolics [14, 20] serving as the most likely sources of electrons. This H_2O_2 burst is apparently distinct from the lower level of H_2O_2 production that functions in the abscission signaling process [18].

Polysaccharide cleavage could occur through direct interaction between the target sugar residue and a high-valent metal-oxo or metal-peroxo species with potent $\cdot\text{OH}$ -like reactivity. This is analogous to the proposal for C—C bond cleavage of the side chain of cholesterol by cytochrome P450 [21]. The $\cdot\text{OH}$ -like activity that has been attributed to peroxidases [22] is most likely actually due to their active site formation of such a metal-bound oxygen species.

A hypothetical model summarizing key activities in free radical-mediated CW loosening is presented in Fig. 2. Apoplastic peroxidases are probably central players in the process. Most peroxidases are heme-containing enzymes having three distinct catalytic modes for metabolizing H_2O_2 depending on their physiological context: consumption or production of H_2O_2 , or formation of a potent oxidizing species [22]. Copper ions complexed by CW histidine-rich glycoproteins have been hypothesized as another source of $\cdot\text{OH}$ that function in CW loosening [23].

The network of lignin and other phenolics that are entwined with CW polysaccharides are primarily considered in their structural role. These phenolics strongly complex with copper

[24], which raises the intriguing possibility that they may also direct the polymer-nicking activity of free radicals [20]. Radical attack is particularly focused on arabinose as seen by ^3H -fingerprinting of weakening cress endosperm cap cells [4]. Both hemicellulose [25] and pectin [26] attach to lignin via arabinose. Thus, release of arabinose could indicate phenolic-associated metals as radical-generating sites in cress endosperm cap whereas the lack of arabinose release in elongating cress radical implies a different positioning of the reactive metal in this tissue.

Another way to generate $\cdot\text{OH}$ is via ONOOH , which can be formed in biological systems via the reaction between nitric oxide (NO) and superoxide (O_2^-), initially generating peroxynitrite (ONOO^-) (Reaction 3). Protonation of ONOO^- ($\text{pK}_a = 6.8$) generates ONOOH (Reaction 4). ONOOH is unstable with respect to rearrangement to nitrate (NO_3^-), which is proposed to occur via the intermediacy of $\cdot\text{OH}$ and NO_2 (Reaction 5) (for example, see [27]).

Most of the $\cdot\text{OH}$ generated via ONOOH decomposition results in NO_3^- generation via rapid reaction with NO_2 . Still, a small portion of $\cdot\text{OH}$ can react with other substrates. This appears to be an inefficient mechanism for $\cdot\text{OH}$ generation. Regardless, the degradation of polysaccharides in the extracellular matrix in the mammalian inflammatory response has been proposed to occur by $\cdot\text{OH}$ produced from the decay of ONOO^- [28, 29]. However, conclusive evidence for ONOO^- production in the extracellular space remains to be demonstrated. Reduction of O_2 by an NAD(P)H oxidase can generate O_2^- [19] that can react with NO and form ONOO^- [30]. Significant levels of ONOO^- are generated only if both substrates are produced at the same place, at the same time and at the same flux [31]. Whether these requirements are met in the plant apoplast has yet to be shown. Although treatment of *Azolla pinnata* plants with the ONOO^- donor 3-morpholinysydnonimine (SIN-1) did not elicit abscission [32] the results of experiments using donor agents on live plants should be interpreted with caution since the intensity and location of production are not likely to match those that may occur *in planta*.

4. Evidence for a thiol regulatory target

The treatment of *A. pinnata* plants with up to 10 mM H_2O_2 alone does not induce root abscission [7]. However, in combination with the known abscission-inducing agent nitrite, coexposure to H_2O_2 exhibited a pronounced effect, either stimulatory or inhibitory, depending on the concentration of H_2O_2 (unpublished results). This may be suggestive of a requirement for a specific oxidative balance at the target rather than a simple on/off switch for triggering abscission (Fig. 2).

The signaling interplay between H_2O_2 and nitrogen oxides in cells is often interpreted at a molecular level through their interaction with thiols [17, 33, 34]. We hypothesize that the combinatorial effect of H_2O_2 and nitrite in inducing root abscission could be indicative of a role for cysteine thiol modification in the abscission process. Such a scenario would be possible if iron in the vicinity of the thiol were to catalyze oxidation of nitrite by H_2O_2 , generating oxidants such as NO_2 . This has been demonstrated for nitration of

deoxyguanosine, phenol and tyrosine (see [35] and refs. therein). The formation of sulfenic acid (RSOH) and other oxidized forms of cysteine can occur through exposure of cysteine to peroxides and nitrogen oxides [36, 37]. Oxygen atom transfer from nitrite, for instance, can also form sulfenic cysteine [38]. Oxidative changes of select cysteine residues, including *S*-nitrosylation, sulfenic acid, sulfinic acid and disulfide formation, permit fine-tuned dynamic regulation of numerous proteins [39]. The findings that induction of rapid root abscission of *A. pinnata* plants by nitrite is inhibited by the thiol-reactive glutathione (GSH) and *S*-methyl methanethiosulfonate (MMTS), which do not cross biological membranes, are consistent with a putative role for apoplastic target thiols in the process (unpublished results).

The high potency of azide as an inducer of rapid abscission is of interest. Ethylene-sensitive abscission, which is accompanied by an increase in respiration, is inhibited by heme-binding azide and cyanide [40]. Conversely both of these molecules induce rapid abscission [8, 41]. Rapid root abscission by *A. filiculoides* is most sensitive to and occurs most rapidly in response to azide treatment [8]. Catalase and peroxidases both oxidize azide to a nitrogen oxide species [42, 43], most likely HNO (nitroxyl) [44]. If indeed HNO is generated via azide oxidation by (for example) catalase, the question remains as to whether HNO chemistry can lead to root abscission. If thiols are involved in the mechanism/signaling associated with root abscission, thiol-reactive nitrogen oxide species could likely be involved. Significantly, one of the most predominate chemical traits of HNO is its extreme thiophilicity (see [45] and refs. therein). HNO is particularly efficient at oxidizing sulfhydryl groups. For example, the rate of reaction of HNO with a thiol is approximately 10^6 faster than with H_2O_2 [17]. Modification of thiols by HNO can occur via two distinct pathways leading to either reversible or irreversible products, depending on the conditions. Thus, the biological effects of HNO can be either reversible or irreversible (as would be the case with root abscission).

Admittedly, the involvement of HNO in these processes is highly speculative at this point as other mechanisms for the actions of azide can be proposed as well. NO, the one-electron product of HNO oxidation, is emitted from azide-treated *A. pinnata* plants [46]. NO does not react with thiols but binds heme groups and can thus influence the behavior of peroxidases and catalases [22]. NO can be produced from nitrite by a variety of reductive mechanisms [34] and thiol-nitrosating NO_2 is an intermediate in the oxidation of NO to nitrite [17].

5. Calcium and polyamines in apoplast-associated protein activities

Calcium ions have variable effects on abscission. They can increase resistance to abscission by forming ionic cross-bridges in the CW but are also required for abscission-related cell signaling (see [47] and refs. therein). We have observed that pre-treatment of *A. pinnata* plants with the 5 mM of the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) caused a strong inhibition of nitrite-responsive induction of root abscission that can be reversed by addition of exogenous Ca^{2+} (unpublished results). The fact that EGTA-treated plants are not more prone to lose roots but rather *retain* them suggests that the cementing function of Ca^{2+} is not essential for root attachment. Instead it appears that removing the Ca^{2+} from the apoplast could prevent their use for other purposes.

The binding to pectins by some apoplastic proteins, including peroxidases, is facilitated by Ca^{2+} (see [48] and refs. therein). Moreover, Ca^{2+} is a component of and necessary for the activity of many peroxidases [49]. Ca^{2+} is needed for the fibrin-binding activity of an extracellular histidine-rich glycoproteins (HRG) in humans and Ca^{2+} controls access to the cysteine of fibrin stabilizing factor that associates with HRG [50]. By analogy, Ca^{2+} may facilitate the proper alignment of putative $\cdot OH$ -producing peroxidases and HRGs.

Polyamines can in some instances displace and substitute for Ca^{2+} [51]. Root abscission following treatment of *A. pinnata* plants with spermine and spermidine [7] could conceivably be related to liberation of Ca^{2+} and resultant alteration of CW-metabolizing enzyme activity by these polyamines; plants treated with the polyamine oxidase inhibitor hydroxyethyl hydrazine abscise roots in response to polyamines as do untreated plants, indicating that H_2O_2 production from polyamines is not a mechanism for inducing abscission [7].

Abscission in *Azolla* and other plants is dependent on the expansion of cells in the abscission zone [11]. It is well established that plant cell expansion requires an influx of Ca^{2+} into the cytoplasm where it acts as a secondary messenger and activates the O_2^- -generating NAD(P)H-oxidase in the plasmodesmata membrane [2, 33]. Whether the Ca^{2+} channels of abscission zone cells are positively regulated by H_2O_2 as they are in guard cells [52] remains to be experimentally demonstrated.

6. Perspectives and future directions

Research accumulated over the past two decades has shown that controlled free radical attack of CW polymers can be a primary cause of CW loosening. The apparent independence of rapid root abscission from ethylene-induced abscission contributes to the usefulness of *Azolla* as a model study system. The induction of plant CW loosening that follows binding of ethylene to a copper-containing membrane receptor is thought to occur primarily through increase in expression of expansins and hydrolytic enzymes [3]. We expect, however, that even this well-studied ethylene response will show the involvement of free radical-mediated CW breakage upon closer investigation. The upregulation of preformed leaf abscission zone peroxidases in response to ethylene treatment in citrus, for instance, may point to such a role [53].

Much progress has been made but we are still far from being able to pinpoint the sequence of regulatory events that result in abscission. The putative involvement of peroxidases illustrates the complexity we face since plants express several dozen apoplastic peroxidases [48]. We could focus on candidates that warrant further investigation by applying deductive reasoning to mutational and physiological analyses. We expect that regulatory targets would possess the following features: (i) localization in the apoplast; (ii) the capacity to metabolize H_2O_2 or O_2^- ; and (iii) modifiable cysteine thiol group(s). With these considerations in mind, an ortholog of the N-type secretory peroxidase of *Arabidopsis* [54] is of interest since it possesses an available cysteine within its putative pectin binding region, perhaps indicative of a regulatory role for this residue. Other candidates include a class of peroxidases called peroxiredoxins, which occur in plant CWs [55] and have an active site cysteine that is oxidized to sulfenic acid in the peroxidase reaction [56]. Alternatively, regulation could take place through a thiol-containing intermediate, such as a thioredoxin. The H_2O_2 -sensitive thioredoxin OsTRXh1 functions within the apoplast in stress responses [57]. Regulatory targets of OsTRXh1 are as yet unknown but a major function for thioredoxins in the cytoplasm is reductive recycling of peroxiredoxins [58, 59].

More broadly, the dynamism of the apoplast and its interconnectedness with cytoplasmic processes is apparent in the CW loosening that accompanies abscission. We hypothesize that signaling molecules, such as H_2O_2 and NO, generated in the environment or in the cytoplasm diffuse into the apoplast where they can both regulate and serve as intermediaries for production of free radicals. Considering the likelihood of protein thiol and heme in the regulatory process, the influence of reduced sulfur species should also be investigated. The treatment of cut flowers with an H_2S donor, for instance, inhibits floral abscission [60]. It should hardly be surprising that plants would evolve to employ small O, N and S molecules

for exerting regulatory control over their extracellular spaces. The cell membrane diffusibility and precise action of these molecules on their targets should allow for tight coupling of cytoplasmic and apoplastic events.

Acknowledgments

We thank the editor and several anonymous reviewers for their constructive criticisms of an earlier version of this manuscript and A. Bermudez and L. Blakney for technical assistance. This work was in part supported by funding from the National Institutes of Health (HL106598) to JMF and a Grant-in-Aid for Scientific Research (B) from Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan to HY.

References

1. Braidwood L, Breuer C, Sugimoto K. My body is a cage: mechanisms and modulation of plant cell growth. *New Phytol.* 2013
2. Wolf S, Hématy K, Höfte H. Growth control and cell wall signaling in plants. *Ann. Rev. Plant Biol.* 2012; 63:381–407. [PubMed: 22224451]
3. Estornell LH, Agustí J, Merelo P, Talón M, Tadeo FR. Elucidating mechanisms underlying organ abscission. *Plant Sci.* 2013; 199–200:48–60.
4. Müller K, et al. In vivo cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. *Plant Physiol.* 2009; 150:1855–1865. [PubMed: 19493972]
5. Uheda E, Nakamura S. Abscission of *Azolla* branches induced by ethylene and sodium azide. *Plant Cell Physiol.* 2000; 41:1365–1372. [PubMed: 11134422]
6. Sexton R, Struthers W, Lewis L. Some observations on the very rapid abscission of the petals of *Geranium robertianum* L. *Protoplasma.* 1983; 116:179–186.
7. Gurung S, Cohen MF, Fukuto J, Yamasaki H. Polyamine-induced rapid root abscission in *Azolla pinnata*. *J. Amino Acids.* 2012; 2012:9.
8. Uheda E, Kitoh S. Rapid shedding of roots from *Azolla filiculoides* plants in response to inhibitors of respiration. *Plant Cell Physiol.* 1994; 35:37–43.
9. Uheda E, Kitoh S, Shiomi N. Response of six *Azolla* species to transient high-temperature stress. *Aquatic Bot.* 1999; 64:87–92.
10. Fukuda K, Yamada Y, Miyamoto K, Ueda J, Uheda E. Separation of abscission zone cells in detached *Azolla* roots depends on apoplastic pH. *J. Plant Physiol.* 2013; 170:18–24. [PubMed: 22940290]
11. Uheda E, Nakamura S, Kitoh S. Events associated with the rapid separation of cells from detached roots of *Azolla filiculoides* depend on pH. *J. Exp. Bot.* 1994; 45:1451–1457.
12. Bowling AJ, Vaughn KC. Leaf abscission in *Impatiens* (Balsaminaceae) is due to loss of highly de-esterified homogalacturonans in the middle lamellae. *Am. J. Bot.* 2011; 98:619–629. [PubMed: 21613162]
13. Fry SC, Dumville JC, Miller JG. Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit. *Biochem. J.* 2001; 357:729. [PubMed: 11463343]
14. Kukavica B, et al. Generation of hydroxyl radical in isolated pea root cell wall, and the role of cell wall-bound peroxidase, Mn-SOD and phenolics in their production. *Plant Cell Physiol.* 2009; 50:304–317. [PubMed: 19098072]
15. Holman H-YN, Bechtel HA, Hao Z, Martin MC. Synchrotron IR spectromicroscopy: Chemistry of living cells. *Anal. Chem.* 2010; 82:8757–8765. [PubMed: 20839782]
16. Lacayo CI, et al. Imaging cell wall architecture in single *Zinnia elegans* tracheary elements. *Plant Physiol.* 2010; 154:121–133. [PubMed: 20592039]
17. Fukuto JM, et al. Small molecule signaling agents: the integrated chemistry and biochemistry of nitrogen oxides, oxides of carbon, dioxygen, hydrogen sulfide, and their derived species. *Chem. Res. Toxicol.* 2012; 25:769–793. [PubMed: 22263838]
18. Sakamoto M, Munemura I, Tomita R, Kobayashi K. Reactive oxygen species in leaf abscission signaling. *Plant Signal. Behav.* 2008; 3:1014–1015. [PubMed: 19704438]

19. Heyno E, Mary V, Schopfer P, Krieger-Liszak A. Oxygen activation at the plasma membrane: relation between superoxide and hydroxyl radical production by isolated membranes. *Planta*. 2011; 234:35–45. [PubMed: 21359959]
20. Sakihama Y, Cohen MF, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology*. 2002; 177:67–80. [PubMed: 12126796]
21. Strushkevich N, et al. Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proc. Nat. Acad. Sci. USA*. 2011; 108:10139–10143. [PubMed: 21636783]
22. Almagro L, et al. Class III peroxidases in plant defence reactions. *J. Exp. Bot.* 2009; 60:377–390. [PubMed: 19073963]
23. Fry SC, Miller JG, Dumville JC. A proposed role for copper ions in cell wall loosening. *Plant Soil*. 2002; 247:57–67.
24. Wieber J, Kulik F, Pethica BA, Zuman P. Sorptions on lignin, wood and celluloses. III. copper(II) and zinc(II) ions. *Colloid. Surface*. 1988; 33:141–152.
25. Goto M, Gordon AH, Chesson A. Changes in cell-wall composition and degradability of sorghum during growth and maturation. *J. Sci Food Agric*. 1991; 54:47–60.
26. Minor JL. Location of lignin-bonded pectic polysaccharides. *J. Wood. Chem. Technol*. 1991; 11:159–169.
27. Gunaydin H, Houk KN. Molecular dynamics simulation of the HOONO decomposition and the HO•/NO2• caged radical pair in water. *J. Am. Chem. Soc*. 2008; 130:10036–10037. [PubMed: 18613683]
28. Duan J, Kasper DL. Oxidative depolymerization of polysaccharides by reactive oxygen/nitrogen species. *Glycobiology*. 2011; 21:401–409. [PubMed: 21030538]
29. Hrabarova E, Juranek I, Soltes L. Pro-oxidative effect of peroxynitrite regarding biological systems: a special focus on high-molar-mass hyaluronan degradation. *Gen. Physiol. Biophys*. 2011; 30:223. [PubMed: 21952432]
30. Vandelle E, Delledonne M. Peroxynitrite formation and function in plants. *Plant Sci*. 2011; 181:534–539. [PubMed: 21893249]
31. Fukuto JM, Ignarro LJ. *In vivo* aspects of nitric oxide (NO) chemistry: does peroxynitrite (-OONO) play a major role in cytotoxicity? *Accounts Chem. Res*. 1997; 30:149–152.
32. Gurung, S. Biochemistry and physiology of root abscission in the water fern *Azolla pinnata* Faculty of Science. Okinawa, Japan: University of the Ryukyus; 2007. p. 98
33. Matilla-Vázquez, M.; Matilla, A. Role of H₂O₂ as signaling molecule in plants. In: Ahmad, P.; Prasad, MNV., editors. *Environmental adaptations and stress tolerance of plants in the era of climate change*. New York: Springer; 2012. p. 361–380.
34. Yamasaki H. The NO world for plants: achieving balance in an open system. *Plant Cell Environ*. 2005; 28:78–84.
35. Castro L, Eiserich JP, Sweeney S, Radi R, Freeman BA. Cytochrome *c* : a catalyst and target of nitrite-hydrogen peroxide-dependent protein nitration. *Archiv. Biochem. Biophys*. 2004; 421:99–107.
36. Poole LB, Nelson KJ. Discovering mechanisms of signaling-mediated cysteine oxidation. *Curr. Opin. Chem. Biol*. 2008; 12:18–24. [PubMed: 18282483]
37. Kettenhofen NJ, Wood MJ. Formation, reactivity, and detection of protein sulfenic acids. *Chem. Res. Toxicol*. 2010; 23:1633–1646. [PubMed: 20845928]
38. Heinecke J, Ford PC. Formation of cysteine sulfenic acid by oxygen atom transfer from nitrite. *J. Am. Chem. Soc*. 2010; 132:9240–9243. [PubMed: 20565124]
39. Spadaro D, et al. The redox switch: dynamic regulation of protein function by cysteine modifications. *Physiol. Plant*. 2010; 138:360–371. [PubMed: 19912563]
40. Addicott FT. Environmental factors in the physiology of abscission. *Plant Physiol*. 1968; 43:1471. [PubMed: 16657013]
41. Uheda E, Nakamura S, Kitoh S. Aspects of the very rapid abscission of *Azolla* branches: anatomy and possible mechanism. *Int. J. Plant Sci*. 1995:756–763.

42. Ogino K, et al. Catalase catalyzes nitrotyrosine formation from sodium azide and hydrogen peroxide. *Free Rad. Res.* 2001; 35:735–747.
43. Tatarko M, Bumpus JA. Further studies on the inactivation by sodium azide of lignin peroxidase from *Phanerochaete chrysosporium*. *Archiv. Biochem. Biophys.* 1997; 339:200–209.
44. Reisz JA, Bechtold E, King SB. Oxidative heme protein-mediated nitroxyl (HNO) generation. *Dalton Trans.* 2010; 39:5203–5212. [PubMed: 20502824]
45. Fukuto JM, Carrington SJ. HNO signaling mechanisms. *Antioxid. Redox Sign.* 2011; 14:1649–1657.
46. Gurung S, Cohen MF, Yamasaki H. Azide-dependent NO emission from the water fern *Azolla pinnata*. *Russ. J. Plant Physiol.* 2014 in press.
47. Xu T, Li T, Qi M. Calcium requirement for ethylene-induced abscission. *J. Plant Nutr.* 2009; 32:351–366.
48. Passardi F, Penel C, Dunand C. Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci.* 2004; 9:534–540. [PubMed: 15501178]
49. Plieth C, Vollbehr S. Calcium promotes activity and confers heat stability on plant peroxidases. *Plant Signal. Behav.* 2012; 7:650–660. [PubMed: 22580695]
50. Leung L. Interaction of histidine-rich glycoprotein with fibrinogen and fibrin. *J. Clin. Invest.* 1986; 77:1305. [PubMed: 3958188]
51. Charnay D, Nari J, Noat G. Regulation of plant cell-wall pectin methyl esterase by polyamines — Interactions with the effects of metal ions. *Eur. J. Biochem.* 1992; 205:711–714. [PubMed: 1572369]
52. Wang P, Song CP. Guard-cell signalling for hydrogen peroxide and abscisic acid. *New Phytol.* 2008; 178:703–718. [PubMed: 18373649]
53. Gaspar T, Goren R, Huberman M, Dubucq M. Citrus leaf abscission. Regulatory role of exogenous auxin and ethylene on peroxidases and endogenous growth substances. *Plant Cell Environ.* 1978; 1:225–230.
54. Mirza O, Henriksen A, Ostergaard L, Welinder KG, Gajhede M. *Arabidopsis thaliana* peroxidase N: structure of a novel neutral peroxidase. *Acta Crystallogr. D.* 2000; 56:372–375. [PubMed: 10713531]
55. Jamet E, et al. Recent advances in plant cell wall proteomics. *Proteomics.* 2008; 8:893–908. [PubMed: 18210371]
56. Rhee SG, Yang K-S, Kang SW, Woo HA, Chang T-S. Controlled elimination of intracellular H₂O₂: regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. *Antioxid. Redox Sign.* 2005; 7:619–626.
57. Zhang C-J, et al. An apoplastic h-type thioredoxin is involved in the stress response through regulation of the apoplastic reactive oxygen species in rice. *Plant Physiol.* 2011; 157:1884–1899. [PubMed: 22010108]
58. Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. *Mol. Cell.* 2007; 26:1–14. [PubMed: 17434122]
59. Tovar-Méndez A, et al. Peroxiredoxins and NADPH-dependent thioredoxin systems in the model legume *Lotus japonicus*. *Plant Physiol.* 2011; 156:1535–1547. [PubMed: 21562331]
60. Zhang H, et al. Hydrogen sulfide acts as a regulator of flower senescence in plants. *Postharvest Biol. Technol.* 2011; 60:251–257.
61. Cohen, MF.; Mazzola, M.; Yamasaki, H. Nitric oxide research in agriculture: Bridging the plant and bacterial realms. In: Rai, AK.; Takabe, T., editors. *Abiotic Stress Tolerance in Plants: Toward the improvement of global environment and food*. New York: Springer Verlag; 2006. p. 71-90.

Highlights

- Abscission of plant organs requires cell wall loosening.
- Rapid cell wall loosening occurs via free radical attack of structural polymers.
- Free radicals are formed in the apoplast by a variety of potential mechanisms.
- Thiol modifications and Ca^{2+} influence in rapid abscission of *Azolla* water ferns.
- O, N and S molecular species potentially function in regulating rapid abscission.

Fig. 1. Rapid abscission of *Azolla filiculoides*. Side view (A) and top view (B) of plants prior to exposure to 5 mM sodium nitrite and 1 mM sodium azide in 10 mM K-phosphate buffer (pH 7), and side view 0.5 h after onset of exposure (C). A dark-field micrograph of the detached end of a root from a plant induced to abscise by treatment with 5 mM sodium nitrite showing expanded cells within the abscission zone (D).

Fig. 2.

A hypothetical scheme for free radical-mediated rapid abscission in *Azolla*. Three possible mechanisms are shown for generating polysaccharide-cleaving free radicals from H_2O_2 via: (i) peroxidase (POX), (ii) metal-bound histidine-rich glycoprotein (HRG), or (iii) metal-bound phenolics. Available reductants, which for simplicity are only shown for the POX-mediated reaction, include ascorbic acid (Asc), O_2^- and hydroxycinnamic acids (HCA). O_2^- may also be converted to H_2O_2 by superoxide dismutase (SOD). Scission events (yellow stars) could be initiated through the activation of a putative preformed target protein in the apoplast regulated by combinatorial oxidative modifications to a cysteine thiol. In the apoplast there are a variety of mechanisms for reducing nitrite back to NO [61]. Azide (N_3^-) and nitrite (NO_2^-) are two of several known exogenous inducers of rapid abscission in *Azolla*. Azide is an especially potent inducer of abscission that can be oxidized by POX to HNO, which reacts rapidly with thiols. We hypothesize that the modification state of a thiol could be the trigger for the onset of rapid abscission. The observed Ca^{2+} dependency of rapid root abscission could be a consequence of many factors, including its role in binding POX to pectin. cytb, cytochrome b; MDA, monodehydroascorbate.