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Review

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*

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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 H_2O_2 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 Ca^{2+} dependency of rapid abscission may reflect the stabilization Ca^{2+} confers to peroxidase structure and binding to pectin. To spur further investigation, we present a hypothetical model for small signaling molecules H_2O_2 and NO and their derivatives in regulating, via modification of putative protein thiols, free radical attack of apoplastic polysaccharides.

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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 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

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[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 h 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

^{3}H-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 ^{3}H-fingerprinting method with NaB_3H_4 to reduce carbonyl groups, leaving the reduced carbons bound to ^{3}H. The tissue is then enzymatically digested and the products analyzed. Exposure of CW polysaccharides to hydroxyl radicals ($^{\bullet}$OH) in vitro results in patterns of degradation products similar to those seen in ripening pear fruit [13]. $^{\bullet}$OH are produced in the apoplasts of elongating radicle cells and of endosperm cells undergoing cap weakening, as ascertained by electron paramagnetic resonance (EPR) with a $^{\bullet}$OH spin trap to complement ^{3}H-fingerprinting results [4]. Similarly, spin traps form adducts with H_2O_2-derived $^{\bullet}$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 $^{\bullet}$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 $^{\bullet}$OH attack (G. Birarda, unpublished results). The presence of a reaction "fingerprint" for $^{\bullet}$OH does not immediately indicate that abscission directly involves $^{\bullet}$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