Trends in Plant Science

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Opinion

How Does pH Fit in with Oscillating Polar Growth?

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Polar growth in root hairs and pollen tubes is an excellent model for investigating plant cell size regulation. While linear plant growth is historically explained by the acid growth theory, which considers that auxin triggers apoplastic acidification by activating plasma membrane P-type H^+-ATPases (AHAs) along with cell wall relaxation over long periods, the apoplastic pH ($_{apo}$pH) regulatory mechanisms are unknown for polar growth. Polar growth is a fast process mediated by rapid oscillations that repeat every ~20–40 s. In this review, we explore a reactive oxygen species (ROS)-dependent mechanism that could generate oscillating $_{apo}$pH gradients in a coordinated manner with growth and Ca^{2+} oscillations. We propose possible mechanisms by which $_{apo}$pH oscillations are coordinated with polar growth together with ROS and Ca^{2+} waves.

Highlights

Recent reports provide new molecular evidence in support of the acid growth theory, which proposes that auxin triggers linear plant cell growth via the TIR1/AFB-Aux/IAA pathway and $_{apo}$pH acidification (e.g., in hypocotyls and root cells).

$_{apo}$pH in linear growing cells is regulated by AHAs via the autoinhibitory C-terminal domain, phosphorylation, and protein interactors.

Root hairs and pollen tubes undergo polar growth in an oscillatory manner as rapidly as several hundred microns over a few hours. This oscillatory polar growth is regulated by Ca^{2+}, ROS, and pH gradients. A complete understanding of how oscillating $_{apo}$pH is regulated remains elusive.

$_{apo}$pH oscillations are coupled Ca^{2+} and ROS waves that coordinate growth by unknown mechanisms.

Control of Polar Cell Expansion

A central question in plant biology is how plant cells integrate endogenous and environmental signals to regulate growth. Plant cells exhibit several types of expansion: isotropic growth in all directions (e.g., meristematic cells), anisotropic growth in two directions with one prevalent over the other (e.g., hypocotyls and root cells), polar growth in a single direction (e.g., pollen tubes and root hairs), or a combination of these (e.g., epidermal cells at the leaf surface). Both root hairs and pollen tubes are single plant cells that expand to a final length that is several hundred-fold their width, and are an excellent model system for studying cell size regulation [1–4]. Root hairs rapidly sense and respond to changes in extracellular nutrient and water status and intracellular hormones (e.g., auxin and ethylene) [4], elongating over the course of approximately 6–8 h in a process controlled by the basic helix-loop-helix transcription factor **ROOT HAIR DEFECTIVE 6-LIKE 4** (RSL4; see Glossary) [1,4]. In the case of pollen tubes, cell elongation is a key process that allows them to penetrate the stigma and grow rapidly through the pistil to deliver the sperm cells for fertilization [5]. This process is controlled by complex autocrine and paracrine signaling mechanisms, some of them only recently discovered [6,7].

The plant cell is surrounded by a rigid cell wall containing polysaccharides and (glyco)proteins, which provides stability and a cell shape linked to a defined function [8,9]. Cell wall expansion is a complex process involving intracellular turgor pressure, relaxation of the existing cell wall, and secretion of new cell wall materials and plasma membrane. Cell expansion is coordinately regulated by dynamic changes in ROS (Box 1), Ca^{2+} gradients (Box 2), and fluctuations in apoplastic/cytoplasmic pH ($_{apo/cyt}$pH) as well as by several other factors, including actin and microtubule cytoskeletal networks, vesicle secretion via actin-mediated exocytosis, and cell wall-modifying proteins [8,10,11]. The detailed molecular mechanisms of how ROS, Ca^{2+}, and pH impact plant growth are described elsewhere [12–17]. Sensors at the interface between the apoplast and the cytoplasmic face of the plasma membrane, such as *Catharanthus roseus RECEPTOR-LIKE KINASE* (*CrRLK1L*) *ANXUR1 and 2* (*ANX1/ANX2*) in pollen tubes [18] and *FERONIA* (*FER*) in root hairs and roots [19,20], control cell expansion (reviewed in [21]).

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Box 1. Key Concepts Relating to $_{apo}$ROS and ROS-Linked Enzymes on Polar Growing Cells

ROS include atmospheric oxygen [singlet oxygen ($^1O_2$)], superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$). H_2O_2 is the most stable ROS species, with a half-life of approximately 1 ms; it acts as an intercellular and intracellular signal triggering downstream responses. SUPEROXIDE DISMUTASES (SODs) enzymatically dismutate $O_2^{\bullet-}$ in the apoplast to $_{apo}H_2O_2$. Oscillations in ROS suggest that the ROS amplitude needs to be close to an optimum and to decline below cytotoxic levels to avoid cell death or to just above cytostatic levels to avoid inhibiting cell proliferation and inducing developmental abnormalities. If ROS levels spike above normal, several ROS-scavenging enzymes in the apoplast, cytoplasm, and organelles restore homeostatic levels, including ASCORBATE PEROXIDASES (APXs), CATALASES (CATs), mitochondrial OXIDASES (AOXs), and Cu-Zn-SUPEROXIDE DISMUTASES (CSDs). Low-molecular-weight antioxidants (e.g., ascorbate, glutathione, and tocopherol) act as redox buffers that determine the lifetime of $_{cyt}$ROS. NADPH OXIDASES (NOXs), also called RESPIRATORY BURST OXIDASE HOMOLOG proteins (RBOHs), catalyze $_{apo}$ROS production (see Figure 1 in the main text). NOXC acts in root hairs, whereas NOXH/NOXJ acts in pollen tubes and root hairs. NOXs are important generators of $_{apo}$ROS during polar growth [3,12,45–47]. Secreted type-III PEROXIDASES (PERs) have key antagonistic roles in $_{apo}$ROS homeostasis (Figure 1) because they can produce ROS in oxidative cycles and metabolize ROS in peroxidative and hydroxylic cycles, leading to either cell wall rigidification or cell wall relaxation, respectively [36]. In the oxidative cycle, PERs reduce dioxygen (O_2) to superoxide radicals ($O_2^{\bullet-}$). In the hydroxylic cycle, PERs generate hydroxyl radicals ($^{\bullet}OH$) from H_2O_2. PERs use H_2O_2 as an oxidant to form covalent linkages between cell wall phenolic compounds and structural proteins (e.g., extensins) [33], or convert H_2O_2 into $^{\bullet}OH$, which cleaves polysaccharides in a nonenzymatic fashion [36]. Other enzymes can produce $_{apo}$ROS, such as amine and oxalate oxidases, which are beyond the scope of this review.

Box 2. Ca^{2+} Transporters Are Key Elements That Generate the Ca^{2+} Gradient in Polar Growing Cells

Ca^{2+} is transported from the apoplast to the cytoplasm or mobilized from intracellular reservoirs to the cytoplasm to generate the Ca^{2+} gradient that functions in polar growth (see Figure 1 in the main text). Given that the Ca^{2+} maximum is oscillatory, excess Ca^{2+} must be actively transported against its electrochemical gradient from the cytosol to the apoplast and endomembrane system. Several different Ca^{2+} transporters and channels were recently characterized in root hairs and pollen tubes. Two different types of plasma membrane channel were identified that transport Ca^{2+} from the apoplast to the cytoplasm in root hairs and pollen tubes: CYCLIC NUCLEOTIDE-GATED CHANNELS (CNGCs) and GLUTAMATE-LIKE RECEPTORS (GLRs). OsCNGC13/AtCNGC18 function in pollen tube guidance [62], whereas AtCNGC14 functions in growing root hairs [60]. No GLRs have been reported in root hairs, but AtGLR1.2 and AtGLR3.7 function in pollen tube growth [62] and PpGLR1/PpGLR2 function in *Physcomitrella patents* reproduction [61]. Ca^{2+} transport from the cytosol to the apoplast is catalyzed by the autoinhibited type-P_{IIB} Ca^{2+}-ATPases (ACAs) ACA8–ACA10, although none of these have been characterized in polar growing cells. Ca^{2+}/H^+ ANTIPORTER (CAXs) could transport Ca^{2+} from the cytosol to the apoplast, but no CAX members have been identified in root hairs. ACAs also transport Ca^{2+} from the cytosol to the endomembrane system, where CAXs, ACAs, and autoinhibited type-P_{IIA} Ca^{2+}-ATPases (ECAs) mediate Ca^{2+} storage in vacuoles, mitochondria, and endoplasmic reticulum (ER)–Golgi. ECA1/ECA2 are localized in the ER, whereas ECA3 is localized in Golgi and endosomes. ACA4–ACA11 are localized in the vacuolar membrane. ACA9 is localized at the pollen tube plasma membrane and modulates Ca^{2+} homeostasis. Currently, no ACAs/ECAs have been reported in root hair cells. The counter ion (H^+) movement provides the driving force for Ca^{2+} transport in the Ca^{2+}/H^+ ANTIPORTER (CAX). CAX1 and CAX3 sequester $_{cyt}Ca^{2+}$ into the vacuole [25]. The fast mobilization of Ca^{2+} from vacuoles is mediated by TWO-PORE CHANNEL1 (TPC1), which functions as a vacuolar Ca^{2+} channel [64].

Plant cell expansion requires $_{apo}$pH acidification from 6–7 to 4.5~5.5, which is triggered by plasma membrane-localized **AHAs**. This is known as the acid growth theory, which was first proposed in 1970 [22] and subsequently revised and updated [23,24]. $_{apo}$pH is also regulated by Ca^{2+}/H^+ (CAXs) [25] and Na^{2+}/H^+ antiporters [26], but this mechanism is beyond the scope of this review. AHA activity is crucial for most plant developmental processes, because the double-null mutant *aha1aha2* (with defects in the two most widely expressed AHAs of the 11 present in the *Arabidopsis* genome) is embryonic lethal [27]. Auxin might control the $_{apo}$pH by local activation of AHAs in expanding root cells [20] and growing hypocotyls [28], but it is important to highlight that auxin promotes or inhibits growth depending on its concentration as well as the underlying cell type. The $_{apo}$pH rapidly changes in response to stresses (e.g., salinity, drought, flooding, and anoxia) and plant interactions with pathogens and symbionts. Here, we present recent advances in our understanding of how $_{apo}$pH oscillations control polar cell expansion in the root hair and pollen tube tips. We also discuss a hypothetical scenario by which AHAs might be regulated in these cells.

Glossary

NADPH OXIDASES (NOXs) or RESPIRATORY BURST OXIDASE HOMOLOG proteins (RBOHs): ROS-producing enzymes are localized in the plasma membrane. NOXs release superoxide anions ($O_2^{\bullet-}$) to the apoplast and require NADPH as a cytosolic electron donor to the extracellular O_2 electron acceptor, which is reduced to $O_2^{\bullet-}$ via FAD. Then, $O_2^{\bullet-}$ can be dismutated to hydrogen peroxide (H_2O_2). For details, see [3,10,44–50].

P-type H^+-ATPase superfamily of cation-transporters (AHAs): pump protons (H^+) out of the cell, which generates a gradient that drives the active uptake of nutrients and controls apoplastic pH. AHA1 and AHA2 are localized at the plasma membrane and control the apoplastic pH in hypocotyl and root cells. The long-standing acid growth theory is based on AHA activities. For details, see [21,27,29,30,71].

PLASMA MEMBRANE INTRINSIC PROTEINS (PIPs): proteins of the aquaporin family involved in responses to biotic and abiotic stresses. PIPs are ubiquitous membrane channels that facilitate the permeation of water and other molecules, such as urea, glycerol, and H_2O_2, across the plasma membrane. For details, see [52–56].

ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4): transcription factor of the basic helix-loop-helix (bHLH) family that is sufficient to promote postmitotic growth in root hair cells. RSL4 is a direct transcriptional target of *root hair defective 6* (*RHD6*) and determines the final root hair length based on its expression level. It is involved in root hair initiation, response to auxin stimulus, positive regulation of transcription, cell growth, and transcriptional regulation. For details, see [1,4].

Secreted type-III PEROXIDASES (PERs): these heme-containing class III plant peroxidases have complex relationships with ROS. They are known primarily as H_2O_2-reducing enzymes that oxidize or polymerize various hydrogen donors and convert H_2O_2 into water. PERs also promote the formation of $^{\bullet}OH$ or $O_2^{\bullet-}$. They are secreted into the apoplast in all organs that control cell proliferation and cell elongation. For details, see [36,40,41].

How Does Auxin Trigger $_{apo}$pH Changes in Anisotropically Expanding Cells?

Linear plant cell growth has been extensively studied in hypocotyls kept in darkness, which are highly responsive to internal and environmental signals, such as plant hormones and light. Auxin activates AHA1 and AHA2 via *SMALL AUXIN UP RNA19* (SAUR19), which inhibits the activity of 2C *PROTEIN PHOSPHATASE* D1 (PP2C-D1). This phosphatase negatively regulates AHA1 activity via 14-3-3 protein binding to AHA1. In this model, SAUR19 and PP2C-D1 act antagonistically to control cell expansion [29]. Auxin promotes phosphorylation of a threonine in the AHA C-terminal region (Thr^{948} and Thr^{947} in AHA1 and AHA2, respectively), which leads to AHA activation, H^+ extrusion, and hypocotyl growth (within ~20 min). Hypocotyl cells require the nuclear TIR1/AFB-Aux/IAA signaling pathway, which activates auxin-mediated *de novo* protein synthesis [28]. Expression of either a stabilized SAUR19 protein or a truncated AHA2 lacking the autoinhibitory C-terminal domain enhance growth independently of auxin. High intracellular auxin concentrations acidify the $_{apo}$pH, enhancing cell wall protonation. Furthermore, FER interacts with the RAPID ALKALINIZATION FACTOR1 (RALF1) peptide to alkalize the apoplast by inhibiting the activity of AHA2 possibly via Ser^{899} phosphorylation, thereby suppressing cell expansion [30], although FER direct or indirect inactivation of AHA2 needs to be experimentally validated. Several regulators modulate AHA activity under different physiological contexts to adjust $_{apo}$pH during growth [16]. AHA1 and AHA2 are highly expressed in root hair cells (see Figure S1 in the supplemental information online), suggesting that they control $_{apo}$pH during polar growth. Further experiments with these candidate proteins are needed to confirm their roles in root hair growth.

How Does $_{apo}$pH Control Rapid Changes in Cell Wall Structure?

$_{apo}$pH acidification is postulated to activate several cell wall-loosening enzymes and hyperpolarize the plasma membrane, resulting in solute and water uptake, an increase in turgor pressure, and cell expansion (Figure 1, Key Figure). Given that turgor pressure is mostly uniform in all directions, wall extensibility must be tightly regulated in a directional manner. α- and β-expansin are activated by low $_{apo}$pH (~4) to regulate the structure and relaxation of the cellulose-xyloglucan network in cell walls [8]. PECTIN METHYLESTERASES (PMEs) modulate the methylesterification of homogalacturonans, thereby modulating pectin matrix viscosity and cell wall stiffness [31,32]. PMEs are inhibited by the formation of a complex containing PME INHIBITOR PROTEIN (PMEI) and PME (PME-PMEI). PME3-PMEI7 complex formation is enhanced by low $_{apo}$pH, which inhibits PME3 activity [32]; however, complex formation with other PMEs could be insensitive to pH. In addition, POLYGALACTURONASES (PGs) and PECTIN/PECTATE LYASES (PLs) also regulate homogalacturonan polymerization [33], thereby affecting cell wall properties. Recent work showed that short galacturonic acid (GalA) $GalA_{3-4}$ fragments triggered hypocotyl growth in darkness, suggesting that specific cell wall components function in cell–cell communication linked to expansion in addition to their structural roles [34]. XYLOGLUCAN ENDOTRANSGLYCOSYLASES/HYDROLASES (XTHs), belonging to the glycosyl hydrolase $(GH16)^{I}$ family, cut and paste xyloglucan chains (XGs) [35], thereby mediating xyloglucan network remodeling. **Secreted type-III PEROXIDASES** (PERs) have antagonistic roles in $_{apo}$ROS homeostasis (Figure 1), triggering either cell wall crosslinking or cell wall relaxation processes [36]. It is unclear whether the same PERs catalyze all reactions in oxidative (OC), peroxidative (PC), and hydroxylic cycles (HC), or whether certain isoforms have specialized activities (Box 1). Recent work characterizing the contribution of three PERs to ROS homeostasis together with three **RESPIRATORY BURST OXIDASE HOMOLOG** (RBOH) proteins, RBOHC, H, and J, during root hair growth suggested that these PERs function downstream of RSL4 [3] to crosslink cell wall proteins (extensins and extensin-like molecules) [14,37,38]. Therefore, this glycoprotein network might interact with the negatively charged pectin network and affect cell elongation [38,39]. In other plant cells, PERs are also regulated at

Key Figure

Selected Molecular Components That Sustain Oscillatory Growth in Root Hair and Pollen Tube Cells

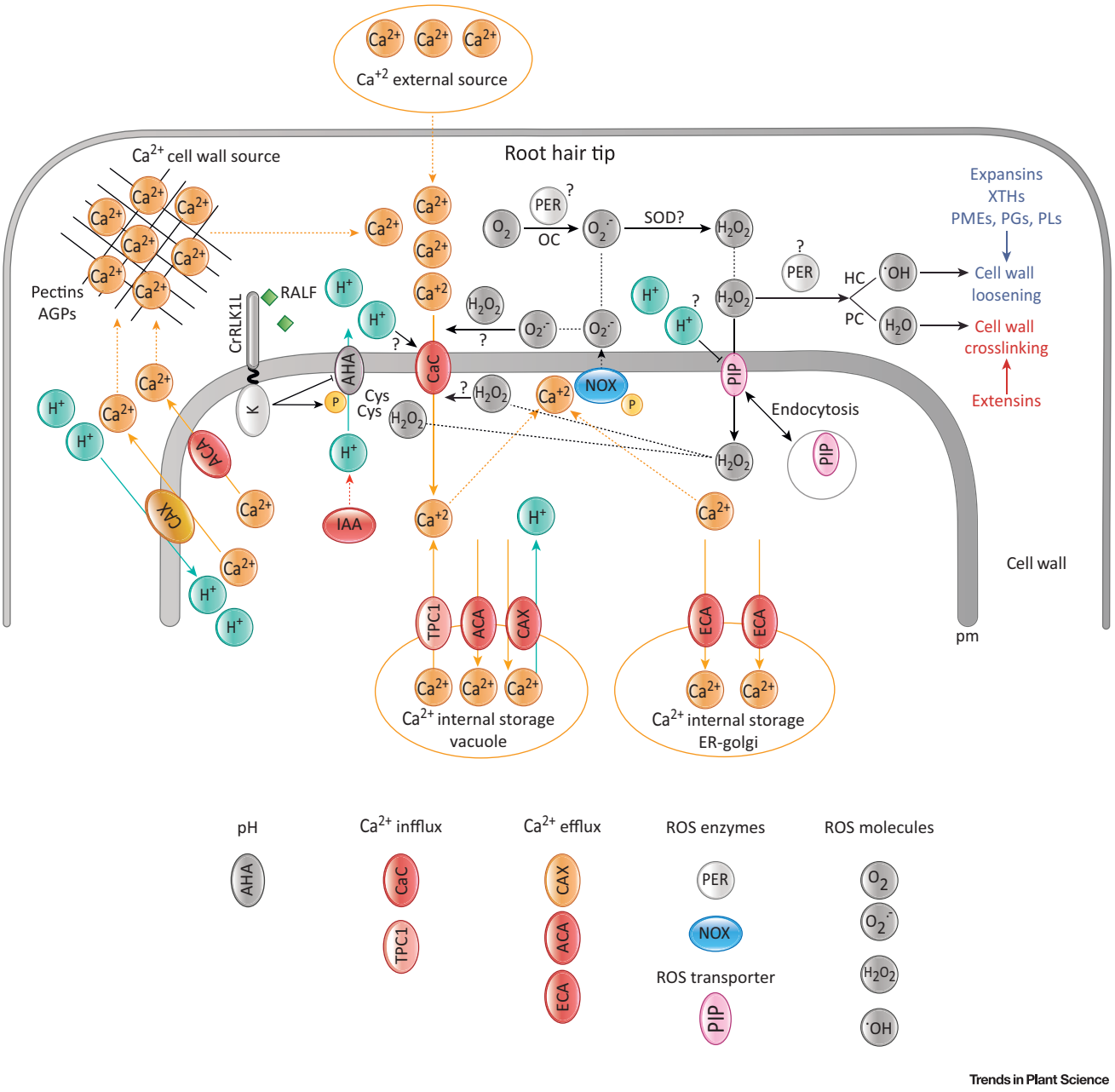

Figure 1. Polar growth in root hairs is oscillatory and involves the generation of tip gradients of reactive oxygen species (ROS), Ca^{2+}, and pH. ROS homeostasis is controlled by the activity of NADPH OXIDASES (NOXs) and apoplastic secreted type-III PEROXIDASES (PERs), and by PLASMA MEMBRANE INTRINSIC PROTEIN (PIP)-mediated H_2O_2 transport to the cytoplasm. Ca^{2+} ions are transported from the apoplast [stored in pectins and arabinogalactan proteins (AGPs) in the cell walls and in the soil] by several types of Ca^{2+} channel (CaC) and mobilized from intracellular organelles to generate an oscillating cytoplasmic Ca^{2+} gradient. Ca^{2+} and ROS

(See figure legend on the bottom of the next page.)

the post-translational level by $_{apo}$pH and Ca^{2+} [40,41]. Nutrient transporters and nutrient availability in the soil microenvironment are also affected by $_{apo}$pH [42]. These combined results indicate that $_{apo}$pH affects cell wall structure and biophysical properties as well as nutrient uptake, which are components of the complex regulatory network orchestrating polar cell growth (Figure 1).

A Positive Feedback Loop That Maintains Polar Growth Induces ROS–Ca^{2+} Oscillations

Anisotropic growth in hypocotyls and root cells is linear, whereas polarized growth in root hairs and pollen tubes is oscillatory [43]. During polar cell expansion, the apical zone contains $_{cyt}Ca^{2+}$ and $_{apo}$ROS (mostly H_2O_2) gradients. High $_{cyt}Ca^{2+}$ levels in the apical region trigger $_{apo}$ROS production via reactions catalyzed by RBOHs or NOXs (NADPH oxidases; Box 1), because Ca^{2+} ions bind to EF-hand motifs in the N terminus of NOXC and other NOXs, thereby enhancing their enzymatic activity [10,11,44–46]. High ROS ($_{apo}$ROS and/or $_{cyt}$ROS) levels transiently elevate the $_{cyt}Ca^{2+}$ concentrations by opening plasma membrane Ca^{2+} channels (Box 2) via an as yet unknown mechanism. It is possible that $_{cyt}$pH oscillations affect the binding affinity of Ca^{2+} as well as the conformation state to the EF-hand motifs, thereby affecting NOX activity (Figure 2). NOXC activity in root hairs and NOXH/NOXJ activities in pollen tubes and root hairs are the main producers of $_{apo}$ROS [3,14,46]. Protein phosphorylation [47], RBOH-lipids [48], and RBOH-specific protein interactions [49] also regulate RBOH activity [50].

Although most ROS are produced by NOXs and related enzymes in the apoplast, it has been postulated that **PLASMA MEMBRANE INTRINSIC PROTEIN** (PIP) aquaporins actively transport H_2O_2 into the cytoplasm. *In vitro* studies confirmed that several PIPs transport H_2O_2 [51]. Recent studies established the physiological roles of PIP2;1 in ABA- and flg22-mediated stomatal closure [52], of PIP1;4 in epidermal cells under pathogen attack [53], and of *AQUOPORIN8* AQP8 in the responses of plants to *Botrytis cinerea* infection [54]. Low $_{apo}$pH inhibits PIP2;1 activity [55], possibly affecting H_2O_2 transport across the plasma membrane. By contrast, high H_2O_2 levels trigger PIP2;1 intracellular sequestration [56]. Specific phosphorylation of two residues (Ser^{280} and Ser^{283}) in the AtPIP2.1 C terminus in response to extracellular H_2O_2 correlated with the redistribution of AtPIP2.1 to intracellular vesicles. High Ca^{2+} levels and low $_{apo}$pH inhibit ionic conductance in AtPIP2.1 [55], suggesting that oscillations in H_2O_2, Ca^{2+}, and $_{apo}$pH affect PIP activity. Plant cells have a high buffering capacity, which controls the $_{apo/cyt}H_2O_2$ levels and avoids cytotoxic oxidative stress [57]. Oscillating $_{cyt}H_2O_2$ levels might affect several other processes linked to polar growth (Figure 2), but the molecular mechanisms are unknown.

Positive feedback loops between Ca^{2+} and ROS sustain polar growth (Figure 1) [10,11,44–47]. The Ca^{2+} ions contributing to the cytoplasmic gradient during polar growth can be stored in three different compartments: the extracellular space in the soil surrounding the root hair cell surface; the cell wall (apoplastic space), linked to arabinogalactan proteins (AGPs) and pectins

oscillations are maintained by a robust positive feedback loop involving concomitant activation of several NOXs and CaC transport. Auxin triggers apoplastic acidification by activating the P-type plasma membrane H^+ ATPases (AHAs) along with cell wall relaxation mediated by expansins, pectin methylesterases, and xyloglucan endotransglycosylases/hydrolases (XTHs). ROS and pH oscillations could affect CaC transport and the subcellular localization of PIPs, suggesting complex crosstalk among pH, Ca^{2+}, and ROS. Abbreviations: Auxin, IAA/Indole 3-acetic acid; CrRLK1L, *Catharanthus roseus* RECEPTOR-LIKE KINASE; CW, cell wall; EXP, expansins; HC, hydroxylic cycle; RBOHs, respiratory burst oxidase homolog proteins; OC, oxidative cycle; P, phosphorylated site; PC, peroxidative cycle; PGs, polygalacturonases; PLs/PNLs, pectin/pectate lyases; PM, plasma membrane; RALF, RAPID ALKALINIZATION FACTOR; SOD, SUPEROXIDE DISMUTASE; XTH, xyloglucan endotransglycosylase/hydrolase. See main text for further details. Solid arrows indicate a signaling pathway, a downstream step, or transport across the plasma membrane; solid lines indicate a close relationship between proteins or ions. Figure layout inspired by [3,4,8,13,24,28,29,36].

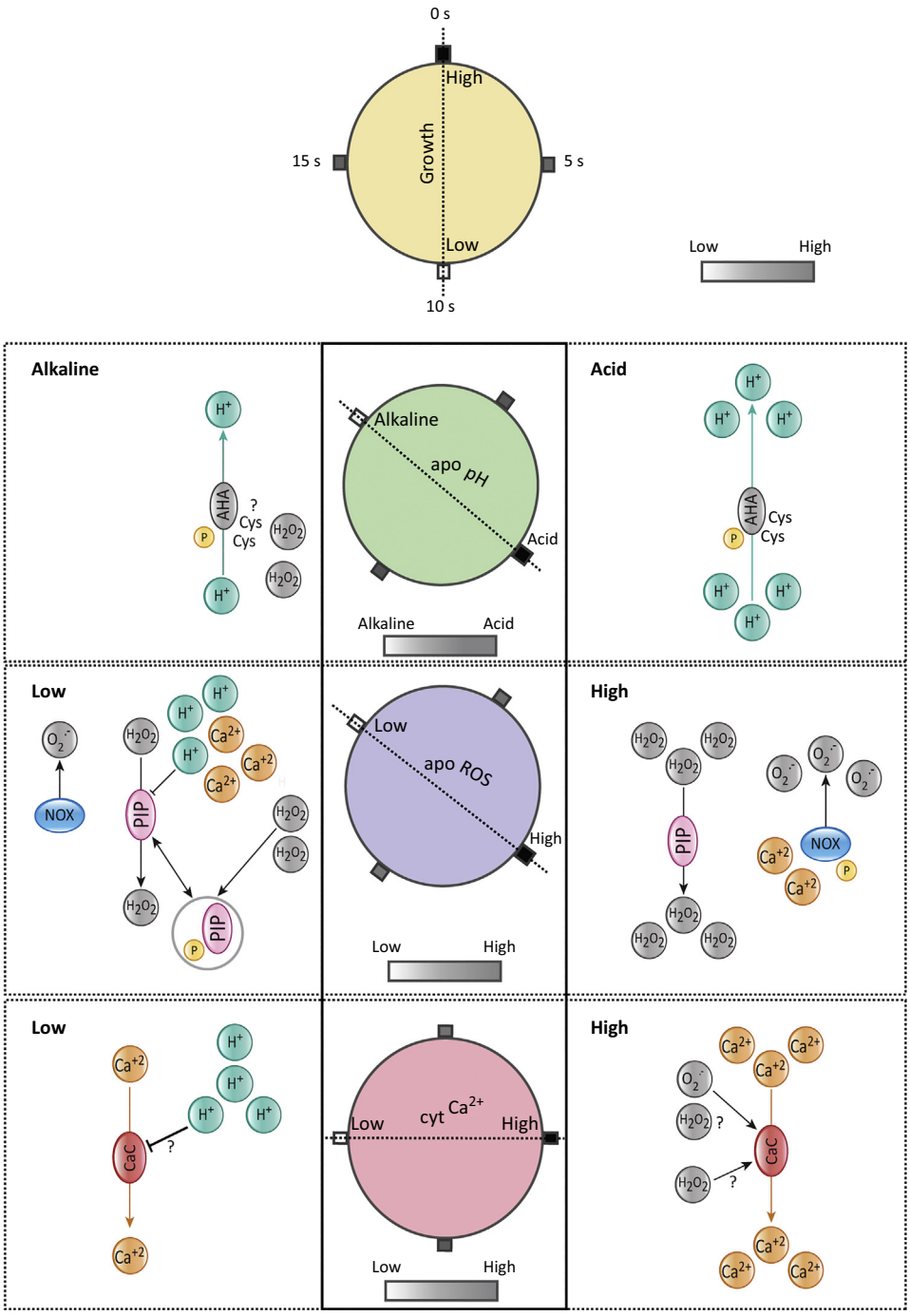

Figure 2. Crosstalk between pH, Reactive Oxygen Species (ROS), and Ca^{2+} Gradients Modulate Polar Growth. Oscillatory growth in root hairs and pollen tubes is presented as a clock; it requires the generation of tip gradients of ROS, Ca^{2+}, and pH. Each component is represented as an individual clock that is delayed with respect to the growth clock. The growth oscillation takes ~20 s for each cycle in root hairs and ~30–40 s for each cycle in pollen tubes, although this can vary according to the growth conditions. ROS homeostasis in the apoplast is controlled by the activity of several enzymes, including NADPH oxidases (NOXs), and by PLASMA MEMBRANE INTRINSIC PROTEIN (PIP)-mediated H_2O_2

(Figure legend continued on the bottom of the next page.)

[58,59]; and in intracellular compartments, such as endoplasmic reticulum (ER)–Golgi, mitochondria, and vacuoles. Recent work [60–63] identified specific Ca^{2+} channels (CaC) involved in polar growth (Box 2), which transport Ca^{2+} from the apoplast into the cytoplasm. In addition, the $_{cyt}Ca^{2+}$ gradient could be also established by rapid release of Ca^{2+} from vacuoles and other organelles, although the mechanism is unknown [64].

Polarized root hair growth is associated with positive feedback loops between oscillatory Ca^{2+} and ROS gradients [11,44,45] and dynamic $_{apo}$pH oscillations [10]. The $_{apo}$pH and ROS oscillations have a time lag of approximately 7–8 s with respect to the growth cycle and approximately ~2–3 s with respect to the intracellular Ca^{2+} gradient [10,11,65]. Oscillations in pollen tubes behave in a similar way, although the frequency is slightly slower and $_{cyt}Ca^{2+}$ and acidified $_{cyt}$pH have longer time lags relative to growth oscillations [66–68]. Root hair and pollen tube growth oscillations require approximately ~20–40 s per cycle. Overall, these results suggest that $_{apo}$ROS and $_{apo}$pH maxima occur before Ca^{2+} induces transient repression of cell growth. Figure 2 presents each oscillatory pathway ($_{apo}$ROS, $_{cyt}Ca^{2+}$, and $_{apo}$pH) as an individual clock and shows how each pathway is coupled to polarized growth (exemplified in root hairs). However, quantification of the frequency and/or period, amplitude, and phase relationships of these pathways is limited by low resolution and technical noise in the acquisition and analysis methods [65,69]. In addition to ROS and Ca^{2+} waves as key players in polar growth, oscillating pH also appears to be important in maintaining growth. In agreement with this, growth and the Ca^{2+} gradient can be rescued in a mutant with deficient ROS production (*noxc*) by transferring cells to a neutral pH environment [10]. It is unclear how a change in pH is able to restore polar growth in a deficient ROS environment.

What Mechanism Produces $_{apo}$pH Oscillations?

Currently, little is known about the mechanism regulating $_{apo}$pH oscillations in polar growth and how this relates to oscillating ROS and Ca^{2+} gradients. Plant plasma membrane AHAs are regulated by the autoinhibitory action of the C-terminal domain, which can be displaced by threonine phosphorylation and subsequent binding of 14-3-3 proteins, resulting in pump activation [70]. AHAs could also be regulated by the cytoplasmic redox state, which is linked to ROS. In this case, the H^+ transport activity of AHAs would be sensitive to $_{cyt}$ROS (H_2O_2), in agreement with linked $_{apo}$pH-$_{cyt}$ROS oscillatory gradients (Figure 2). AHA (de)phosphorylation regulates its own activities [16], and it could control the $_{apo}$pH oscillations. Single-molecule studies of AHA2 function suggest that inactive states and protein-mediated H^+ leaks must be considered as part of the functional AHA2 cycle [71]. In addition to (de)phosphorylation states, one additional putative mechanism could involve cysteine (Cys) residues in the cytoplasmic face of AHAs acting as sensors to modulate its activity. These Cys residues may undergo reversible thiol oxidation in response to changes in the oxidation status of the cytoplasm and their reactivity might highly affected by the intimate environment of the protein [72]. The reactivity of Cys residues towards ROS can differ, and this reactivity is strongly correlated with their pKa, an acid dissociation constant that indicates their acid strength [72]. Five Cys residues are conserved in all 11 AHAs in *A. thaliana* and related plants (see Figure S2A in the supplemental information online), and three of these are located in the first three cytoplasmic

transport to the cytoplasm. High Ca^{2+} levels and phosphorylation trigger NOX activity, whereas apoplastic acidification might inhibit PIP activity. Ca^{2+} transport from the apoplast to the cytoplasm is conducted by several Ca^{2+} channels (CaC), and $_{apo/cyt}$ROS enhances their activity, whereas acidic pH might inhibit Ca^{2+} transport. P-type H^+-ATPase superfamily of cation-transporters (AHAs) primarily generate apoplastic acidification. We hypothesize that cytoplasmic ROS levels would affect the redox status of AHAs (possibly by oxidation of Cys) and thereby affect their structure and activity. Figure layout inspired by [9,10,64,65,83,84].

loops, which could be exposed to oscillating $_{cyt}H_2O_2$. Two neighboring Cys residues are also conserved and located in one transmembrane domain. We hypothesize that H_2O_2 oxidizes any of these Cys residues to produce sulfenic acid (Cys-SOH) or intra- and/or intermolecular disulfide bonds, which would cause reversible structural and enzymatic alterations [73]. ATPases undergo conformational changes from their native state, and ATPase homologs have different conformations. Accordingly, an AtAHA2 (Uniprot accession PMA2_ARATH) crystal structure (5KSD) was recently subjected to homology model analyses (see Figure S2B in the supplemental information online) [74,75]. Furthermore, an AHA from *Neurospora crassa* (1MHS, 37% identity) was used to model AtAHA2 in another (extreme) conformational state [76] (see Figure S2C in the supplemental information online), although it is still not clear which is the active state and which is the inactive state. The resulting conformations were evaluated to determine whether the crucial Cys residues were exposed at the structural surface of the proteins. Three Cys residues (Cys^{28}, Cys^{202}, and Cys^{327}) become more exposed at the surface in only one of the possible conformations (AHA from *Neurospora*, 1MHS), whereas they are essentially buried under the surface in the protein conformation of AHA2 in the crystal structure (see Figure S2B,C in the supplemental information online). An additional two Cys residues (Cys^{247} and Cys^{249}) are nearby in both conformations. Cys residue mutations in an AHA from *Saccharomyces cerevisiae* (Pma1) reduce or inhibit enzymatic activity [77]. Thus, Cys residues in AHAs are important structural components that may control their enzymatic activity. Therefore, $_{cyt}H_2O_2$ oscillations in the root hair tip may trigger oscillations in AHA (de)activation, thereby controlling $_{apo}$pH. The K^+ channel SKOR is activated by exogenous H_2O_2, which targets Cys^{168} and other Cys residues in the voltage sensor complex [78]. In the yeast homolog of the pore-forming α_1 subunit of the mammalian voltage-gated Ca^{2+} channel (VGCC) Cch1p, H_2O_2 activates the channel by a redox-dependent mechanism involving four conserved Cys residues [79]. The Ora1 Ca^{2+} channel appears to be inhibited by intramolecular hydrogen bond formation [80]. Redox mechanisms involving Cys-modified disulfide bonds control the function and structure (from intrinsically disordered to organized domains) of several chaperones, such as bacterial Heat Shock Protein33 (HSP33), Copper Chaperone 17 (COX17), and the yeast dimeric ATPase Get3 [81]. Future work should test whether $_{cyt}H_2O_2$ oscillations reversibly control AHA2 (and AHA1) activity and directly affect $_{apo}$pH. Other mechanisms might act in parallel to trigger rapid $_{apo}$pH oscillations, such as H^+ leaking across the plasma membrane or H^+ transport through acidified vesicles, similar to synaptic vesicles during neurotransmission [82]. A symport transporter of $Cl^-/2H^+$ from the apoplast to the cytoplasm and H^+ restoration to the cytoplasm by the Ca^{2+}/H^+ ANTIPORTER (CAX) and Na^{2+} ANTIPORTER (Na^{2+}/H^+) [25,26] are strong candidates to promote apoplastic alkalinization after the acidic pH peak [83].

Concluding Remarks

This review highlights recent work aimed at understanding how $_{apo}$pH controls polar growth in root hairs and pollen tubes. The molecular mechanism producing $_{apo}$pH oscillations remains to be determined, along with understanding how $_{apo}$pH, ROS, and Ca^{2+} oscillations are coordinately integrated. Advanced methods to analyze polar growth oscillations have been developed recently [65,67], along with advanced sensors to track dynamic changes in Ca^{2+}, ROS, pH, and apical growth [20,84–87]. Future work will identify and characterize the interacting regulatory networks. This knowledge could be used to develop more efficient crops with engineered roots and root hairs that can grow under stress conditions.

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Outstanding Questions

Oscillatory growth mediates cell elongation in root hairs and pollen tubes in response to environmental and endogenous signals. How is this complex process coordinated? Is there a central regulator of growth oscillations? Several missing links remain to be discovered that might integrate oscillating Ca^{2+}, ROS, and pH gradients.

Although secreted type-III peroxidases (PERs) are important in $_{apo}$ROS homeostasis, they catalyze antagonistic reactions that relax or rigidify cell walls at the tip. How are PERs regulated in the apoplast of growing root hair cells? Do the same PERs catalyze oxidative, peroxidative, and hydroxylic reactions *in vivo*? Do some PERs have enzymatic specificity?

ROS (H_2O_2) homeostasis involves apoplastic reactions linked to the cell wall structure and cytoplasmic reactions linked to cell signaling and catalysis. How is $_{apo}$ROS homeostasis linked to ROS-mediated cytoplasmic signaling? What are the direct targets of $_{cyt}$ROS signaling in polar growth?

There are three Ca^{2+} transport pools, including soil, apoplast, and intracellular compartments. Ca^{2+} transport needs to be tightly coordinated during polar growth. How are Ca^{2+} influx and Ca^{2+} mobilization from cytoplasmic reservoirs synchronized at the molecular level?

How are $_{apo}$pH oscillations generated in the root hair tip? Do AHAs generate $_{apo}$pH oscillations? Do other mechanisms (e.g., fusion of acidified vesicles) or plasma membrane proteins control $_{apo}$pH oscillations?

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Author Contributions

S.M. and J.M.P. reviewed the text, references, and figures. C.M.B modeled and wrote sections on the structural aspects of AHAs and reviewed text. J.M.E conceived the project, wrote the review, and designed the figures with contributions from all authors.

Resources

[i]www.cazy.org classification system

Appendix A Supplementary data

Supplemental information associated with this article can be found online at https://doi.org/10.1016/j.tplants.2018.02.008.

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