RBOH-Dependent ROS Synthesis and ROS Scavenging by Plant Specialized Metabolites To Modulate Plant Development and Stress Responses

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Abstract

Reactive oxygen species (ROS) regulate plant growth and development. ROS are kept at low levels in cells to prevent oxidative damage, allowing them to be effective signaling molecules upon increased synthesis. In plants and animals, NADPH oxidase/respiratory burst oxidase homolog (RBOH) proteins provide localized ROS bursts to regulate growth, developmental processes, and stress responses. This review details ROS production via RBOH enzymes in the context of plant development and stress responses and defines the locations and tissues in which members of this family function in the model plant *Arabidopsis thaliana*. To ensure that these ROS signals do not reach damaging levels, plants use an array of antioxidant strategies. In addition to antioxidant machineries similar to those found in animals, plants also have a variety of specialized metabolites that scavenge ROS. These plant specialized metabolites exhibit immense structural diversity and have highly localized accumulation. This makes them important players in plant developmental processes and stress responses that use ROS-dependent signaling mechanisms. This review summarizes the unique properties of plant specialized metabolites, including carotenoids, ascorbate, tocochromanols (vitamin E), and flavonoids, in modulating ROS homeostasis. Flavonols, a subclass of flavonoids with potent antioxidant activity, are induced during stress and development, suggesting that they have a role in maintaining ROS homeostasis. Recent results using genetic approaches have shown how flavonols regulate development and stress responses through their action as antioxidants.

Graphical Abstract

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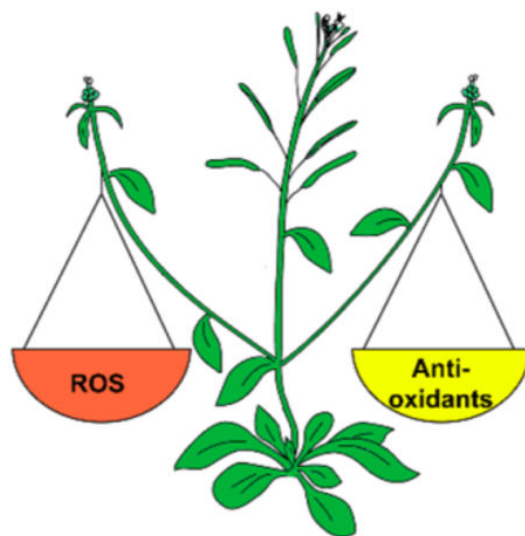
1. INTRODUCTION

Reactive oxygen species (ROS) function as signaling molecules in both plant and mammalian cells. ROS homeostasis is an important feature of productive ROS signaling. High levels are toxic to the cell, but keeping ROS at low levels allows small increases in ROS to initiate signaling events in plant growth, development, and stress responses.[1,2] ROS are a group of highly reactive oxygen-containing molecules that can readily accept electrons from more stable compounds. Each reactive oxygen species has unique reactivity, signaling properties, and sites of production.[3] Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), also called respiratory burst oxidase homologues (RBOHs) in plants, are critical enzymes that produce ROS in response to hormone and environmental signals.[4] Furthermore, plants have class III peroxidases, which are able to produce superoxide compounds.[5] Superoxide can be dismutated into H_2O_2 by superoxide dismutase (SOD),[6] and in the presence of iron, H_2O_2 can be made into hydroxyl radicals by the Fenton reaction. [6] In this review, we focus on highlighting how distinct members of the RBOH family display tissue-specific localization and regulation of plant development and stress responses.

Plants have evolved diverse enzymatic and non-enzymatic machineries to prevent ROS from reaching damaging levels.[7] This review summarizes the mechanisms that plants share with animal cells but also highlights plant-derived specialized metabolites that are used to scavenge ROS, with a focus on carotenoids, ascorbate, tocochromanols, and flavonoids. These metabolites exhibit large structural diversity and function. We highlight recent advances in using genetic approaches to dissect the *in vivo* antioxidant role of one of these metabolite classes, termed flavonols, in regulating a number of ROS-dependent processes.

2. NADPH OXIDASES/RBOHS AS ROS SIGNALING HUBS

NOX enzymes were first identified in human phagocytic cells for their role in generating ROS during pathogen defense.[8,9] The human NADPH oxidase family has seven members, NOXI–NOX7.[10] Most NADPH oxidase proteins catalyze the reduction of oxygen to a superoxide anion ($O_2^{\bullet-}$) using NADPH as an electron donor. The superoxide anion can subsequently produce H_2O_2 through enzymatic or non-enzymatic dismutation. NOX4 is an exception, producing H_2O_2 directly.[11,12] Mammalian NADPH oxidases are membrane protein complexes composed of the catalytic membrane-bound flavocytochrome b_{558} and additional subunits. NOX2, the enzyme most similar to the plant RBOH family, has a catalytic subunit gp91[phox] and a p22[phox] subunit along with three additional cytosolic subunits: p40[phox], p47[phox], and p67[phox] [13]

RBOH enzymes were identified in plants on the basis of their sequence similarity to the gp91[phox] subunit of NOX2.[14] Plant RBOH proteins are also integral plasma membrane proteins with six transmembrane domains. The third and fifth transmembrane domains contain two pairs of histidine residues, which are attachment sites for the heme groups that facilitate electron transport.[15] Although plant RBOH proteins have NADPH and flavin adenine dinucleotide binding domains on the C-terminus, similar to the mammalian phagocytic gp91[phox],[16,17] they also contain an extension of approximately 300 amino acids at the N-terminus. This region contains two calcium-binding EF hand motifs and two phosphorylation sites that participate in regulation of enzyme activity.[14] The role of RBOH proteins in ROS signaling was previously reviewed,[4] but new findings, summarized below, provide more information on the distinct role of these enzymes in developmental and stress contexts.

The *RBOH* gene family is best characterized in the model plant *Arabidopsis thaliana* because of the availability of molecular genetic tools. The *Arabidopsis RBOH* family has 10 members, *RBOHA* to *RBOHJ*,[18,19] with distinct expression patterns and functional roles in development and stress response. Functions of the different RBOHs in plant growth and development are illustrated in Figure 1. The tissue-specific expression of transcripts encoding all of the isoenzymes has been examined using genome-wide transcriptional profiling and is summarized in Figure 2. The distinct RBOH isoenzyme expression patterns across plant tissues are generally linked to their reported functions.

Substantial information about RBOH function has been gathered using null mutants or plants with other genetic manipulations, such as site-directed mutations to alter protein activity. For two isoenzymes, RBOHA and RBOHG, neither mutant phenotypes nor function *in planta* have been reported. The following sections are organized by tissue and integrate current knowledge about each isoenzyme's function, expression, and regulation. These findings are also summarized by isoenzyme in Table 1.

2.1. Roots.

The *Arabidopsis* root has been well-characterized because of its simple organization, with genetically defined development regulated by hormonal and environmental stimuli; ROS have emerged as signals that modulate root development. There are four root tissue layers

organized radially: the epidermis, endodermis, cortex, and central vascular cylinder.[20] In addition, there are four distinct longitudinal zones arranged by maturity (Figure 3).[20]

The meristematic zone is at the root tip and contains the stem cell niche, a pool of actively dividing stem cells and newly differentiated cells.[21] These root meristem cells divide asymmetrically, where one daughter cell differentiates to make up one of the four tissue layers.[22] The final cell identity of these daughter cells is largely dependent on positional cues of neighboring cells.[22,23] Above the meristematic zone is the transition zone, where newly differentiated cells leave the meristem to undergo slow growth and are defined by isodiametric shape (equal length and width).[24,25] Rapid expansion of the root occurs in the elongation zone, where cells increase in length in a matter of hours.[26,27] Finally, the maturation zone, which encompasses the rest of the root, is delineated from the elongation zone by the initiation of root hair bulges, which extend from epidermal cells and elongate into root hairs.[28,29] In addition to producing root hairs, the maturation zone is also the site of lateral root initiation. Lateral root primordia form from pericycle cells in the center of the primary root and develop into lateral roots after emergence through the epidermis.[28] The intricate higher-order root architecture is formed through continuous branching of lateral roots. The lateral root formation zone can be loosely defined as the length of the primary root encompassing the first emerged lateral root to last lateral root primordium.[30,31]

Reactive oxygen species have been implicated in both developmental and environmental responses in roots, with established roles for RBOHC, RBOHD, and RBOHF. The function of these specific RBOH in roots is consistent with the high levels of accumulation of the transcripts encoding these enzymes in roots (Figure 2).

2.1.1. Primary Root Development and Elongation.—Plant root growth and elongation enables exploration of soil/growth media for nutrients.[32] RBOHF-mediated primary root elongation is dependent on nutrient and sucrose levels.[33,34] In complete nutrient media with 3% sucrose, primary root elongation in *rbohd* and *rbohf* single mutants and the *rbohf/d* double mutant is comparable to the wild-type.[34] Under these conditions, treatment with the plant hormone abscisic acid (ABA) inhibits primary root growth in all lines, with a smaller effect seen in mutant plants.[34] Another study using sucrose-free and low-nutrient media showed that *rbohf* and the double mutants had reduced primary root length in the absence of ABA compared with *rbohd* and the wild-type.[33] When ABA was applied to these lines, primary root length was inhibited in *rbohd* and the wild-type, but *rbohf* and *rbohd/f* showed no change compared to control conditions, suggesting that *rbohf* mutants are insensitive to ABA.[33] These studies point to a specific role of RBOHF in ABA-mediated signaling under low-nutrient conditions.

In addition to adapting their growth to nutrient levels, plant roots also alter their architecture to maximize water uptake from the soil/growth medium. Hydrotropism, the growth of roots toward water (or away from low water potential), is negatively regulated by RBOHC.[35] H_2O_2 accumulation in the elongation zone is presumably a signal for hydrotropic bending, as the sites of bending and H_2O_2 accumulation colocalize in this area in wild-type plants.[35] Assays exposing the *rbohc* mutant (which will be denoted by its more common name, *rhd2*) to low water potential showed that *rhd2* roots are more effective in bending away from low

water potential than wild-type roots.[35] Enhanced bending in *rhd2* is attributed to decreased H_2O_2 content in the elongation zone, as measured by Amplex Red in extracts from root apices. The *rbohd* mutant, which also had lower H_2O_2 levels in whole seedlings but wild-type H_2O_2 levels in root apices, had a similar hydrotropic bending response to wild-type plants.[35] These results suggest that RBOHC provides superoxide that is then converted to H_2O_2 and that this elevated H_2O_2 reduces hydrotropic bending.

Roots contain an endodermal barrier called the Casparian strip that aids in selective uptake of nutrients and water.[36,37] Surrounding the vascular tissue of the root, the Casparian strip is an impermeable layer adjacent to the endodermis in the maturation zone of the root and is composed of lignin and suberin, which are phenolic and lipophilic polymers, respectively.[36,37] Radial diffusion into the root system through the cell wall is prevented by the Casparian strip, which forces water and nutrients to enter the vascular system through plasma membrane-localized transporters.[36,37] In *Arabidopsis*, formation of the Casparian strip requires controlled deposition of lignin formed by oxidative coupling of monolingol compounds by laccases and peroxidases.[38] Lignification of the Casparian strip is regulated by RBOHF, which was the only NADPH oxidase found in a large-scale forward genetic screen of mutants with an impaired endodermal barrier.[39] Consistent with this function, *RBOHF* transcripts accumulate in the maturation zone in the stele, cortex, and endodermis (Figure 2), and RBOHF–mCHERRY protein fusions under control of the native promoter shows distinct fluorescence at the Casparian strip domain.[39] The *rbohf* mutant has delayed Casparian strip formation, consistent with the idea that superoxide produced by RBOHF is necessary to form lignin.[39] RBOHF activity coordinates with peroxidase 64 (PER64) to increase efficiency of Casparian strip lignification.[39] Collectively, these observations show distinct roles of RBOH proteins in modulation of primary root architecture and development.

2.1.2.   Lateral Roots.—The initiation and elongation of lateral roots, which are root branches that develop from the primary root, lead to a robust root system that anchors plants and maximizes moisture and nutrient uptake. Recent evidence implicates ROS as a developmental signal in this process. Lateral root primordia initiate in the pericycle, a cell layer within the primary root, and must emerge through several other cell layers, including the epidermis.[40] The inhibitory effect of RBOHD and RBOHF on lateral root development has been reported. Single mutants of *rbohd* and *rbohf* revealed no difference in lateral root development, while *rbohd/f* double mutants had increased lateral root density in comparison with the single mutants and the wild-type.[41,42] The homeostasis of different ROS compounds is pertinent to lateral root development. The double mutant had reduced levels of H_2O_2 throughout the root relative to the wild-type, as demonstrated by 3,3-diaminobenzidine (DAB) staining, and increased levels of superoxide in the mature region of the root compared with the wild-type, as measured using dihydroethidium (DHE) and nitro blue tetrazolium (NBT).[41] The increased levels of superoxide in the root maturation zone of the double mutant are inhibited by treatment with peroxidase inhibitors, reducing superoxide levels and lateral root density to wild-type levels.[41] This phenotype was shown to be independent of auxin, the plant hormone that stimulates lateral root initiation.[41] These data suggest that superoxide produced by RBOHD and RBOHF is directly channeled toward

H_2O_2, which then inhibits lateral root development. The increased superoxide in the *rbohd/f* mutant is attributed to increased activity of class III peroxidases, a unique class of plant-specific enzymes.[41] Consistent with the role of class III peroxidases as positive regulators in lateral root development, overexpression of *PER7* and *PER57* results in increased lateral root emergence, while mutants have the opposite effect.[43] The balance of superoxide/H_2O_2 or H_2O_2 itself produced from RBOHD and RBOHF likely inhibits these peroxidases. Taken together, these results suggest that specific reactive oxygen species have opposing effects on lateral root number: H_2O_2 produced downstream of RBOHD and RBOHF enzymes has an inhibitory effect on lateral root number, while superoxide produced by class III peroxidases promotes lateral root number.

A second study reported impaired lateral root emergence in roots treated with diphenylene iodonium (DPI), an RBOH inhibitor, and in higher-order RBOH mutants lacking RBOHD and/or RBOHE in particular.[44] In roots, RBOHE is expressed in the three layers overlying the lateral root primordium: the endodermis, cortex, and epidermis.[44] A loss-of-function mutant of RBOHE has delayed lateral root emergence. *RBOHE* transcripts accumulate across the cell types in the maturation zone (Figure 2), and its transcript abundance increases after treatment of roots with auxin, as judged by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of whole roots.[44] However, the effect of auxin did not depend on the auxin influx carriers AUX1 and LAX3, as RBOHE was expressed at the same level as in the wild-type in *aux1* and *lax3* mutants in the absence of auxin treatment.[44] A *RBOHE* promoter-driven β-glucuronidase (GUS) reporter accumulated above the site of lateral root initiation, suggesting that localized ROS synthesis may facilitate emergence of lateral roots. Targeted overexpression of RBHOD in lateral root primordia and overlying tissue induced lateral root emergence.[44] The authors suggest that this localized ROS may drive changes in cell wall structure to facilitate the emergence of roots through surrounding tissues.[44]

2.1.3. Root Hair Elongation.—Root hairs are single-cell extensions of epidermal cells that increase root surface area to maximize water and nutrient uptake (Figure 1).[29,32] A screen for root hair developmental defects identified the *root hair defective 2 (rhd2)* mutant, which has shorter root hairs than the wild-type (Figure 3).[45,46] This mutation is mapped to the RBOHC gene, suggesting a role for ROS in regulation of this process.[45] *RBOHC* transcripts accumulate in epidermal cells and elongating root hairs, and RBOHC proteins fused to green fluorescent protein (GFP) localize to root hair tips,[47] consistent with ROS accumulation promoting polar tip growth.[45] Further studies showed that hydroxyl radicals ($^\bullet$OH), formed downstream of superoxide, are the specific ROS that stimulate root hair elongation.[45] Localized synthesis of ROS at the tips of root hairs via RBOHC is essential for proper root hair morphology, as global application of $^\bullet$OH did not complement the short root hair phenotype of *rhd2* but rather, led to spherical root hairs as a result of depolarized cell expansion.[45] Hydroxyl radicals facilitate cell wall loosening[48] and are required to form a tip-focused Ca^{2+} gradient in the root hair, both of which are necessary for elongation.[45,49] Staining the wild-type and the *rhd2* mutant with calcium green dextran showed that, unlike the wild-type, the *rhd2* mutant did not form a Ca^{2+} gradient.[45] The Ca^{2+} gradient was restored in *rhd2* after exposure to $^\bullet$OH via the Fenton reaction (by addition of Cu^{2+}, H_2O_2,

and ascorbate).[45,50] Detailed below are the processes that control localization of *RBOHC* transcripts and proteins and regulation of RBOHC activity.

Chromatin immunoprecipitation (ChIP) was used to identify proteins that bind to the *RBOHC* promoter.[51] This assay identified the root hair cell-localized transcription factor root hair defective six-like 4 (RSL4),[51] which was previously found to promote root hair length.[52] The *rsl4–1* mutant has decreased ROS levels and shorter root hairs compared to wild-type plants, although transcription levels of *RBOHC* were not measured in the mutant. [51,52] *RSL4* expression was found to be controlled by several auxin response factors (ARFs), including ARF5, ARF7, ARF8, and ARF19.[51,52] As auxin has been shown to induce root hair formation,[53,54] this ARF–RSL network may induce localized ROS synthesis that drives root hair formation in response to auxin.[53,54]

Root hairs emerge outward from existing epidermal cells through polarized cell growth. Polarized cell growth requires cell wall expansion, which is a turgor-driven process where pressure exerted by the plasma membrane is met with resistance at the cell wall, stimulating cell wall elongation to accommodate the increase in volume.[55] RBOHC trafficking and localization is a highly regulated process that is controlled by the FERONIA (FER) receptor-like kinase[56] and members of the Rho-related GTPases of plants (ROP) family.[57–60] The extracellular domain of FER binds to pectin components of the cell wall and activates Ca^{2+} channels that maintain cell wall integrity during cell elongation.[56,61,62] Accordingly, *fer* and *rhd2* single mutants have similar impairments in root hair morphology and decreased ROS accumulation in root hairs, suggesting that they function in the same pathway.[56] Pull-down assays suggest that FER interacts with ROP2,[56] a protein that promotes root hair tip growth upstream of RBOHC.[58] Plants that have impaired ROP2 function or altered expression of *ROP2* have deformed root hairs.[58,59] These phenotypes are RBOHC-dependent, as crosses between the *rhd2* mutant and constitutively active ROP2 lines fail to produce superoxide and have short root hairs.[59] Finally, endosomal cycling of RBOHC at the plasma membrane may occur through fine-tuning of ROP2 localization and stability through GDP dissociation inhibitor (GDI) proteins that participate in RAC/ROP recycling and post-translational modification.[47,60,63]

RBOHC activity is also regulated by binding of Ca^{2+} at its N-terminal EF hand motifs and by serine phosphorylation. Site-directed mutagenesis to block the function of the EF hand motifs reduced ROS production by RBOHC in HEK293T cells,[47] a mammalian cell line that lacks endogenous NOX2 and NOX5 activity.[64,65] Serine phosphorylation can occur through the interaction of RBOHC and the calcineurin B-like-interacting protein kinase 26 (CIPK26)–calcineurin B-like protein 1 (CBL1) sensor complex.[47,66] Heterologous expression of wild-type or serine-to-alanine mutated versions of RBOHC showed that ROS production was dampened in serine-mutated RBOHC proteins, suggesting that phosphorylation at S318 and S322 amplifies ROS production in response to Ca^{2+} influx.[66] The authors suggested that phosphorylation of RBOHC may act in concert with Ca^{2+} oscillation to modulate ROS production in developing root hairs.[49,66] Interestingly, root hair development in *cbl1/cipk26* double mutants and single mutants and in *rhd2* mutant lines harboring the serine-mutated RBOHC protein displayed wild-type root hair morphology,

suggesting that the two conserved serine residues are not required *in planta* or that other phosphorylation sites/kinases are involved in RBOHC activity.[66]

Maize (*Zea mays*) and rice (*Orzya sativa)* genomes encode RBOH proteins named *Zea mays* roothairless5 (Zmrth5), and *Orzya sativa* NADPH oxidase 3 (OsNOX3) with high sequence similarity to *Arabidopsis* RBOHH and RBOHJ, respectively.[67,68] Although these proteins have greater sequence similarity to pollen tube-localized *Arabidopsis* RBOHH and RBOHJ (described below), Zmrth5 and OsNOX3 localize to root epidermal cells and root hairs. These monocot proteins have four transmembrane domains,[67,68] suggesting that RBOH topology differs among monocots and dicots.[19] Superoxide and hydrogen peroxide levels, as visualized by NBT and DAB, were reduced in root hairs of *Zmrth5* and *Osnox3* mutants relative to parental lines. Mutations in *ZmRTH5* and *OxNOX3* result in reduced root hair initiation and elongation.[67,68] This phenotype differs from the phenotype of the *Arabidopsis rhd2* mutant that forms near wild-type numbers of root hairs, but with reduced elongation. [45,67,68] These results support the role of RBOH-dependent ROS synthesis in root hair formation across the plant kingdom, despite structural differences in these particular RBOH proteins.

2.2. Guard Cell Signaling.

Stomata are pores found on leaves and other aerial organs that enable gas exchange between plants and the atmosphere. Stomata are surrounded by two guard cells that are able to expand, causing the stomatal pore to open, or to become flaccid, closing the pore,[69,70] in a process requiring a ROS burst. Stomatal closure and opening is highly regulated, as open stomata allow exchange of gases for photosynthesis, but also allow water loss. Stomatal closure is stimulated by either the hormone ABA or environmental signals, which trigger an influx of Ca^{2+} into the guard cells.[69,70] This Ca^{2+} influx causes an efflux of potassium and anions through K^+ channels and slow anion channel 1 (SLAC1), respectively.[71] Water then exits the guard cells, and the loss of turgor pressure causes the cells to shrink, closing the pore. The opposite is true for opening of stomata: K^+ ions move into the guard cell vacuole, causing water influx into the vacuole and expanding the cells around the stomatal pore.[72]

ABA activates RBOH enzymes, creating a ROS burst that induces stomatal closure[33,73–76] and reduces water loss. ABA-induced increases in ROS, as observed with chloromethyl-2$'$7$'$-dichlorofluorescein diacetate (CM-H_2DCFDA) and peroxy orange 1 (PO1), precede stomatal closure, as shown in Figure 4 for tomato.[76] The increase in ROS and the resulting changes in stomatal closure are blocked by treatment with DPI, which blocks RBOH enzymes (Figure 4).[76]

The antioxidant glutathione has also been implicated in modulating ROS-induced stomatal closure, as mutations that impair its synthesis have elevated ROS and guard cell closure.[77,78] In *Arabidopsis*, the RBOH enzymes that regulate stomatal opening are RBOHD and RBOHF.[73–75] The single mutant *rbohf* shows partial reduction of stomatal closure after ABA treatment, while *rbohd* is comparable to the wild-type.[33] In the presence of DPI[76] or in the *rbohd/f* double mutant,[33] the ABA-mediated ROS burst and subsequent stomatal closure is impaired. The *rbohd/f* double mutant has impaired Ca^{2+} influx and stomatal closure that are rescued by exogenous H_2O_2 treatment[33] via activation of Ca^{2+} ion channels.[79] Mutation

of guard cell hydrogen peroxide resistant 1 (GHR1), a receptor kinase-like protein, results in impaired ABA- and H_2O_2-induced stomatal closure, indicating that GHR1 is upstream of RBOH but downstream of ABA.[80,81] GHR1 has been shown to regulate SLAC1 through CPK3,[82] highlighting its importance in activating key players in stomatal closure.

In addition to ABA, stomatal closure is also regulated by phosphatidic acid (PA)-induced synthesis of reactive nitrogen species (RNS), including nitric oxide (NO).[83] Induction of the phospholipase D a1 (PLDa1) by H_2S increased PA levels and elevated ROS compared with untreated plants.[84] Disruption of PLDa1 reduced PA, impairing ROS and NO production in response to ABA.[85] Furthermore, PA binding sites on RBOHD and RBOHF were identified in the 300 amino acid N-terminal extension; disruption of these binding sites resulted in impaired ROS synthesis and reduced ABA-dependent stomatal closure.[85]

RBOH-dependent ROS bursts modulate calcium channels, but Ca^{2+} signals also regulate RBOHF activity through CBL, Ca^{2+} sensor proteins and CIPKs.[86,87] CIPK26 has been identified as interacting with RBOHF by a yeast two-hybrid screen and confirmed by co-immunoprecipitation.[86] In HEK293T cells, coexpression of CIPK26 and RBOHF resulted in a decrease in ROS levels, suggesting that CIPK26 directly binds to RBOHF to negatively regulate ROS production.[86] However, expression of CIPK26, RBOHF, and CBL1 or CBL9 strongly enhances ROS production.[87] These results reinforce the positive regulation of calcium channels by RBOH-produced ROS bursts.[87]

2.3.   Reproduction.

Several RBOHs have been linked to plant sexual reproduction, highlighting a role for ROS in this process. Plant reproduction occurs in flowers when pollen, the male gametophyte, fertilizes the female gametophyte located within the ovule to initiate seed and fruit development.[88] During pollination, pollen grains are transferred from the anther to the stigma (female reproductive tissue) of the flower, where they germinate and generate a pollen tube that navigates long distances through female reproductive tissues.[89] Fertilization is completed when the pollen tube reaches the ovule and releases sperm cells to fertilize the female gametes.

A role for ROS has been identified during several stages of pollen development and fertilization,[90–95] with localized ROS synthesis needed for fertilization, but with stress-induced ROS inhibiting this process.[96] Multiple RBOH enzymes have been implicated in this process in *Arabidopsis* and other plants, with specific roles defined for RBOHB, –E, –H, and –J (as illustrated in Figure 1 and summarized in Table 1). Transcripts encoding these proteins have been detected in plant reproductive structures, and the phenotypes of these mutants suggest distinct developmental roles for each RBOH enzyme.

2.3.1.   Anther and Pollen Development.—RBOHE has expression patterns and functions tied to plant reproduction, with a role in the formation and development of pollen grains (Figures 1 and 2).[90] *RBOHE* transcripts are detected in mature pollen (Figure 2) and in the tapetum, a layer of cells in the anther that surrounds the developing pollen.[90] During pollen development, programmed cell death of tapetal cells releases lipids, flavonoids (specialized plant metabolites with antioxidant activity) and proteins that are utilized by

developing pollen.[97] In *Arabidopsis*, programmed cell death of the tapetum coincides with the stages of flower development when pollen mitotic division occurs. The *rbohe* mutant had reduced ROS accumulation in the anther, as judged by NBT staining, and delayed programmed cell death of the tapetum.[90] The *rbohe* mutant has impaired pollen viability, with mutant pollen displaying an abnormal outer cell wall layer and a collapsed shape.[90] *RBOHE* was found to be transcriptionally regulated by the transcription factors MYB80 and ABORTED MICROSPORES,[90] both of which control tapetum development and pollen formation.[98–100]

2.3.2. Pollen Tube Growth and Fertilization.—Fully developed, mature pollen grains are released from the anther and transferred to the stigma of the flower, where they germinate to generate a pollen tube that will deliver sperm cells to the ovules for fertilization. The involvement of RBOHs in pollen germination and tube growth has been demonstrated using genetic approaches in tobacco and *Arabidopsis*. Potocky et al.[91] showed that in tobacco a pollen-specific RBOH is necessary for pollen tube growth and production of superoxide. In *Arabidopsis*, RBOHH and RBOHJ, whose transcript abundances are at the highest levels in mature and germinating pollen (Figure 2), are involved in apical growth of the pollen tube (Figure 1) and thus plant sexual reproduction.[92–94] While single mutants in RBOHH and RBOHJ have phenotypes comparable to the wild-type, double mutants display reduced pollen tube growth and integrity and seed set, consistent with redundant roles of these two pollen tube-expressed RBOHs.

Tip growth of the pollen tube occurs in an oscillatory manner and requires precise coordination between cell expansion and deposition of new cell wall materials through exocytosis.[101] Compromised coordination between cell expansion and deposition of new cell wall materials leads to reduced tube growth and cell wall integrity, resulting in premature tube bursting. In *Arabidopsis*, ROS generated by RBOHH and RBOHJ dampen growth rate oscillations.[93] In the wild-type, the oscillatory growth dynamics do not change over time, leading to ordered tip growth. In the double mutant, however, two types of oscillation patterns are observed: high-amplitude oscillations with short growth pulses occur initially, and slower and dampened growth oscillations are observed prior to tube bursting.[93] The growth behavior in the *rbohh/j* double mutant indicates that ROS reduce maximal growth rates, thereby allowing timely deposition of cell wall materials through exocytosis. Mutations in the Ca^{2+}-binding EF hand motifs of RBOHH and RBOHJ resulted in impaired Ca^{2+}-activated ROS production.[92] In addition to Ca^{2+}, ROS production by RBOHH and RBOHJ was shown to be activated by phosphorylation, with the two signals operating synergistically.[92]

A critical feature of the function of RBOHH and RBOHJ in tip growth is targeting of these proteins to the plasma membrane at the tip of pollen tubes. These two RBOHs are localized to distinct but overlapping domains,[93] with RBOHH being targeted to the subapical region of the plasma membrane and RBOHJ accumulating in more distal regions of the pollen tube plasma membrane. Similarly to RBOHC, RBOHH and RBOHJ are targeted to the plasma membrane by exocytosis and undergo endosomal recycling.[93] Treatment with brefeldin A, which blocks endosomal transport, revealed that RBOHH and RBOHJ are inserted into the

plasma membrane in the region behind the tip and then internalized at the pollen tube tip, with lower internalization rates for RBOHH than for RBOHJ.[93]

The pollen-specific RBOHH and RBOHJ are regulated by the receptor-like kinases (RLKs) ANXUR1 and ANXUR2, which are colocalized in the same plasma membrane domain at the pollen tube tip.[94] ANXUR1 and ANXUR2 were proposed to detect changes in cell wall architecture and initiate responses if alterations to the cell wall architecture are too profound. Their loss results in decreased pollen tube integrity, while their overexpression leads to increased deposition of cell wall materials, exocytosis, and arrested tube growth.[94] By regulating RBOHH and RBOHJ, they coordinate cell wall/pollen tube integrity with the internal pollen tube growth machinery.

ROS also function in later stages of the plant reproductive process. RBOH-derived ROS play a role in pollen tube rupture and sperm cell discharge during fertilization of the female gametophyte.[95] Pharmacological inhibition of RBOH enzymes with DPI prevented RBOH-dependent ROS production in the synergid cells located within the ovule.[95] These cells are essential in pollen tube guidance toward the ovule[102] and for the release of sperm cells from the pollen tube, with ROS generated in the synergid cells inducing rupture of pollen tubes to discharge sperm cells to the ovule.[95]

2.3.3. Seed Development.—ROS also function during seed development. Seeds undergo a process called "after-ripening" when they are stored under dry conditions at room temperature. During this process, seeds are released from their dormant state, and seed germination rates are enhanced.[103] Seed after-ripening is modulated by ROS.[104] *RBOHB* transcripts accumulate in embryos[104] and whole seeds with a greater than 5-fold increase when seeds are imbibed (Figure 2). The RBOHB protein functions to generate superoxide and regulate the germination kinetics of seeds (Figure 1).[104] Fresh seeds of an *Arabidopsis rbohb* mutant germinated faster than wild-type seeds, and this difference was abolished in after-ripened seeds.[104] This highlights a role for RBOHB-generated ROS in slowing germination in fresh seeds.

RBOHB is post-transcriptionally regulated to control seed development and hormone-regulated dormancy.[104] *RBOHB* mRNA in embryos has two splicing variants: the smaller *RBOHB-α* variant has intron 1 spliced out, while this intron is retained in the larger *RBOHB-β* splice variant leading to premature termination and a nonfunctional protein.[104] Expression of the two variants was developmentally and hormonally regulated in seeds, with after-ripened seeds containing the functional *RBOHB-α*.[104] Additionally, imbibition, which is treatment of seeds with moisture, to initiate germination increases the abundance of the *RBOHB-α* variant and ROS levels (as judged by NBT staining), while accumulation of the *RBOHB-α* transcript variant was blocked by ABA, a hormone that induces seed dormancy.[104] These results suggest a mechanism by which ROS can be generated specifically in after-ripened seeds and its synthesis can be blocked by ABA to maintain seed dormancy.

2.4. Stress Responses.

Elevations in ROS in response to biotic and abiotic stress are some of the best-characterized redox responses in plants.[2] ROS increases have been reported in response to bacterial

infection and shown to protect the plant from bacterial invasion.[105] These studies have identified pertinent RBOH enzymes and dissected the molecular mechanisms by which these enzymes are activated. ROS is elevated through RBOH enzymes in response to multiple abiotic stresses, including ozone, salt, and moisture and iron deficiency.[42,106–109] In some cases these ROS bursts are protective, and in other cases they appear to trigger programmed cell death. ROS, along with Ca^{2+} and electrical signals, orchestrate long-distance signal transduction in plants.[122] Insight into cell-to-cell signal transmission via ROS was previously reviewed in detail.[110] This section summarizes the roles of specific RBOH isoenzymes in biotic and abiotic stress.

2.4.1.   Biotic Stress Response.—Plants are constantly exposed to bacterial and fungal pathogens and mount elaborate defense mechanisms against them. RBOHD has been implicated in ROS synthesis as a defense against pathogenic bacteria and fungi, including *Pseudomonas syringae* and *Peronospora parasitica*.[111] In *rbohd* and *rbohd/f* mutants, pathogen-induced ROS synthesis is reduced in guard cells, and plants are more susceptible to both pathogens.[105] The role of RBOHs in biotic stress response is tied to pathogen-associated molecular pattern (PAMP)-triggered immune responses.[112,113] There is an enhanced ROS burst during PAMP in the absence of mitogen-activated protein kinase 4 (MAPK4), suggesting that MAPK4 works to downregulate a RBOHD-mediated ROS burst.[114] Major PAMP-triggered immune responses are activated by bacterial flagellin protein 22 (flg22) and elongation factor TU (EF-Tu), which activate pattern recognition receptors (PRRs), including flagellin sensitive 2 (FLS2) and elongation factor receptor TU (EFR). Upon recognition of flg22 and EF-Tu, PRRs recruit a coreceptor, brassinosteroid insensitive1 (BRI1)-associated kinase 1 (BAK1), which initiates a pathogen defense signaling cascade[115] including phosphorylation of botrytis-induced kinase 1 (BIK1) and AVRPPHB susceptible-like 1 (PBL1). The N-terminal domain of RBOHD directly interacts with BIK1,[112,113] PBL1,[113] FLS2,[112] and EFR in a ligand-independent manner.[113] BIK1 is able to directly transphosphorylate RBOHD.[113] BIK1 is negatively regulated by calcium-dependent protein kinase 28 (CPK28), ensuring an optimal level of BIK1 accumulation.[116] FLS2, BAK1, BIK1, CPK28, RBOHD, and cysteine-rich receptor-like kinase 36 (CRK36), which enhances the ROS response,[117] work together to create a feedback loop regulating pathogen defense signaling.

The RBOHD protein has multiple phosphorylation sites that modulate the enzyme activity during pathogen response. The N-terminal domain of RBOHD has six serine phosphorylation sites.[112,113] Three serines are phosphorylated solely by BIK1, while one serine can be phosphorylated by BIK1 or CPKs and two other serines are phosphorylated by CPKs.[113] To determine the impact of BIK1 phosphorylation of RBOHD, serine-to-alanine mutants were made for BIK1-specific phosphorylation sites. While the single mutations did not have a significant effect on the flg22-induced ROS burst, double and triple site mutations diminished this response.[113] Similarly, in the *rbohd* null mutant, the ROS burst is abrogated upon treatment with flg22 or EF-Tu.[118] The triple site mutant showed no differences in ABA-induced stomatal closure, indicating that ABA-dependent activation of RBOHD and RBOHF is not mediated by BIK1 phosphorylation.[113]

Pathogen response elicits both Ca^{2+} and ROS induction, where ROS is required to maintain prolonged Ca^{2+} signaling.[119] HEK293T cells transfected with *FLAG:RBOHD* show that ROS levels increase after ionomycin-induced Ca^{2+} accumulation, suggesting that RBOHD is regulated by Ca^{2+} binding.[119] Furthermore, mutations in Ca^{2+} binding sites (EF-hand motifs) in *FLAG:RBOHD*-transfected cells abolished ionomycin-induced ROS production.[119] Calcium measurements in wild-type guard cells treated with flg22 show an initial Ca^{2+} peak followed by a second peak or a plateau.[118] In *rbohd*, the initial peak is comparable to that for the wild-type while the second peak/plateau is reduced, suggesting that an initial ROS burst is required to maintain Ca^{2+} signaling.[118] These results indicate a feedback loop between Ca^{2+} and RBOHD. BIK1 is able to phosphorylate RBOHD upon treatment with Ca^{2+} channel inhibitors,[112,113] suggesting Ca^{2+}-independent BIK1 phosphorylation within guard cells during pathogen attack.

Ethylene, a gaseous plant hormone, is involved in biotic stress signaling pathways upstream of RBOHD activation. Ethylene treatment increases transcript accumulation of *FLS2*, the gene encoding the flg22 receptor.[120,121] FLS2 protein accumulation was reduced in two mutants with defects in ethylene signaling: ethylene response 1 (ETR1), an ethylene receptor, and ethylene insensitive 2 (EIN2), an essential signaling protein.[120] Similarly, flg22-induced ROS detected using luminol and horseradish peroxidase is decreased in *etr1–1* and *ein2–1* mutants relative to the wild-type.[120]

2.4.2. Abiotic Stress Response.—Plants experience constant fluctuations in their environment, such as light, water, salt, nutrients, and temperature, and are subject to abiotic stress when these factors are not at optimal levels. Reactive oxygen species produced in plants in response to abiotic stress can lead to two outcomes: acclimation to stress through ROS signaling events or ROS-induced cell death. Abiotic stress can elicit rapid responses, where local and systemic changes occur in seconds to minutes, or slow responses, where changes take place in tens of minutes to hours and affect the whole plant.[122] A recent review integrates data suggesting that metabolic and physiological changes induced by abiotic stress are mediated through both rapid and slow responses, where pulses of transcript abundance changes are regulated by ROS and Ca^{2+} waves to prime and/or acclimate plants to fluctuations in their environment.[122] RBOHD and RBOHF play major roles in ROS production in response to multiple abiotic stresses,[123] while RBOHI plays a role in root abiotic responses.[106]

An example of detrimental ROS accumulation occurs in plants exposed to ozone, an atmospheric pollutant.[107] Ozone treatment of wild-type plants elevated ROS levels in guard cells, which then spread to neighboring mesophyll cells.[107] ROS levels in mesophyll cells, measured by DCF fluorescence, were decreased in the *rbohd, rbohf*, and *rbohd/f* mutants, suggesting that RBOHD and RBOHF propagate the ROS signal to nearby cells.[107] Ozone treatment caused similar levels of cell death and necrotic lesions in leaves of wild-type and *rbohd* plants, while *rbohf* single and *rbohd/f* double mutants exhibited smaller lesions.[107] Resistance to ozone stress in *rbohf* and *rbohd/f* suggest that the ROS accumulation in response to ozone is not an acclamatory signal.[107] In other cases, such as hypoxia and salt stress, ROS is a necessary signal to initiate expression of acclimation genes.[42,108]

While ROS produced by RBOHD and RBOHF accelerate cell death in response to ozone,[107] these same RBOHs facilitate plant survival in saline-rich[42] and hypoxic environments.[108] In wild-type plants, salt stress increases ROS levels in roots[42,108] and hypoxia increases ROS in leaves,[42] leading to higher levels of Ca^{2+}.[42,108] In contrast, these stresses result in lower ROS accumulation in *rbohd/f* double and single mutants, as measured by DAB or DCF.[42,108] Leaf damage in response to salt stress (chlorosis and browning) or hypoxia stress (pale leaves with necrotic lesions) was accentuated in *rbohd/f* double mutants compared with wild-type or single mutant plants.[42,108] RBOHI also contributes to hypoxia tolerance.[124] Although ROS levels were not measured, *rbohi* mutants had paler leaves and lower seedling survival than the wild-type under hypoxic conditions.[124]

RBOHD and RBOHF function in response to multiple stresses, but there are different physiological outcomes for each stress. Salt-stressed *rbohd/f* mutants had elevated sodium and reduced potassium contents compared with wild-type plants, suggesting that ROS regulates Na^+ and K^+ homeostasis.[42] Treatment with DPI to inhibit RBOH activity in the wild-type increased sodium to similar levels as *rbohd/f* while exogenous H_2O_2 decreased the sodium content in *rbohd/f* mutants, consistent with ROS mediating salt stress response. Hypoxia-induced genes involved in anaerobic central metabolism were downregulated in *rbohi*[124] and *rbohd/f* relative to the wild-type, suggesting that the RBOHD- and RBOHF-dependent ROS burst initiates transcriptional cascades to shift aerobic metabolism to anaerobic respiration.[108]

A specific role of RBOHD was found in response to iron deficiency.[109] Iron deficiency increases transcript accumulation of root-specific genes that orchestrate iron uptake to decrease leaf chlorosis.[125,126] Like other stresses, ROS signaling is central in the iron deficiency response.[127] Iron-deficient growth conditions increase transcript accumulation of *RBOHD* and *RBOHF* in wild-type plants.[109] Similar to RBOHC, RBOHD interacts with a member of the ROP family, ROP6.[109] *RBOHD* transcript accumulation is dependent on ROP6, and leaves of *rop6* and *rbohd* mutants were more chlorotic compared with wild-type plants and *rbohf* mutants under iron deficiency.[109] Consistent with a decrease in *RBOHD* transcript levels, ROS production is decreased in *rop6*, and the mutant is unable to induce expression of iron uptake genes,[109] consistent with a ROS-dependent transcriptional response cascade.

Ethylene increases in response to stress[128] and modulates growth and development through transcriptional networks.[129,130] Responses to multiple stresses, such as heat, high aluminum levels, flooding, high light, and drought, are linked to the transcription factor ethylene response factor 74 (ERF74), which was shown to increase expression of a luciferase reporter driven by a *RBOHD* promoter sequence.[128] The *erf 74* mutant had decreased ROS production, while the *35S::ERF74* overexpression line had increased ROS-dependent DCF fluorescence during response to these stresses. Wild-type plants treated with DPI or in *rbohd* and *erf 74* mutants had decreased transcript abundance of genes known to respond to the different stresses, suggesting that RBOHD-synthesized ROS is necessary for the activation of these genes, presumably through a ROS-regulated transcription factor.[128] While ERF74 regulates *RBOHD* expression, *ERF6* expression is induced downstream of RBOHD-mediated ROS signals.[131] The *rbohd* mutant has reduced levels of *ERF6* transcripts, and

these transcripts increased after treatment with H_2O_2.[131] The *erf6* had decreased accumulation of transcripts encoding antioxidant proteins, suggesting that ERF6 may play a role in protecting plants from excess ROS.[131]

RBOHI, which is also expressed in seeds and roots (Figure 2), was recently shown to play a role in seed germination and plant survival under drought conditions.[106] Germination of *rbohi* mutant seeds was delayed relative to the wild-type upon exposure to mannitol, which mimics drought stress conditions. When subjected to water withholding, leaves of soil-grown *RBOHI*-overexpressing plants were more resistant to wilting and exhibited better recovery upon rewatering compared with wild-type plants.[106] The *rbohi* mutants and *RBOHI*-overexpressing plants were indistinguishable from the wild-type under control conditions, indicating a stress-specific function for RBOHI.[106] Collectively, the expression patterns of *RBOHs* show specific (in the case of RBOHI) or broad (as seen in RBOHD and RBOHF) functions in response to abiotic stress.

3.   MECHANISMS THAT SCAVENGE ROS

3.1.   Enzymatic Machineries That Scavenge ROS.

Plant cells utilize many of the same enzymatic pathways to reduce or neutralize ROS that have been described for animal cells. Multiple enzyme classes are involved in direct or indirect scavenging of ROS.[132–135] Catalase in particular catalyzes the conversion of H_2O_2 to H_2O and O_2.[132] In *Arabidopsis*, there are three catalase proteins: CAT1, CAT2, and CAT3.[136,137] Genetic studies have shown that deletion of *CAT2* reduces catalase activity by 90% in *Arabidopsis* leaves, while there is only a slight reduction in catalase activity with the deletion of *CAT1* or *CAT3*.[132] *CAT2* and *CAT3* transcript abundances in seedlings are regulated by the circadian clock, with *CAT2* transcript abundance peaking in the morning[138] and *CAT3* in the evening.[139] Catalase enzymes are highly involved in the stress response and also have developmental implications, which have been previously reviewed.[132,140]

Localization of catalase proteins was determined using GUS reporters and qRT-PCR, with *CAT1* localizing mainly in the cotyledon, rosette leaves, roots, and the siliques. In contrast, *CAT2* is expressed in germinating seeds, cotyledons, flowers, bolts, and siliques,[141] while *CAT3* is found in roots and young leaves.[141] Subcellular localization of catalase enzymes using biochemical fractionation has demonstrated that all three catalase enzymes are in the mitochondria[142] and in the peroxisome, consistent with the presence of a peroxisomal targeting sequences.[143] Although one study found catalase activity in chloroplast fractions,[144] other authors have suggested that there may have been peroxisomal contamination of the chloroplast fraction.[143]

Peroxidases are enzymes that function to reduce peroxide and have been shown to modulate ROS regulation and redox signaling in *Arabidopsis*.[140,145] In many of these peroxidases, the catalytic activity depends on a peroxidatic cysteine and a resolving cysteine.[146] These enzymes are able to detect the levels of ROS on the basis of their oxidation status.[135] There are many families of peroxidases, including peroxiredoxins, ascorbate peroxidase, and glutathione peroxidase, each of which functions as an enzymatic antioxidant. After reduction of H_2O_2, each peroxidase is reduced back to the active form by a pathway of enzymes or

small molecules. For example, glutathione peroxidases are oxidized after reducing H_2O_2. They are reduced by glutathione (GSH), which is thereby converted to its oxidized form, glutathione disulfide (GSSG). GSSG is then reduced by glutathione reductase (GR), with reduced GR regenerated by electrons from NADPH. In *Arabidopsis*, glutathione peroxidases[147] and GSH[148] have been reviewed with respect to their antioxidant ability and role as redox sensors.[149,150]

Another peroxidase in *Arabidopsis*, ascorbate peroxidase, utilizes L-ascorbic acid to reduce H_2O_2, converting the ascorbate to monodehydroascorbate, which can either be reduced by monodehydroascorbate reductase (MDAR) back to ascorbate or become dihydroascorbate.[151] Dihydroascorbate is recycled back to L-ascorbic acid by dehydroascorbate reductase (DHAR) using GSH.[151] The role of ascorbate peroxidase in maintaining ROS homeostasis in the chloroplast[152,153] and in response to abiotic stress[154] has recently been reviewed.

Thioredoxin and glutaredoxin are found at multiple subcellular localizations and are reduced by NADPH and glutathione, respectively. Thioredoxin has been implicated in the ability to reduce peroxiredoxins. Peroxiredoxins are able to become sequentially oxidized, resulting in a cysteine sulfinic acid (-SOOH) that can be reduced to a cysteine sulfenic acid (-SOH) by sulfiredoxin. The localization of peroxiredoxins as well as their catalytic function and physiological significance in plants was recently reviewed in detail,[133,146] as was the function of both glutaredoxin and thioredoxins.[134,149,150,155,156] The detailed mechanisms of action and regulation of these pathways are beyond the scope of this review and have been previously reviewed.[133,134,146,149,150,155,156]

3.2. Plant-Specific Non-enzymatic ROS Scavengers.

To assume their signaling roles, ROS must reach certain threshold levels to oxidize their downstream target proteins. These protein oxidation events will then propagate the ROS signal to initiate the specific growth and developmental programs and stress responses described above.[157,158] However, to prevent ROS from reaching concentrations that cause oxidative damage, ROS levels must be tightly regulated by antioxidants.[159–161] Below we highlight plant specialized metabolites with ROS scavenging activity and the diversity of their known roles in plant growth, development, and stress responses. These metabolites are often beneficial to human health, making their biosynthetic pathways targets of metabolic engineering for biofortification. Two well-known examples are golden rice, which has increased carotenoid levels,[162] and purple tomatoes, with high abundance of anthocyanins.[163]

3.2.1. Carotenoids.—Carotenoids are lipid-soluble pigment molecules of the terpenoid family that produce orange and red colors in plants.[164,165] Carotenoids in the chloroplast increase photosynthetic efficiency by acting as accessory pigments, protein stabilizers, and antioxidants.[164–166] Carotenoids are essential, as homozygous mutations in early pathway enzymes cause seedling lethality.[167,168] There are two classes of carotenoids: carotenes (α-carotene and β-carotene) are pure hydrocarbon molecules, as opposed to their oxygen-containing derivatives, xanthophylls (lutein, zeaxanthin, antheraxanthin, violaxanthin, and

neoxanthin).[164,165] The biosynthetic pathway of carotenoids is well-established and was recently reviewed elsewhere.[164,165]

Carotenoids accumulate in the light-harvesting centers of Photosystems I and II (PSI and PSII), which are two complexes that mediate photosynthesis, where they scavenge ROS byproducts of photosynthesis.[169] High light intensity increases production of singlet oxygen ($^1O_2$),[169] a short-lived, highly damaging ROS responsible for the majority of photooxidative damage.[170] High light intensity can also excite chlorophyll to the triplet state (^3Chl), which interacts with O_2 to form $^1O_2$.[169] Xanthophylls directly quench 95% of ^3Chl, leaving a small percentage available for $^1O_2$ formation.[169,171] A small fraction of xanthophylls are found in the lipid bilayer, where they also reduce lipid peroxidation by $^1O_2$.[171–174]

Non-photochemical quenching (NPQ), a process that allows excess light energy absorbed by chlorophyll to be dissipated as heat, is crucial in preventing $^1O_2$ formation.[169,175,176] In wild-type plants, NPQ depends on zeaxanthin, which is produced in response to excess light.[173,177–179] NPQ is repressed in the *non-photochemical quenching 1* (*npq1*) mutant, which does not synthesize zeaxanthin because of a defect in violaxanthin de-epoxidase.[173,178] As a consequence, $^1O_2$ levels are higher in the *npq1* mutant, as measured by the fluorescent probe Singlet Oxygen Sensor Green (SOSG).[180] Constitutive production of zeaxanthin in the zeaxanthin epoxidase-defective *npq2* mutant leads to faster NPQ induction compared with wild-type plants and *npq1*.[178] This is presumably due to higher antioxidant activity from increased zeaxanthin levels, although $^1O_2$ levels have not been reported for *npq2*.

Normal xanthophyll composition in the light harvesting centers is altered in mutants with defective carotenoid enzymes.[181] Mutation of lycopene *e*-cyclase in *lut2* mutants abolishes lutein production and increases flux into the β-carotene branch.[182–184] As a consequence, lutein is replaced by violaxanthin, leading to destabilization of the light harvesting complex and increased ^3Chl in the *lut2–1* mutant.[171,183,184] Compared with wild-type plants, decreased NPQ or unstable light-harvesting complexes have been reported for the majority of carotenoid mutants,[181,185] and increased $^1O_2$ production has been reported in *lut2*, *lut5*, and *chy1/chy2* mutants, which have defects in the xanthophyll-producing enzymes cytochrome P450 enzyme CYP973A/LUT5 and carotenoid hydroxylase 1 and 2 (CHY1 and CHY2).[186,187] Higher-order mutants tend to have more compromised NPQ and photoprotection, with increased $^1O_2$ levels reported in more recent articles.[187–190] A quadruple mutant that does not produce any xanthophyll compounds, *no xanthophyll* (*nox*, defective in CHY1, CHY2, LUT1, and LUT5) has extreme sensitivity to light and decreased photosynthetic efficiency.[191] Although *nox* does synthesize α- and β-carotenes, $^1O_2$ levels were dramatically higher in the mutant compared with the wild-type, suggesting that xanthophylls play a major role in ROS scavenging.[191] Moreover, *nox* mutants exhibited extreme sensitivity to light, as photoinhibition studies and $^1O_2$ measurements were performed at a light intensity of 200 μmol photons s^{-1} m^{-2}, a level 5-fold lower than commonly used for "excess light".[191] As a consequence, *nox* mutants could survive only under very low light (20 μmol photons s^{-1} m^{-2}).[191] Collectively, these studies suggest that optimal $^1O_2$ scavenging requires a mixture of xanthophyll compounds.

Binding of β-carotene affects the stability of the photosystem core complex.[192] The *suppressor of zeaxanthin-less 1* (*szl1*) mutant has decreased lycopene β-cyclase (LycB) activity, increasing flux into the α-carotene branch.[186,189] Replacement of β-carotene with α-carotene in PSI increases photosensitivity and $^1O_2$ production in *szl1* compared with the wild-type.[186] Additionally, an oxidation product of β-carotene, β-cyclocitral (β-CC), is involved in acclimation to excess light. Changes in transcription levels in response to β-CC are specific to $^1O_2$ stress and participate in retrograde signaling to promote acclimation to excess light.[193,194] Lipid peroxidation and decreased photosynthetic efficiency caused by high light stress are mitigated in plants exposed to β-CC prior to high light exposure compared with untreated plants.[194] Collectively, carotenoids assume a number of distinct and important roles to mitigate high light stress by direct scavenging of $^1O_2$ and promotion of acclimation.

3.2.2. Ascorbate.—L-Ascorbic acid (vitamin C) is a water-soluble, plant-derived antioxidant that is essential to humans and serves diverse functions in plants, especially during abiotic and biotic stresses. Besides acting as a cofactor in plant enzymatic reactions, it can donate electrons and thereby act as a ROS scavenger.[195,196] However, the iron-reducing activity of L-ascorbic acid can lead to the formation of Fe^{2+} from Fe^{3+} and thus to increased $^•OH$ production by the Fenton reaction.[197] In humans, dietary vitamin C is required for collagen and carnitine biosynthesis,[198,199] and its deficiency in humans, better known as scurvy, results in weakness, joint pain, gum bleeding, and skin hemorrhages.

The L-ascorbic acid biosynthetic pathway in plants converts L-galactose to L-ascorbic acid. The final reaction occurs on the inner mitochondrial membrane. From there, L-ascorbic acid is transported to different subcellular compartments, including the cell wall, to assume its different functions.[200] L-Ascorbic acid is present at high concentrations in the different subcellular compartments (mM range in the chloroplast and cytosol)[201,202] and thus serves as a major antioxidant in plants.

L-Ascorbic acid reacts with free radicals and Fe^{3+} to produce monodehydroascorbate or can be converted to monodehydroascorbate by ascorbate peroxidase, which concomitantly catalyzes the conversion of H_2O_2 to water.[203] Monodehydroascorbate can either be recycled to L-ascorbic acid through MDAR[204,205] or be non-enzymatically converted to dehydroascorbate. Dehydroascorbate is converted to L-ascorbic acid by DHAR, which uses glutathione a reducing agent.[205]

Several mutants with altered L-ascorbic acid levels, termed *vtc* mutants, were isolated in *Arabidopsis*, enabling characterization of L-ascorbic acid functions in plants.[206,207] A 5-fold decrease in L-ascorbic acid levels in the *vtc2* mutant, which has a defect in the synthesis of the L-ascorbic acid precursor l-galactose, did not result in reduced plant growth.[206] This indicates that in the absence of stress, only minimal levels of l-ascorbic acid are necessary to support plant growth. However, the *vtc* mutants, as well as mutants defective in ascorbate peroxidase or ascorbate recycling, are more sensitive to environmental stresses than the wild-type, in particular to photooxidative stress.[160,208] During high light stress, removal of H_2O_2 by ascorbate peroxidases and 2-cysteine peroxidases reduced superoxide levels, as shown by NBT staining of leaves, to protect the photosynthetic machinery.[209] In heat-

stressed leaves with an inactivated oxygen-evolving complex, L-ascorbic acid acted as an electron donor to PSII, thereby sustaining electron transport.[210] The roles of L-ascorbic acid against biotic stresses are more complex, as protective effects from certain pathogens were observed at reduced L-ascorbic acid levels,[211,212] while in other cases elevated ascorbate increased pathogen resistance.[213]

3.2.3. Tocochromanols.—Tocochromanols, such as vitamin E, include tocopherols and tocotrienols, which are lipid-soluble compounds produced mainly by photosynthetic organisms. They are synthesized by condensation of tyrosine-derived homogentisate and a polyprenyl side chain; the side chain is saturated in tocopherols and contains three unsaturated double bonds in tocotrienols.[214] The homogentisate moiety forms a chromanol ring and has antioxidant capacities,[215,216] while the polyprenyl side chain anchors tocochromanols into membranes. Tocochromanols accumulate in photosynthetic membranes and plastoglobules inside the chloroplast.[217] Because of their presence in photosynthetic membranes, the main antioxidant activity of tocochromanols is to scavenge lipid peroxyl radicals generated by lipid peroxidation[218] through $^1O_2$. Additionally, tocochromanols have excellent *in vitro* $^1O_2$ quenching and scavenging capacities.[219,220] These compounds are also important in the human diet, as tocochromanols are essential for reproduction.[221]

Arabidopsis mutants with altered tocochromanol biosynthesis, termed *vte* mutants, have provided insight into the roles of tocochromanols. The *vte1* mutant does not exhibit any differences in growth, photosynthetic quantum yield, or chlorophyll content under normal light conditions compared to the wild-type, and only minimal differences were observed under high light stress.[222] Similar results were observed in *Synechocystis*, a unicellular photosynthetic cyanobacteria that synthesizes tocopherols.[223] In a tocopherol-deficient *Synechocystis* strain with a mutation in the homogentisate phytyltransferase, growth under high light conditions did not change from the wild-type to the mutant.[223] Treatments that induce superoxide or $^1O_2$ production, combined with high light stress, slowed the growth of the wild-type and the tocopherol-deficient *Synechocystis* mutant,[223] suggesting that tocopherols are not essential in scavenging these ROS. However, treatment with polyunsaturated fatty acids, which generate lipid peroxyl radicals by auto-oxidation in the presence of oxygen, slowed the growth of the tocopherol-deficient *Synechocystis* mutant,[223] indicating that tocopherols protect cells from lipid peroxidation. A similar function for tocopherols in protecting lipids from oxidation during seed germination and early seedling development was uncovered in *Arabidopsis thaliana* using the tocopherol-deficient mutants *vte1* and *vte2*.[224,225]

Beyond their role in protecting plant cells against lipid peroxidation, tocopherols have also been shown to scavenge $^1O_2$ formed by PSII.[226] Singlet oxygen formation can induce degradation of the D1 protein, a protein that forms the reaction center of PSII. The role of tocopherols as $^1O_2$ scavengers was shown to be particularly important when the turnover rate of the D1 protein is high.[226]

3.2.4. Flavonoids.—Flavonoids are a large class of plant metabolites that act as antioxidants to lower ROS levels in plants. Flavonoid antioxidants have been studied to have positive effects on human health including in cancer and age-related diseases.[227,228]

Flavonoids regulate a wide array of plant developmental programs and stress responses, prompting intense study of their biosynthesis and regulation.[229–232] The flavonoid biosynthetic pathway, which is shown in Figure 5, begins with the condensation of *p*-coumaroyl-CoA with three malonyl-CoAs, resulting in synthesis of multiple flavonoid subgroups, including flavonols (described in detail in the next section), flavones, flavan-3-ols, flavanones, anthocyanins, and isoflavonoids.[228,233] Specific pathway details have been reviewed elsewhere.[229,232]

The first committed step of flavonoid biosynthesis is catalyzed by chalcone synthase (CHS) to produce naringenin chalcone. Naringenin, isomerized from naringenin chalcone, is a precursor for the flavone and isoflavonoid branches. Flavonols are produced from dihydrokaempferol, an intermediate downstream of naringenin. The flavan-3-ol branch is produced from leucoanthocyanidins downstream of dihydrokaempferol, as is the first committed step in the production of anthocyanins, the final subclass produced in the flavonoid pathway. The flavonoid compounds can be further modified by glycosylation of hydroxyl groups with different carbohydrates, which further increases the chemodiversity of flavonoid compounds.

Flavonoid composition is species-specific, with some plant species being able to produce all of the flavonoid subclasses while in others only some subclasses accumulate.[234] For instance, flavones[235] such as apigenin, luteolin, baicalein, and chrysin are found in herbs such as parsley and thyme, while the flavanones eriodictyol, hesperetin, and naringenin accumulate to high levels in citrus fruits.[236] Anthocyanins are red, blue, and purple pigments found in most plant species, with high concentrations found in many berries, fruits, and flowers.[237,238] Flavan-3-ols, which include catechins and epicatechins, are most commonly associated with tea leaves,[228] but are also found in berries, apples, and cacao. Finally, isoflavonoids are most commonly found in soybeans and other legumes,[234] although they are present in other plant species. The differences in concentrations and types of flavonoids found across the plant kingdom are consistent with species-specific regulation and modulation of the biochemical pathway.

What is particularly striking is the difference in the antioxidant activities of these subclasses of flavonoids, suggesting that some flavonoid subclasses with higher antioxidant capacity have a more significant role in redox-regulated processes. Flavonoids share a similar chemical backbone with two benzene rings linked by a heterocyclic pyran ring, enabling them to donate one or more electrons to less stable molecules such as ROS.[239] Many of the flavonoid backbones are decorated by hydroxylation, methylation, glycosylation, and/or acylation, which alters their ability to scavenge ROS, thus leading to a range of antioxidant activities *in vivo* that reflect the immense flavonoid chemodiversity. Flavan-3-ols, flavonols, and anthocyanins are able to act as potent antioxidants for lipid peroxidation *in vitro*.[240,241] Studies investigating the flavonoid scavenging ability of H_2O_2 and $^1O_2$ found that anthocyanins and flavonols are the most effective in reducing these ROS,[241] while flavones are the least effective, with no antioxidant capacity for H_2O_2 and very little capacity for $^1O_2$. Other classes show selectivity: flavonones show no antioxidant capacity for $^1O_2$ and very little capacity for H_2O_2, whereas flavan-3-ols have high scavenging ability for H_2O_2 and

very low ability to scavenge $^1O_2$.[241] Because of their high antioxidant activity, flavonols and anthocyanins are some of the most interesting and best-studied members of this family.

Flavonoid accumulation is induced by a variety of ROS-inducing abiotic stresses, hormones, and developmental signals.[229,231,232,242,243] Expression of genes encoding flavonoid metabolic enzymes is highly regulated by transcription factors belonging to the R2R3-MYB, basic helix–loop–helix (bHLH), and WD40-type families of transcription factors. These factors associate in a ternary complex (termed the MYB–bHLH–WD40 complex)[244] and mediate the changes in flavonoid synthesis in response to environmental factors[229,231] and hormones.[242,243]

Anthocyanins, the most downstream products of the flavonoid pathway, are the best-studied flavonoid subclass because their pigmentation allows easy visualization of their stress- or developmentally-induced synthesis. The transcriptional machinery that drives stress-induced anthocyanin synthesis includes a number of MYB proteins, including MYB75, also called production of anthocyanin pigment 1 (PAP1), PAP2,[245] MYB113, and MYB114.[246] A variety of abiotic stresses, including high light, drought, high sucrose, and oxidative stress, have been shown to cause increased anthocyanin synthesis via PAP1.[247–249] An *Arabidopsis* line that overaccumulates *PAP1 (pap1-D)* has constitutively high anthocyanin accumulation driven by increased transcription of dihydroflavonol-4-reductase (DFR), the metabolite branch point channeling dihydroflavonols into anthocyanins.[250,251] The presence of elevated anthocyanins offers protection from the damage caused by these stresses.[247,252] To demonstrate the protective role of flavonoids, flavonoid-deficient *transparent testa 4* (*tt4*), which has a defect in CHS, was shown to be more sensitive to drought and oxidative stress and to lose the protective effect of *pap1-D* in a double mutant.[252] Consistent with flavonoids acting as antioxidants to reduce ROS overaccumulation, *pap1-D* has reduced H_2O_2 levels in leaf tissue during drought stress compared with the wild-type, while *tt4* has elevated levels, as detected by DAB.[252] H_2O_2 treatment was shown to induce transcription levels of zinc finger of *Arabidopsis thaliana 6* (ZAT6), which is a positive regulator of flavonoid synthesis, suggesting that ROS can also stimulate antioxidant production.[251] Knockdown of ZAT6 showed decreased anthocyanin accumulation, while ZAT6 overexpression resulted in increased anthocyanin levels.[251] However, these studies did not look at changes in endogenous ROS levels after anthocyanin accumulation/reduction in these mutant lines.

Anthocyanin accumulation is a typical phenotype associated with high light stress. One regulatory process in facilitating the response to high light is to deactivate negative regulators of anthocyanin biosynthesis. The transcription factor TCP15 negatively regulates anthocyanin biosynthesis under normal conditions.[253,254] However, during prolonged exposure to high light, TCP15 is rendered inactive.[253] This deactivation is facilitated by increased ROS levels, as oxidation of a cysteine residue in the protein prevents binding of TCP15 to DNA.[253] Once TCP15 is inactivated, anthocyanin accumulation can occur, which may be a protective mechanism to prevent damage from high light stress.[253] In contrast, two NAC transcription factors, ANAC032 and NAC019, are induced in response to high light/increased sucrose[254] and drought,[255] respectively. These transcription factors have also been shown to be involved in repression of anthocyanin biosynthesis. Treatments of ANAC032 overexpression lines with the catalase inhibitor 3-amino-1,2,4-triazole (3-AT), with the goal

of increasing ROS accumulation, reduced the anthocyanin accumulation compared with 3-AT-treated wild-type and ANAC032 repressor plants. These results suggest that ANAC032 represses anthocyanin synthesis under high light and in the presence of elevated ROS levels. [254]

Despite visible changes in anthocyanin levels upon environmental stresses or during development, changes in anthocyanins could reflect altered flux through the entire pathway and affect levels of metabolites belonging to the flavonoid subclasses outlined above. In particular, the dihydroflavonols are precursors of both flavonols (through the action of flavonol synthase (FLS)) and anthocyanins (through the action of DFR) (Figure 5), so these two steps represent important branch points. Flavonol compounds synthesized from dihydrokaempferol include kaempferol, quercetin, isorhamnetin, and myricetin, which have distinct antioxidant activities.[256–260] In *Arabidopsis*, 32 different flavonol derivatives with distinct glycosylation patterns have been identified by liquid chromatography-mass spectrometry (LC-MS),[261] but the antioxidant capacities of only a few of these compounds have been measured. *In vitro* data suggest that quercetin may be the best ROS scavenger.[240] Quercetin 3-*O*-Glc has a lower IC_{50} value for superoxide ions than kaempferol 3-*O*-Glc, indicating that quercetin is a better superoxide scavenger *in vitro*. Similarly, quercetin is able to scavenge $^1O_2$ better than kaempferol, although myricetin and isorhamnetin have been reported to be better at scavenging $^1O_2$ than quercetin.[241] Among the flavonols, quercetin has the highest antioxidant capacity for H_2O_2, followed by kaempferol, myricetin, and isorhamnetin.[241] These insights into regulation of flavonol synthesis, flavonol localization, and their range of antioxidant activity set the stage for understanding the specific functions of these molecules.

4. GENETIC APPROACHES THAT UNCOVERED THE ROLE OF FLAVONOLS AND ROS IN PLANT DEVELOPMENT AND STRESS RESPONSES

Multiple stress responses increase anthocyanin levels,[250] which are readily detected because of changes in the concentrations of these pigments, but few studies have investigated how this impacts accumulation of earlier pathway products like flavonols. Additionally, in many publications the effect of changes in anthocyanin accumulation on ROS homeostasis or flavonol levels has not been examined. Recent experimental advances have made it possible to study flavonols directly. LC-MS can be used to quantify changes in accumulation of specific flavonols, while the identification of diphenylboric acid 2-aminoethyl ester (DPBA), which binds flavonols and becomes fluorescent, allows their visualization *in planta*.[263] Both methods have revealed developmental, hormonal, and stress-induced synthesis of these compounds. Finally, the power of genetic systems like those found in *Arabidopsis* is that we can begin to elucidate the specific functions of individual flavonoid molecules through examination of genetic mutants or tissues in which these flavonols and anthocyanins are uncoupled.

Flavonols are particularly interesting because of their cellular localization. In plant cells, ROS accumulates in the cytoplasm, nucleus, and cell wall, as determined using confocal

imaging. This localization overlaps with flavonol accumulation patterns and subcellular localization of enzymes involved in flavonol synthesis.[96,242,264,265] Since anthocyanins localize to the vacuole,[266,267] flavonols are more likely to be the active compounds in modulating cytoplasmic and nuclear ROS.[76,242,264,268] In support of the hypothesis that flavonols act as an important class of ROS scavengers, recent studies have shown that flavonols alter growth and development by locally regulating ROS levels. Using well-established genetic systems, this section examines the function of flavonols as ROS scavengers in a variety of stress and developmental contexts.

4.1. Roots.

Root tissues synthesize and accumulate flavonols, but do not synthesize later flavonoid pathway intermediates such as anthocyanins because of the absence of transcripts encoding DFR, a branch-point enzyme controlling the conversion of dihydroflavonol precursors into anthocyanins.[242] The role of flavonols in modulating root architecture was initially tied to their ability to negatively regulate transport of the hormone auxin. Mutants that do not make flavonols have elevated auxin transport compared with the wild-type,[269–272] and these studies revealed that quercetin derivatives are the active flavonols that negatively regulate auxin transport in roots.[242] Additionally, auxin treatment of wild-type plants increased accumulation of transcripts encoding flavonoid transcription factors and enzymes of the early flavonol biosynthetic pathway, thereby increasing flavonol biosynthesis, perhaps as part of a negative feedback loop.[242] Recent reports have uncovered a role of flavonols in modulating root architecture via antioxidant activity, as summarized below. Additionally, several lines of evidence suggest that auxin transport is regulated by the levels of ROS and RNS,[273] suggesting that the regulation of auxin transport by flavonols might be linked to flavonol modulation of ROS status.

ROS gradients define the root meristematic and elongation zones, which are areas of cell division and elongation, respectively.[274] The ROS gradient is characterized by elevated superoxide accumulation in the meristem, which decreases toward the elongation zone, while H_2O_2 accumulates in the elongation zone and is reduced in the meristem.[274] The spatial balance of superoxide and H_2O_2 is maintained by the transcription factor UPBEAT1 (UBP1), which represses accumulation of transcripts encoding peroxidases in the elongation zone.[274] The *tt4* mutant had increased superoxide levels in the meristem compared with wild-type roots, as measured by NBT staining, which was suggested to override the superoxide/H_2O_2 gradients in the mutant.[275] Treatment of wild-type roots with H_2O_2 induced flavonol accumulation, which is hypothesized to reduce meristem size.[275]

Light induces accumulation of anthocyanins as described above and also increases flavonol accumulation in roots through rapid induction of transcripts encoding enzymes of the early flavonol biosynthetic pathway.[233,276] Visualization of flavonols using DPBA and flavonol metabolite profiling by LC-MS show that in plants where shoots are exposed to light and roots are either shielded (dark-grown) or exposed to light (light-grown), flavonol levels are higher in light-grown roots.[275,277] Wild-type roots exposed to light have reduced cell number in the meristem and shorter primary roots compared with roots shielded from light. [275] In the *tt4* mutant, the effect of light on root growth is lost, with light-grown *tt4* seedlings

resembling the dark-grown wild-type.[275] The longer root length and increased cell number in *tt4* are reversed by application of the flavonol quercetin.[275] In the wild-type, exposure of the roots to light leads to lower superoxide levels compared with dark-grown roots, which may be due to increased flavonol synthesis.[275] Consistent with this finding, superoxide levels in the meristem of *tt4* are comparable to those of dark-grown wild-type roots, suggesting that light-dependent flavonol antioxidant activity regulates root growth.[275] The authors suggest that regulation of flavonol synthesis may occur through cytokinin- and UPB1-H_2O_2-mediated pathways, as *UPB1*-overexpressing plants had increased flavonol levels and H_2O_2.[275] We note, however, that the meristem cell number in *UBP1*-overexpressing seedlings was not quantified in response to light or darkness and that overexpression of *UBP1* was previously reported to reduce meristem cell number and primary root length compared with the wild-type.[274,275] It would be interesting to measure meristem cell number and ROS in crosses between *tt4* and *UPB1*.

The relationship between flavonols, ROS, and root hair development has been explored in tomato (*Solanum lycopersicum*).[272] Roots of the *anthocyanin reduced (are)* mutant, which contains a point mutation in flavonoid-3-hydroxylase (F3H),[259,260] exhibit reduced flavonol accumulation as measured by LC-MS and increased ROS levels as measured by DCF fluorescence.[272] ROS accumulates in epidermal cells and root hairs, with higher ROS levels in the *are* mutant linked to greater root hair density compared with the wild-type (Figure 6).[272] Treatment with a general antioxidant, L-ascorbic acid, reduced ROS levels and root hair number in the *are* mutant, while H_2O_2 treatment of wild-type plants increased ROS and root hair number.[272] Genetic complementation of the *are* mutant restored flavonol synthesis, reduced ROS accumulation, and returned root development to wild-type patterns, consistent with the role of flavonols as antioxidants to modulate root development.[272]

Bending of roots away from light (negative phototropism) may also be modulated by localized synthesis of flavonol antioxidants.[275] Wild-type roots grown in the dark and then exposed to directional light show increased flavonol accumulation on the light-exposed side, which also has a higher elongation rate to allow bending away from the light.[275] Accumulation of GUS activity driven by the *CHS* promoter in transgenic lines and flavonol products overlapped with the convex (outer) side of bent roots.[275] Localized flavonol production is suggested to fine-tune bending, as a reduced bending angle was observed in wild-type roots treated uniformly with quercetin and in *UPB1*-overexpressing lines.[275] Additionally, the absence of flavonols in the *tt4* mutant also resulted in reduced bending angle.[275] Consistent with this result is the finding that ROS are an important signal in asymmetric growth during root gravitropism.[278] Despite these lines of evidence, we note that ROS localization or quantification was not reported in bent roots, which is an important question for future studies on root tropism.

4.2. Guard Cells.

The role of an ABA-dependent ROS burst in guard cells to initiate stomatal closure is clearly established.[73,279,280] Elevated ABA triggers H_2O_2 production through activation of RBOH enzymes as described above.[33,76,281] ROS levels then must reach a certain threshold to oxidize their downstream targets, which may include ion channels that initiate stomatal

closure.[79,282] The localized accumulation of flavonols in guard cells suggested the testable hypothesis that these molecules may act to modulate this ROS to fine-tune guard cell closure and prevent oxidative damage.

Flavonol accumulation in guard cells suggests a role in regulation of ROS homeostasis in a diversity of species, including fava bean (*Vicia faba*), pea (*Pisum sativa*), onion (*Allium cepa*), tomato (*Solanum lycopersicum*), and *Arabidopsis*.[76,264,283–286] Transcripts of flavonoid biosynthetic enzymes CHS and FLS were shown to accumulate in *Arabidopsis* guard cells using a GUS promoter and GFP construct, respectively.[264,271] The accumulation of flavonols in guard cells but not in the surrounding pavement cells of *Arabidopsis* and tomato has been shown using DPBA visualized through confocal microscopy.[76,264,287] DPBA signal accumulates in the nucleus, consistent with prior reports,[76,242,264,268] where it may regulate redox-sensitive transcription factors and/or prevent ROS-induced DNA damage. The *Arabidopsis tt4–2* mutant did not exhibit a DPBA signal and showed increased ROS in guard cells, as visualized by DCF fluorescence.[264] Consistent with enhanced ROS signaling in *tt4–2*, ABA-induced stomatal closure was greater in this mutant.[264] Similarly in tomato, flavonoid production is reduced in the *are* mutant, resulting in decreased DPBA signal in guard cells (Figure 7). In contrast, the tomato *anthocyanin without (aw)* mutant, which has elevated levels of flavonols, has enhanced DPBA signal in guard cells.[76] These results are consistent with flavonol synthesis and accumulation in guard cells and their function to reduce ROS in these cells.[288]

The relationship between ROS, flavonols, and ABA-dependent stomatal closure was studied in greater detail in tomato.[76] Levels of total ROS, as detected by DCF fluorescence, and H_2O_2, as detected by PO1, were shown to increase in response to ABA treatment preceding stomatal closure.[76] The levels of DCF and PO1 were shown to be inversely proportional to the flavonol levels, with increased DCF and PO1 signals in the *are* mutant and decreased DCF signal in the *aw* mutant compared with the wild-type (Figure 7).[76] The ABA-induced DCF signal increases are dependent on RBOH enzymes, as they are not detected after treatment with DPI (Figure 4). Time course experiments showed that the rates of ABA-dependent stomatal closure were proportional to the rate of change in H_2O_2 in these flavonol mutants (Figure 7). Additionally, this study also examined the process of light-dependent stomatal opening. In wild-type plants, ROS decreases substantially after exposure to light. Elevated flavonols in the *aw* mutant facilitated this decrease, and reduced flavonols in the *are* mutant impaired this process.[76] Finally, consistent with a direct relationship between flavonol-modulated ROS and stomatal aperture, the water loss from *are* mutant leaves was greater compared with the wild-type, while the *aw* mutant had less water loss.[76]

The accumulation of flavonols in guard cells to control ROS-induced stomatal closure is regulated by the hormone ethylene. Increasing ethylene levels by treating wild-type plants with ethylene gas, or comparing untreated plants to the *ethylene overproducing 1 (eto1)* mutant, which has endogenous overproduction of ethylene,[289] increased flavonol accumulation in guard cells.[76,264] Coincident with elevated flavonols, there was a substantial reduction in ROS accumulation, as shown with DCF in guard cells of *eto1*[264] or ethylene-treated wild-type *Arabidopsis* or tomato plants.[76,264] Consequently, ethylene-elevated flavonols and decreased ROS resulted in decreased rates of ABA-induced stomatal closure

compared with untreated plants.[76,264] Similarly, induction of flavonol accumulation in *Arabidopsis* guard cells was observed after treatment with the plant growth regulator 5-aminolevulinic acid, which decreased ROS levels and ABA-induced stomatal closure.[287]

In addition to their function in guard cell signaling, flavonols may also modulate the development of stomata and surrounding pavement cells. The altered flavonol glycosylation profiles in the *Arabidopsis rol1* mutant, which has a defect in a rhamnose synthase gene, reducing flavonol rhamnosylation and increasing flavonol glycosylation, are responsible for multiple phenotypes, including pavement and guard cell development, resulting in larger stomata and decreased stomatal density.[265] These phenotypes were rescued when the *rol1* mutation was crossed into the flavonoid-deficient *tt4–2* mutant, verifying that these phenotypes are linked to elevated glycosylated flavonols.[265] Additionally, soybean isolines containing elevated concentrations of kaempferol-tri-*O*-glucoside showed decreased stomatal density in response to blue light.[290,291] However, these studies did not examine the changes in ROS status to ask whether these effects were mediated via flavonol action as antioxidants. Taken together, these experiments implicate flavonols in control of stomatal closure and development, with many of these developmental changes mediated by alterations in ROS signaling

4.3. Pollen.

During plant sexual reproduction, pollen grains germinate on the stigma of flowers to generate a pollen tube that grows through female reproductive tissues to fertilize ovules. As mentioned above, many stages of plant reproduction–from pollen development to fertilization of ovules–are modulated by ROS. Flavonols and their glycosylated derivatives accumulate in plant reproductive structures, specifically in pollen, anthers, and stigmas, [96,292–294] raising the intriguing possibility that flavonols in reproductive structures serve as regulators of ROS-dependent processes. Flavonols were found to regulate pollen viability, germination, and tube growth and integrity.[96,292,295–299] *CHS-RNAi* tomato plants had reduced pollen tube growth, which led to reduced or no production of seeds.[297] Pollen germination and tube growth also were severely inhibited in a petunia *CHS* mutant, leading to reduced seed set.[292,295] Pharmacological complementation of petunia *CHS* mutant pollen with flavonols or an extract from wild-type anthers rescued germination and tube growth *in vitro*,[295] while treatment with other flavonoids such as flavones, flavanones, and dihydroflavanols did not,[299] pointing to a specific role for flavonols. Addition of flavonols to the germination medium also promoted germination and tube growth of wild-type tobacco pollen.[299] *In situ* application of kaempferol or quercetin onto stigmas during self-crosses of the petunia *CHS* mutant or a maize *CHS* mutant resulted in a complete seed set, while self-crosses without flavonol application failed to do so.[296]

Paradoxically, flavonols were not found to be required for fertilization in *Arabidopsis*,[300,301] despite the presence of significant flavonol metabolite pools in *Arabidopsis* pollen.[293,294] Indeed, the *Arabidopsis tt4* mutant had undetectable flavonol levels in reproductive organs yet exhibited pollen tube growth and a seed set similar to the wild-type.[300,301] Surprisingly, the flower flavonoid transporter (FFT), which is expressed in anthers, is required for pollen development, viability, and release and thus for the formation of a complete seed set.[302] It is

still unclear whether this transporter has specificity toward flavonols or promiscuous activity toward other compounds necessary for pollen development. The latter could explain discrepancies between the dispensability of flavonols for pollen development in *Arabidopsis* and the reliance of reproduction on functional transport of flavonols.

While the studies mentioned above hypothesized that flavonols act as signaling molecules in pollen, their function to reduce the levels of ROS in pollen was only recently reported.[96] Muhlemann et al.[96] showed that the tomato *are* mutant had reduced levels of flavonols in pollen grains and tubes. This mutation does not affect anthocyanin accumulation in pollen, as this metabolite is absent in this tissue in both the wild-type and the *are* mutant.[96] The *are* mutant had impaired pollen viability, germination, and tube growth and integrity (Figure 8). ROS levels in *are* pollen grains and tubes, as measured using DCF and PO1 fluorescence, were inversely proportional to the flavonol levels, pointing to a role of flavonols as ROS scavengers in pollen (Figure 8).[96] Treatment with the antioxidant L-ascorbic acid or inhibition of ROS synthesis with the RBOH inhibitor DPI rescued pollen tube growth and integrity, respectively, in the *are* mutant, which confirmed that elevated ROS in the mutant caused inhibition of pollen tube growth and integrity.[96]

Heat stress has profound inhibitory effects on pollen viability[303–305] and pollen tube growth,[306–308] and a recent study by Muhlemann et al.[96] demonstrated that this inhibitory effect is linked to elevated ROS. These authors found that elevated temperatures increased ROS in tomato pollen tubes and resulted in reduced pollen viability and tube growth and integrity.[96] The detrimental effects of elevated temperature on pollen tube growth and integrity were alleviated by supplementing the pollen germination medium with the antioxidant L-ascorbic acid, demonstrating that elevated ROS generated during high-temperature stress underlie the inhibition of pollen tube growth and integrity.[96] In the flavonol-deficient *are* mutant, the high-temperature effects on pollen viability and tube growth and integrity were exaggerated,[96] suggesting that flavonols protect pollen from heat-induced ROS.

5. CONCLUSIONS

ROS are potent signals that can oxidize proteins, thereby changing their activity and leading to growth and developmental changes. Plants have tissue-specific expression of the RBOH family of enzymes that can be activated by hormones or other environmental signals to mediate a range of developmental responses. Equally important to productive ROS signaling is maintenance of ROS homeostasis to prevent its concentration from reaching damaging levels. Regulation of ROS levels in plants relies on a large network of specialized metabolites with antioxidant activities, such as carotenoids and flavonoids. These metabolites originate from different biosynthetic pathways and exhibit distinct subcellular localizations and functions in plant growth, development, and stress responses. Because of their human health-promoting antioxidant activities and visually attractive appearance, metabolites of the flavonoid class have received much attention. Recent experiments using genetic approaches have revealed the *in planta* functions of flavonols as ROS scavengers and their impact on ROS-dependent developmental and stress response programs.

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Biography

Jordan M. Chapman is a Ph.D. candidate at Wake Forest University. She received her B.S. in Chemistry with a concentration in Biochemistry from Wake Forest University. Her current work focuses on identification of plant proteins that undergo reversible oxidation, the role of flavonols in maintaining ROS homeostasis, and how flavonols and oxidized proteins modulate root architecture.

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Sheena R. Gayomba is a postdoctoral researcher at Wake Forest University. She received her M.S. and Ph.D. from Cornell University. Her research uses a genetic approach to uncover the role of flavonol antioxidants in root development in the model plant *Arabidopsis thaliana*. She is also interested in understanding the role of flavonols and reactive oxygen species in modulating plant responses to environmental stresses.

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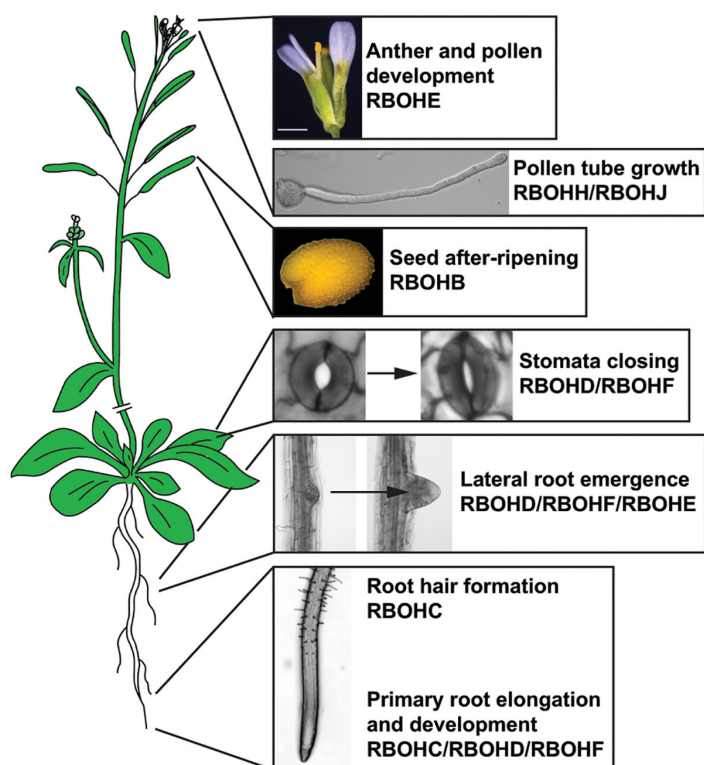
Figure 1.
Respiratory burst oxidase homologues (RBOHs) have developmental roles in distinct plant organs and tissues in *Arabidopsis thaliana*. This figure illustrates the plant model species *Arabidopsis thaliana* (left) and highlights plant tissues and developmental responses, in which the different RBOHs are implicated in controlling signaling or development (right). The image of the *Arabidopsis* plant was reprinted with permission from ref 311.
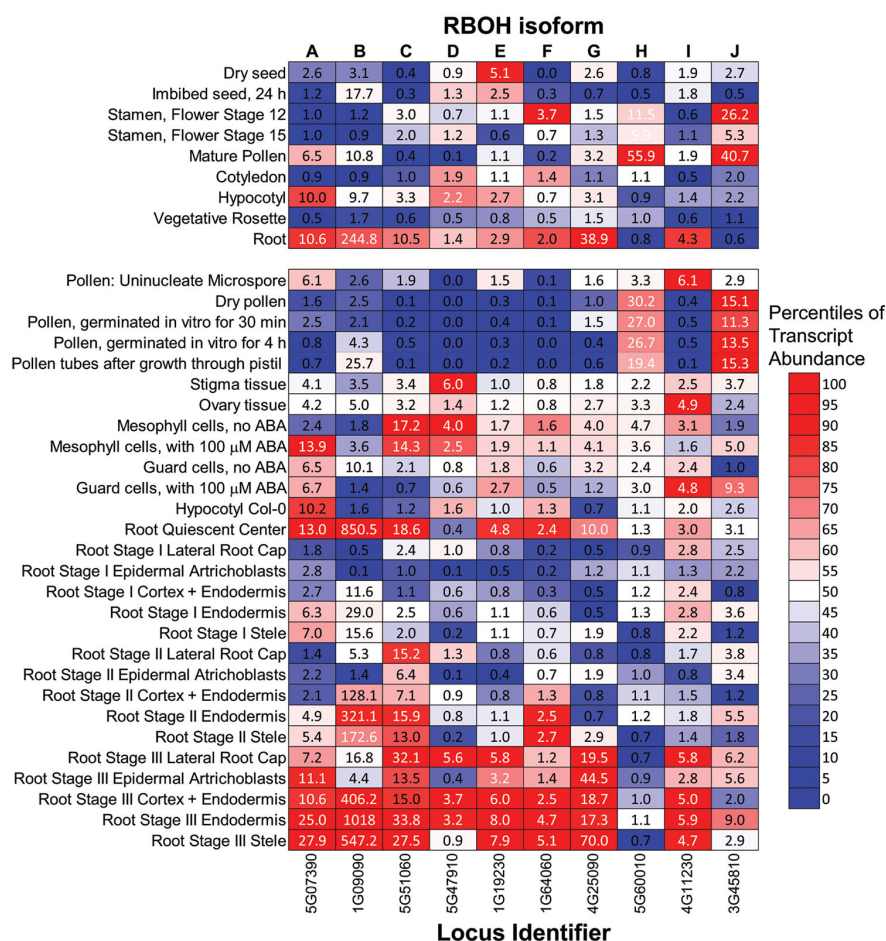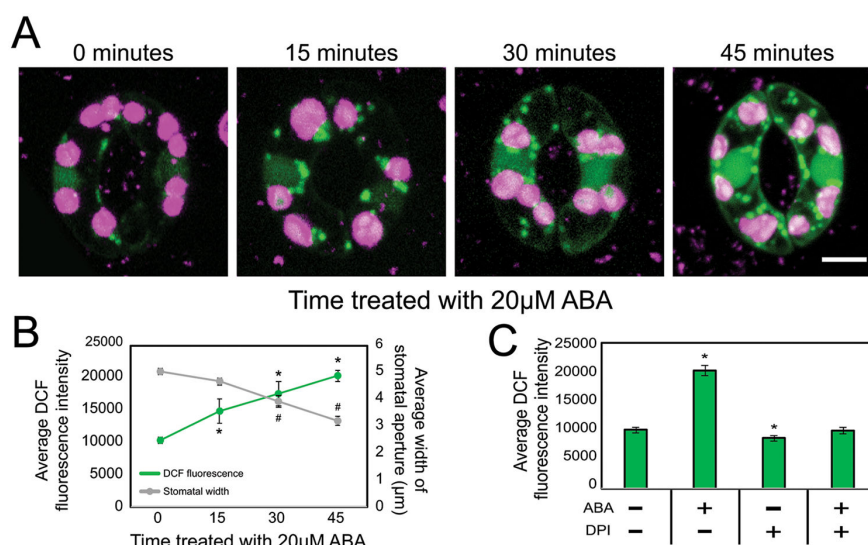
Figure 2.

Tissue and developmental patterns of *Arabidopsis RBOH* transcript abundance. The abundance of the transcripts encoding all the RBOH enzymes were quantified by microarray and were extracted from the eFP Browser using the developmental map and tissue-specific data sets.[311] The values were normalized relative to the median transcript abundance within the eFP Browser, and the fold changes from that value are reported. Conditional formatting was used within each transcript column to highlight their abundances across tissues and cell types, with values in the lowest 10% shown in dark blue, those in the top 10% in red, and those at the 50th percentile in white. The font color was changed to white for the values in the highest 20%. The locus identifiers for the individual genes are listed at the bottom of the figure.
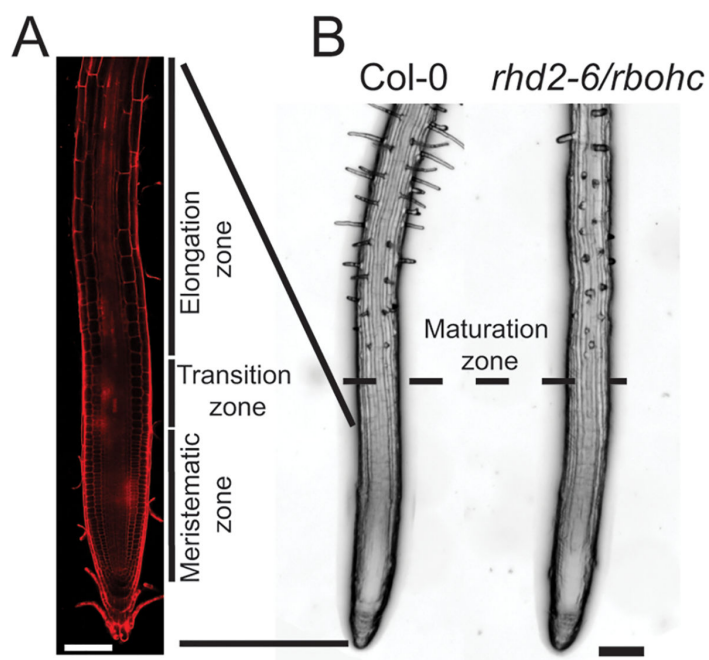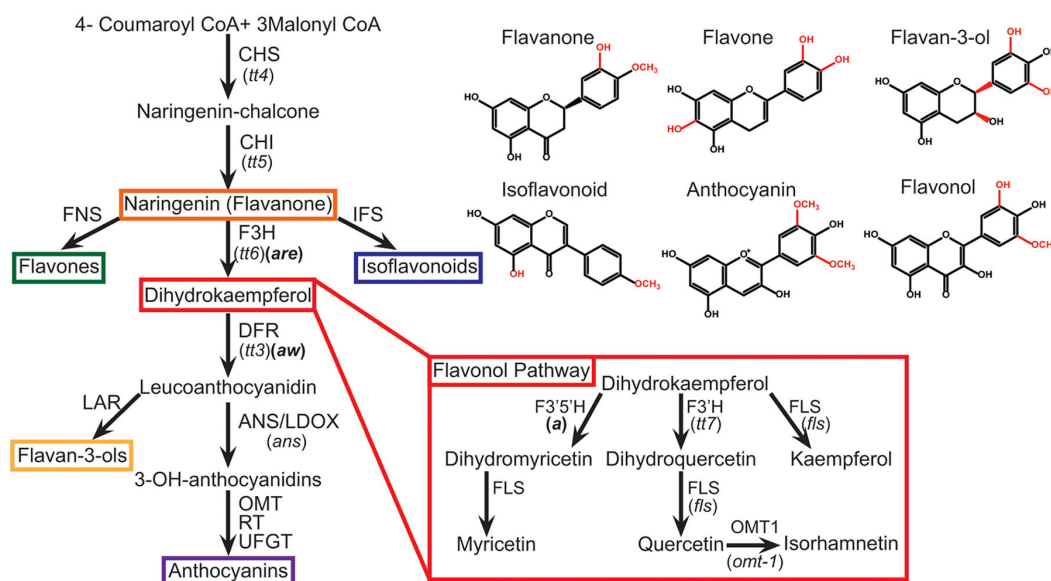
Figure 3.

The *rhd2–6/rbohc* mutant has impaired root hair elongation compared with the wild-type (Col-0). (A) An *Arabidopsis* root stained with propidium iodide, showing cell outlines found in the meristematic, transition, and elongation zones. Scale bar =100 μm. (B) Comparison of young *Arabidopsis* root tips for the wild-type (Col-0) and the root-hair-defective (*rhd2*) mutant, which has a mutation in the *RBOHC* gene. The area above the dashed lines indicates the maturation zone, where root hairs form. Scale bar = 200 μm.
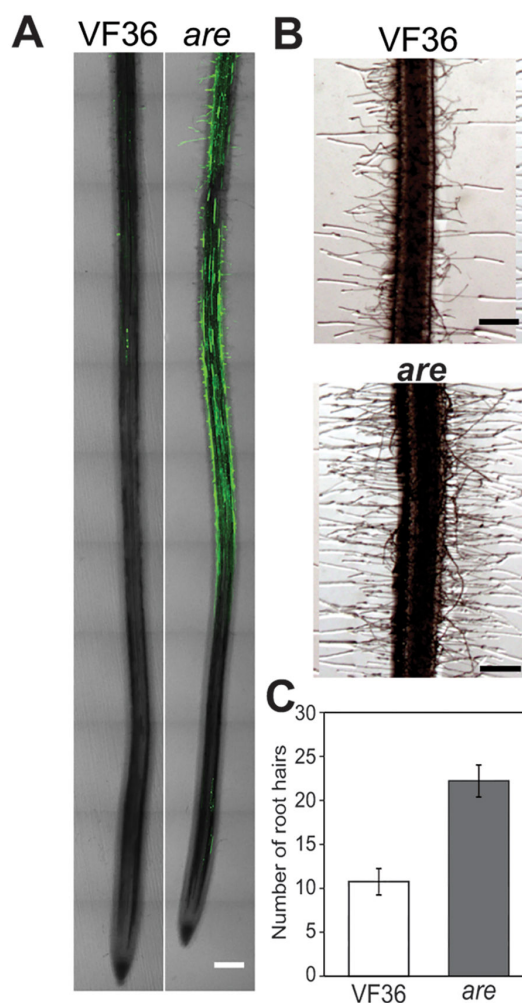
Figure 4.

ABA-induced ROS burst in guard cells precedes stomatal closure in wild-type tomato leaves and requires RBOH activity. (A) Increases in DCF fluorescence were visualized in guard cells across a 45 min time course of ABA treatment in wild-type tomato leaves. Scale bar = 5 μm. (B) DCF fluorescence in entire guard cells and stomatal aperture shown as functions of time after treatment with 20 μM ABA. Asterisk and number signs represent significant differences in DCF fluorescence ($P < 0.001$) and stomatal aperture ($P < 0.009$) between time 0 and indicated times or treatment. (C) DCF fluorescence were quantified with and without 100 μM DPI at 0 and 45 min of treatment with ABA. Asterisks and number signs represents significant differences ($P < 0.001$) in DCF fluorescence between time 0 and the indicated times or between untreated and treated, respectively. Statistics were determined using two-way analysis of variance followed by Tukey's posthoc test with $N = 70$. Reproduced with permission from ref 76. Copyright 2017 American Society of Plant Biologists.
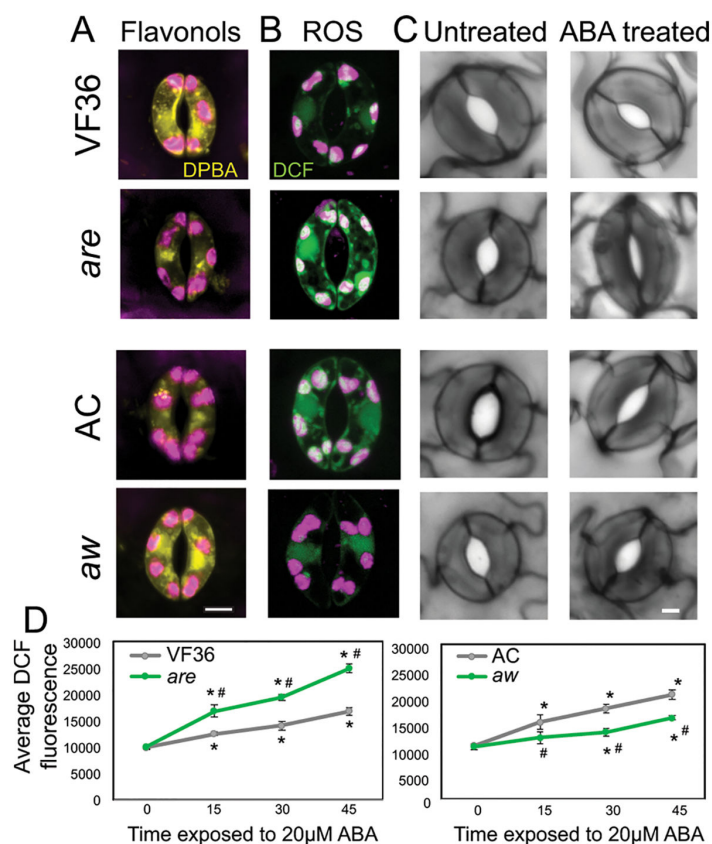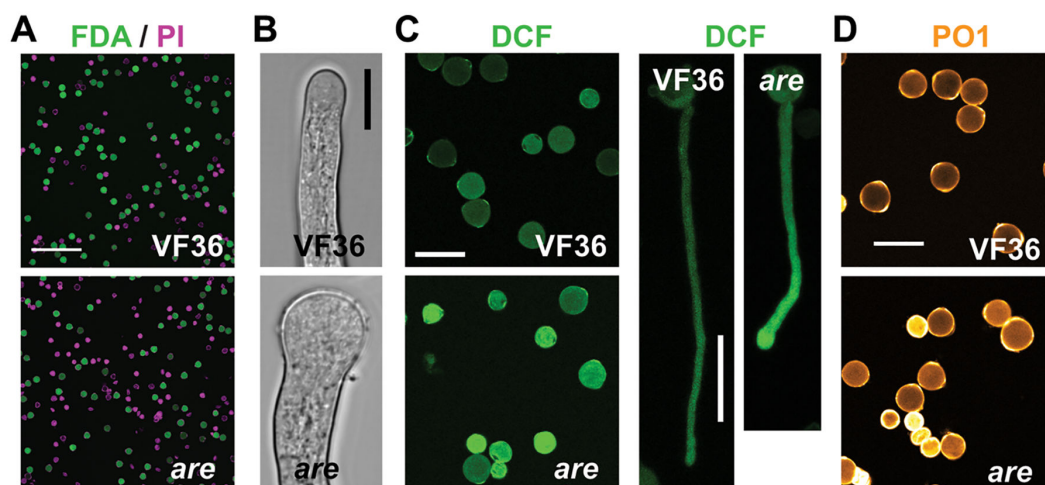
Figure 5.

Flavonoid biosynthesis pathway highlighting the six major subclasses of flavonoids. Mutants are noted in parentheses under the enzyme abbreviation, with *Arabidopsis* mutants in italics and tomato mutants in bold italics. The flavonol biosynthesis branch is further detailed in the red box. General flavonoid structures are presented for the six subclasses of flavonoids. The chemical bonds and/or substituents highlighted in red indicate sites of structural variation within each subclass. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; IFS, isoflavone synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS/LDOX, anthocyanidin synthase/leucoanthocyanidin dioxygenase; OMT, *O*-methyltransferase; (RT), rhamnosyl transferase; UFGT, UDP-glucose flavonoid 3-*O*-glucotransferase; F3′5′H, flavonoid 3′5′-hydroxylase; F3′H, flavonoid 3′-hydroxylase; (FLS), flavonol synthase; OMT-1, *O*-methyltransferase-1.

Figure 6.
ROS levels and root hair numbers are greater in roots of the *are* mutant, which is impaired in flavonol synthesis, compared with the wild-type (VF36). (A) ROS levels are concentrated in the epidermal cells and root hairs of the maturation zone of roots, where root hairs initiate. ROS is elevated in the mutant, as shown in tile-scan confocal images of the *are* mutant and wild-type (VF36) roots stained with CM-H$_2$DCFDA. Scale bar = 300 μm. (B) The higher number of root hairs in the *are* mutant than the wild-type is evident in bright-field images of primary roots. Scale bars =150 μm. (C) Quantification of root hair numbers in mature regions of primary roots grown on control media. Reproduced with permission from ref 272. Copyright 2014 American Society of Plant Biologists.

Figure 7.
Levels of flavonols are inversely proportional to the levels of ROS in tomato guard cells, with more rapid closure and higher ROS. (A) Confocal micrographs show DPBA-bound flavonols in yellow and chlorophyll autofluorescence in magenta in the *are* mutant with reduced flavonols and the *aw* mutant with increased flavonols compared with the parental lines (VF36 and AC, respectively). Scale bar = 5 μm. (B) DCF fluorescence is shown in green, and the levels are inversely proportional to flavonol levels. (C) ABA closes guard cells with closure proportional to levels of ROS. (D) Changes in DCF fluorescence upon ABA addition were quantified and are greater in the *are* mutant with low flavonols than the wild-type, while elevated flavonols in the *aw* mutant show the lowest rate of DCF changes. Reproduced with permission from ref 76. Copyright 2017 American Society of Plant Biologists.

Figure 8.
Flavonols regulate pollen development by scavenging ROS. (A) Confocal micrographs of the wild-type tomato (VF36) and the flavonol-deficient *anthocyanin reduced (are)* mutant show reduced pollen viability in the mutant. Pollen grains were stained with FDA (live pollen grains, shown in green) and PI (dead pollen grains, shown in magenta). Scale bar = 200 μm. (B) Bright-field images of pollen tube tips of VF36 and the *are* mutant show that flavonols reduce swelling of the pollen tube tip. Scale bar = 10 μm. (C) Confocal micrographs of VF36 and *are* mutant pollen grains and tubes stained with the general ROS sensor CM-H$_2$DCFDA show that flavonols reduce ROS levels (as evidenced by DCF fluorescence) in pollen grains and tubes. Scale bars = 50 μm. (D) Confocal micrographs of VF36 and *are* mutant pollen grains stained with the hydrogen peroxide-specific sensor peroxy orange 1 (PO1) show elevated hydrogen peroxide in the flavonol-deficient *are* mutant. Scale bar = 50 μm. These images were reprinted from ref 96.

Table 1.

Arabidopsis Respiratory Burst Oxidase Members[a]

Gene name Locus ID	Localization	Mutant phenotype	Regulation	Orthologues	References
AtRBOHA AT5G07390	Primary Root: Maturation zone (endodermis, stele) Lateral root primordia Basal meristem of primary root	Unknown	Unknown		39,44
AtRBOHB AT1G09090	Maturation zone in primary root Seed embryo	Faster germination of fresh seeds	ABA		39,104
AtRBOHC/RHD2 AT5G51060	Root epidermal cells Root hairs Columella, elongation, and differentiation zone in primary root	Short root hairs Reduced superoxide production at root hair apoplast	Transcription: RSL4 Subcellular localization: FER, ROP2, SCN1 Protein modification: CBL1, CIPK26	OsNOX3 (rice) Zmrth5 (maize)	44,45,47,51,52,56,59,60,66,67,309
AtRBOHD AT5G47910	Root maturation zone Lateral root primordia	Reduced primary root elongation Increased lateral root number* Reduced ABA-mediated stomatal closure* Reduced pathogen response (flg22) Resistance to ozone stress* Reduced survival under salt and hypoxia stresses Increased survival under salt or hypoxia stress* Response to iron deficiency, heat, high aluminium, flooding, high light, and drought	Protein modification: BIK1, PBL1, FLS2, ERF74 Alternate binding: PA, Ca^{2+}	StRBOHB (potato) NbRBOHB	33,34,41,42,44,85,105,107–109,112–114,118,119,128,310
AtRBOHE AT1G19230	Tapetum Root: Basal meristem Cortex Endodermis Epidermal cells overlying lateral root primordia	Delayed degeneration of tapetum Reduced pollen viability Abnormal pollen grain shape and exine layer Delayed emergence of lateral roots	Transcription: MYB80 and AMS (tapetum), NAA (roots)		44,90
AtRBOHF/SGN4 AT1G64060	Periphery of lateral root primordia Elongation to lateral root formation zone in the primary root Vascular tissue in leaves Guard cells	Delayed Casparian strip formation Reduced primary root elongation Increased lateral root number* Reduced ABA mediated guard cell closure* Resistance to ozone stress* Increased survival under salt or hypoxia stress*	Protein modification: CIPK26 Alternate binding: PA		33,34,39,41,42,44,79,85–87,107,108,111
AtRBOHG AT4G25090	Mature roots	Unknown	Unknown		311
AtRBOHH AT5G60010	Pollen	Reduced pollen tube growth and integrity*	ANXUR1/ANXUR2 Ca^{2+} Phosphorylation		92–94

Gene name Locus ID	Localization	Mutant phenotype	Regulation	Orthologues	References
AtRBOHI AT4G11230	Seeds Leaves Roots	Delayed germination under drought stress conditions Decreased survival under hypoxia stress	Unknown		3,11
AtRBOHJ AT3G45810	Pollen	Reduced pollen tube growth and integrity*	ANXUR1/ANX UR2 and Ca²⁺ Phosphorylation		92–94

*a*Commonly used gene names and locus numbers are shown along with expression patterns and phenotypes of mutants. Known regulatory events are listed. Orthologues in other species are included. Asterisks indicate phenotypes in *rbohd/f* or *rbohh/j* double mutants.