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Authors

Linden, Katrina J

Callis, Judy

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The ubiquitin system affects agronomic plant traits

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Katrina J. Linden and Judy Callis*

From the Department of Molecular and Cellular Biology and the Integrative Genetics and Genomics Graduate Group, University of California, Davis, California, USA

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In a single vascular plant species, the ubiquitin system consists of thousands of different proteins involved in attaching ubiquitin to substrates, recognizing or processing ubiquitinated proteins, or constituting or regulating the 26S proteasome. The ubiquitin system affects plant health, reproduction, and responses to the environment, processes that impact important agronomic traits. Here we summarize three agronomic traits influenced by ubiquitination: induction of flowering, seed size, and pathogen responses. Specifically, we review how the ubiquitin system affects expression of genes or abundance of proteins important for determining when a plant flowers (focusing on *FLOWERING LOCUS C*, FRIGIDA, and CONSTANS), highlight some recent studies on how seed size is affected by the ubiquitin system, and discuss how the ubiquitin system affects proteins involved in pathogen or effector recognition with details of recent studies on FLAGELLIN SENSING 2 and SUPPRESSOR OF NPR CONSTITUTIVE 1, respectively, as examples. Finally, we discuss the effects of pathogen-derived proteins on plant host ubiquitin system proteins. Further understanding of the molecular basis of the above processes could identify possible genes for modification or selection for crop improvement.

Thousands of proteins participate in ubiquitination, recognition, and processing of ubiquitinated proteins and in regulation of the 26S proteasome, indicating ubiquitin's broad effects in plant cellular physiology and in plant responses to environmental conditions (1). Ubiquitin is attached to substrate proteins by a ubiquitin-activating enzyme (E1), one or more ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). The E3s fall into different groups based on catalytic mechanisms or conserved domains (RING, HECT, plant U-box (PUB), and RBR), and through their interactions with a substrate and promotion of ubiquitin transfer, they are key substrate specificity determinants. Ubiquitination can lead to changes in the activity, protein-protein interactions, subcellular localization, and stability of substrates. Proteins involved in the attachment, recognition, and removal of ubiquitin have been aptly coined as writers, readers, and erasers, respectively, of a ubiquitin code (2) (Fig. 1).

As elucidated in animals and yeasts, the ubiquitin system in plants also regulates the cell cycle, protein quality control, and intracellular trafficking. It plays decisive roles in all plant hormone signaling pathways, with ubiquitin E3s serving, surprisingly, as part of several hormone-sensing complexes or modulating the abundance of early downstream signaling proteins and/or balancing hormone abundance by fine-tuning enzyme levels in hormone biosynthetic, inactivation, and/or conjugation pathways. Through modulating hormone signaling and synthesis, the impact of the ubiquitin system on plant growth is extensive and of great consequence.

Our understanding of the ubiquitin system's role in shaping plant characteristics that impact agronomic traits is increasing as protein players are identified and their molecular mechanisms of action are elucidated. Studies in crop species are revealing proteins whose activities can be subjected to modification through breeding and/or directed genome modification efforts to improve productivity. Identifying characteristics that can make plants more robust and resilient in the face of changing growth conditions is of critical importance as climate change alters the environment experienced during a growing season. We must understand the nature of the ubiquitin system's influence on these traits, both qualitatively and quantitatively, to take an informed approach. In this review, we focus on three important agronomic traits relevant to a large number of crops and whose intersections with the ubiquitin system are best understood and fairly direct: induction of flowering, seed size, and pathogen sensing.

The ubiquitin system affects induction of flowering, an important agronomic trait

In the angiosperm plant life cycle, initiation of flowering represents the transition from vegetative to reproductive growth and results in fertilization and seed development. Flowering at the appropriate time of the year is critical for wild and domesticated plants. It ensures that flowers form when conditions are optimal for pollination and that seeds or subsequent seedlings have time to develop when weather is conducive to growth. In agriculture, flowering time influences sowing and harvest schedules and affects crop yield and quality. For crops whose products are vegetative organs, such as leaves, stems, or roots, flowering prior to harvest can reduce product quantity or quality as plants redirect their resources to reproduction at the expense of vegetative growth. For those plants whose product is the seed, late flowering, for example, reduces time for seed production and maturation. Induction of flowering is regulated by environmental factors, including light and temperature, and by genetic variation at loci that control responses to light or temperature. Much of the current knowledge about mechanisms regulating flowering induction was acquired by studying the non-crop model plant *Arabidopsis thaliana*. The following examples illustrate how the ubiquitin system regulates some of the major genes and proteins that control flowering (see Fig. 2).

* For correspondence: Judy Callis, jcallis@ucdavis.edu.

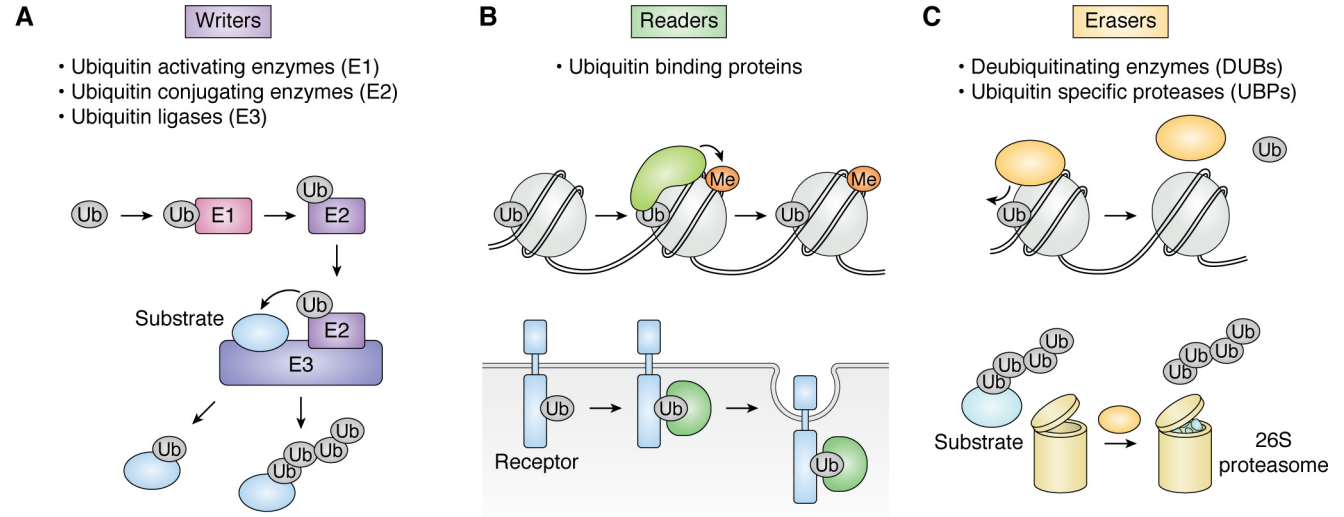

Figure 1. The ubiquitin system. *A–C*, summary of components of the ubiquitin system involved in attaching ubiquitin (*Ub*) to substrate proteins (the writers) (*A*), binding to and targeting the ubiquitinated substrate to a new biological fate (the readers) (*B*), and removing ubiquitin, either through deubiquitination by DUBs independent of degradation or coordinated with degradation of the substrate by the proteasome (the erasers) (*C*). Terms are from Komander and Rape (2). The diagram in *A* depicts ubiquitination of a substrate protein by E1, E2, and E3 enzymes. Diagrams in *B* depict two examples of ubiquitin-binding proteins (represented in *green*): a ubiquitin-dependent histone methyltransferase (*top*) and the involvement of a ubiquitin-binding protein in endocytosis (*bottom*). Histones and DNA represented by gray balls and rope, respectively. *Me*, methylation. Diagrams in *C* depict histone deubiquitination by a ubiquitin-specific protease (*top*) and proteasomal removal of ubiquitins from a proteolytic substrate (*bottom*).

Ubiquitination and deubiquitination regulate FLOWERING LOCUS C expression

FLOWERING LOCUS C (*FLC*) encodes an *Arabidopsis* MADS-box transcription factor that represses flowering (3, 4) by binding to regulatory elements of flower-promoting transcription factor genes, particularly *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), and reducing their transcription. Both monoubiquitination and deubiquitination of histone H2B at the *FLC* locus are necessary for appropriate *FLC* expression.

The RING-type ubiquitin ligases HISTONE MONOUBIQUITINATION 1 and 2 (HUB1 and HUB2) act together with ubiquitin-conjugating enzymes UBC1 and UBC2 to monoubiquitinate histone H2B (H2Bub1) in chromatin throughout the genome, including at the *FLC* locus (5–7). Likely the result of its genome-wide presence in WT plants, the complete loss of H2B ubiquitination as seen in the *hub1/2* mutants affects multiple aspects of plant growth, including leaf development, seed dormancy, the cell cycle, and the timing of meristem identity switch from vegetative growth (leaf production) to reproductive growth (flower production) (8, 9). *hub1* and *hub2* single loss-of-function mutants, as well as the *ubc1 ubc2* double mutant, initiate flowering after producing fewer vegetative leaves than WT plants, indicating an earlier developmental switch (5–7). The effect of histone ubiquitination specifically on the transition to flowering stems from the requirement for H2Bub1 in the chromatin at the *FLC* gene for *FLC* expression. *FLC* mRNA is dramatically lower in the *ubc1 ubc2* double mutant than in WT plants, and expression of *FLC* and/or several *FLC* family members is reduced in *hub1* and *hub2* mutants (5–7).

It is possible that H2B monoubiquitination activates *FLC* transcription by enhancing activity of methyltransferases directed to the *FLC* locus. Methyltransferases from the ATX (ARABIDOPSIS TRITHORAX) and ATXR (ARABIDOPSIS TRITHORAX-RELATED) classes catalyze trimethylation of H3 at lysine 4 (H3K4me3). ATX1 interacts with *FLC* chromatin and is required for increased H3K4me3 at *FLC* (10). Similarly, ATXR7 interacts with chromatin near the *FLC* transcription start site and is important for the presence of H3K4me3 in *FLC* chromatin (11). In *hub* mutants, loss of global H2B monoubiquitination is correlated with local loss of the transcriptionally activating H3 lysine methylations H3K4me3 and H3K36me3 at *FLC* chromatin (5, 7).

It has been demonstrated in a few crop species that HUB orthologs play similar roles in histone H2B ubiquitination. The tomato (*Solanum lycopersicum*) orthologs SlHUB1 and SlHUB2 monoubiquitinate H2B *in vitro* (12). Flowering-related RING protein 1 (FRRP1), a rice (*Oryza sativa*) ortholog of HUB2, monoubiquitinates H2B and affects rice flowering initiation (13). Under long-day conditions, plants in which *FRRP1* expression is reduced with RNAi flower several weeks earlier than WT rice plants. *FRRP1*-reduced expression plants also have lower levels of monoubiquitinated histone H2B than WT plants and have increased expression of *Hd3a*, the rice homolog of *Arabidopsis FT*. Rice *FRRP1* complements the *hub2 Arabidopsis* mutant, providing further evidence that rice FRRP1 and *Arabidopsis* HUB2 have similar functions (13).

Deubiquitination of H2B at the *FLC* locus by UBP26 (UBIQUITIN-SPECIFIC PROTEASE 26) is also necessary for appropriate *FLC* expression (14). *ubp26-1* mutants have increased levels of H2Bub1 at *FLC*, flower earlier, and have lower *FLC* expression than WT plants (14). The nature of H3 modifications is also affected at the *FLC* locus in *ubp26-1*; there is increased H3K27me3, which is associated with transcriptional repression, and lower levels of H3K36me3, which is associated with transcriptional activation (14). The similar down-regulation of *FLC* expression from both loss of H2Bub1 and increased

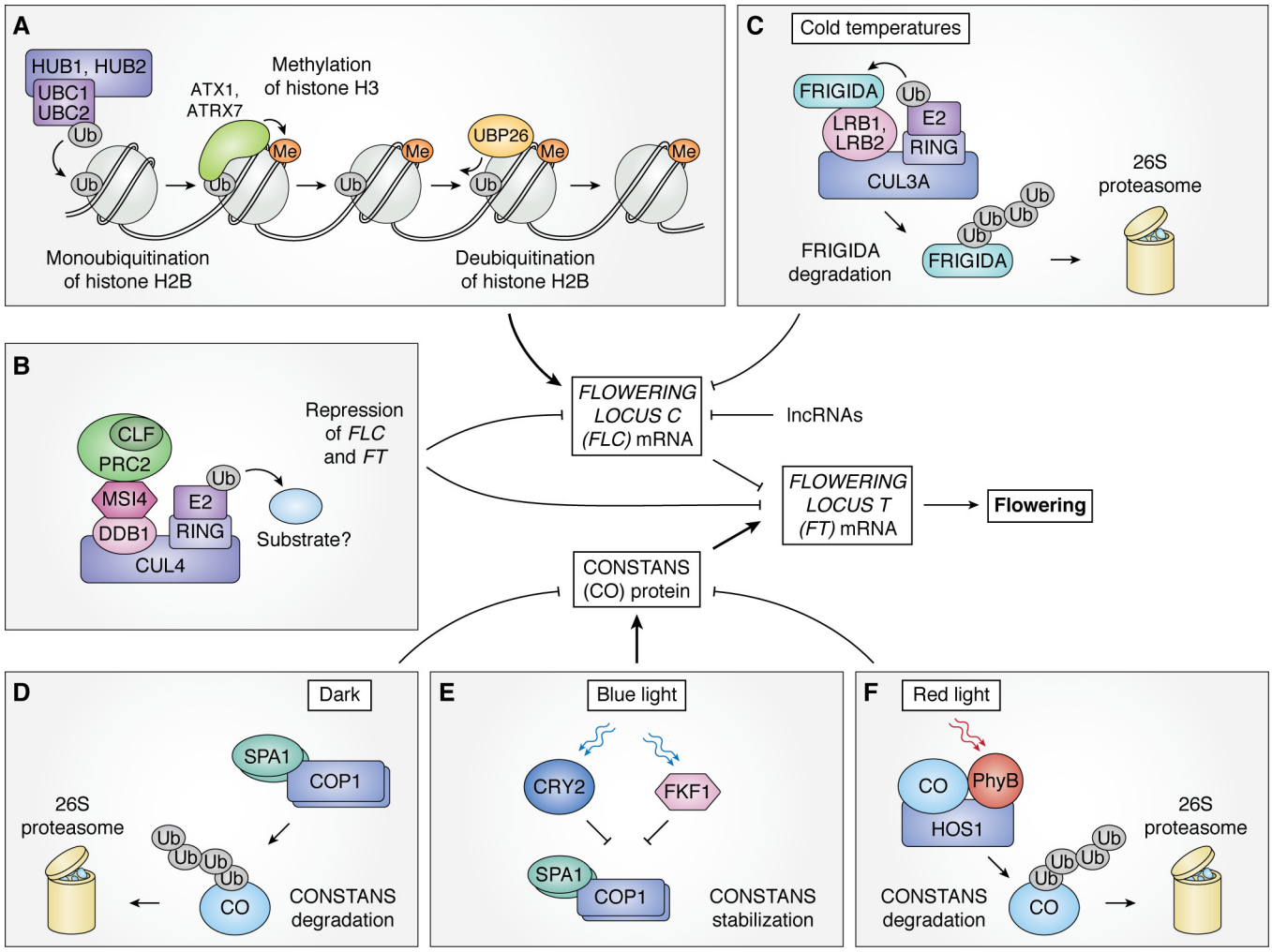

Figure 2. The ubiquitin system affects induction of flowering by regulating *FLC* and *FT* expression and CO protein degradation. *A*, the RING-type E3 ligases HUB1 and HUB2 act with the ubiquitin (Ub)-conjugating enzymes UBC1 and UBC2 to monoubiquitinate histone H2B on chromatin. H2B ubiquitination might promote activity of methyltransferases such as ATX1 and ATXR7, which interact with *FLOWERING LOCUS C* (*FLC*) chromatin and add methylation (*Me*) marks on histone H3. FLC is a negative regulator of flowering through repressing *FLOWERING LOCUS T* (*FT*) expression, a positive regulator of flowering. Deubiquitination by the ubiquitin-specific protease UBP26 is necessary for appropriate *FLC* expression. *B*, the CUL4-DDB1^{MSI4} E3 ligase complex associates with polycomb repressive complex 2 (PRC2), containing the methyltransferase CURLY LEAF (CLF), and reduces mRNA of *FLC* and *FT*. CUL4-dependent ligases may affect expression of other genes, including *CONSTANS* (*CO*) (see "Multiple E3 ligases regulate CONSTANS protein abundance"). *C*, FRIGIDA acts as a scaffold in a large complex that promotes *FLC* expression. After exposure to cold temperatures, a CUL3 E3 ligase complex promotes degradation of FRIGIDA protein. *D*, the E3 ligase COP1 associates with SPA proteins to promote CONSTANS (CO) protein degradation in the dark. *E*, the blue light receptor CRY2 interacts with SPA and COP1 and inhibits CO degradation by competing with COP1 substrates. In addition, CO protein is stabilized by the F-box protein FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1). FKF1 is an adapter protein in an SCF E3 ligase complex and acts as a blue light receptor. *F*, the E3 ligase HOS1 promotes degradation of CO protein in a red light–dependent manner.

H2Bub1 at *FLC* indicates a requirement for the dynamic cycling of H2B monoubiquitination to ensure appropriate deposition of activating methylations on histone H3.

CUL4-dependent ligases also regulate *FLC* expression but have a complex relationship in their regulation of time to flowering. The CUL4–DDB1^{MSI4} E3 ligase complex (consisting of the CULLIN 4 (CUL4) scaffold protein, the substrate adaptors DNA-BINDING PROTEIN 1 (DDB1A or DDB1B), and the WD40 protein MULTI-COPY SUPPRESSOR OF IRA1 4 (MSI4)), is a ubiquitin ligase complex that represses *FLC* expression. *msi4* mutants flower later than WT plants, consistent with the observed increased *FLC* and reduced *FT* and *SOC1* mRNAs in this mutant (15). CUL4–DDB1^{MSI4} interacts with *FLC* chromatin and represses *FLC* expression by associating with CLF-PRC2, a polycomb repressive complex 2 (PRC2) containing the histone H3 Lys-27 methyltransferase CURLY LEAF (CLF) (15). Although the mechanism is unclear, the CUL4-based MSI4 complex might regulate proteins involved with the CLF-PRC2 complex via ubiquitination, or it might modify chromatin associated with CLF-PRC2 (15). Interestingly, *ddb1a* null mutants and *cul4* mutants with reduced CUL4 protein flower earlier, not later, than WT plants. Whereas these mutants also exhibit elevated *FLC* mRNA as expected, *cul4* mutants have the opposite effects on the downstream flower-promoting genes, *FT* and *SOC1*, with elevated mRNAs as well (15), which likely negates any effect on the upstream *FLC*. Likely through other substrate specificity subunits, different CUL4-dependent ligases also reduce mRNAs for FT, SOC, and possibly other positive regulators of flowering (15), explaining the opposite effects of *cul4* reduced and *msi4* mutants on time of flowering induction. The

role of *Arabidopsis* CUL4-based E3 complexes in chromatin remodeling and gene expression has been reviewed recently (16).

In addition to regulating *FLC* transcription, the ubiquitin system might also regulate FLC protein more directly. The E3 ligase SINAT5 ubiquitinates FLC protein *in vitro* (17), but whether or how SINAT5 affects FLC protein abundance is still unknown.

FRIGIDA protein abundance affects initiation of flowering

FRIGIDA (FRI) is a scaffold protein in a transcriptional activation complex that represses flowering by promoting *FLC* expression (18). FRI recruits chromatin remodeling factors to the *FLC* promoter (18), where it is part of a large complex that up-regulates *FLC* expression (19). During exposure to cold temperatures, *FLC* expression is reduced partly due to increased FRI degradation by the proteasome (20). This regulation of FRI protein occurs through cold-induced expression of the transcription factor WRKY34, which binds to the promoter of the E3 ligase complex component *CUL3A* and increases its transcription (20). FRI interacts with CUL3A and its BTB adapter proteins LIGHT RESPONSE BTB 1 and 2 (LRB1 and -2) *in vitro* and *in vivo*, and CUL3A, LRB1, and LRB2 are important for FRI protein degradation. Recombinant FRI protein degradation is slower in extracts from *cul3a* or *lrb1 lrb2* mutant plants than in extracts from WT plants (20). This mechanism in part explains why *Arabidopsis FLC* mRNA is undetectable in shoots after prolonged cold treatment and flowering is promoted (3). Other important sensors of prolonged cold are the cold-induced long noncoding RNAs that bring PRC2 to the *FLC* locus for deposition of transcriptionally repressive histone methylations (21–23).

Arabidopsis thaliana individuals collected from diverse natural environments, termed accessions, have different growth habits; some are winter annuals that germinate in the fall and flower in the spring. Most of the winter annual accessions have a functional *FRI* allele that represses flowering until after prolonged exposure to cold temperatures, when a CUL3-based E3 targets FRI protein for degradation. Other accessions are summer annuals that germinate in the spring and flower in the fall. Most summer annuals have a nonfunctional *FRI* allele (or a nonfunctional *FLC* allele, or both) (24).

FRI similarly affects flowering time in the oilseed crop *Brassica napus*. *B. napus* has four *FRI* homologs, and allelic variation in *BnaA.FRI.a* (renamed as *BnaA3.FRI* (25)) contributes to differences in flowering time among *B. napus* lines (25, 26). *FRI* and *FLC* are also important for determining flowering time in *Brassica rapa* leafy vegetables (27). The role of *FLC* in vernalization and flowering time of *Brassica* crops has been reviewed (28).

Multiple E3 ligases regulate CONSTANS protein abundance

The *Arabidopsis* transcription factor CONSTANS (CO) promotes flowering under long-day conditions (16 h light/8 h dark) by direct enhancement of *FLOWERING LOCUS T* (*FT*) expression. As mentioned above, FT protein is a key floral inducer; the gene and protein are expressed in leaf phloem cells, and the protein travels to the apical meristem to initiate the switch to development of floral organs. CO protein abundance is regulated in a light- and temperature-dependent manner to prevent flowering under short-day conditions, and multiple E3 ligases are involved in the light-dependent regulation.

The RING-type E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) ubiquitinates CO and promotes CO protein degradation in the dark (29, 30). COP1 associates with SPA (SUPPRESSOR OF PHYTOCHROME A) proteins in a tetrameric complex that consists of a COP1 homodimer and two SPA proteins (31). SPA proteins interact with CO and are necessary for appropriate degradation of CO (32). As blue light increases at sunrise, COP1-dependent degradation of CO is suppressed by the interaction of the blue light receptor CRY2 (CRYPTOCHROME 2) with SPA1 and COP1 (33). It was recently shown that CRY2 repression of COP1 activity occurs when CRY2 binds to COP1 and outcompetes COP1 substrates (34).

A rice ortholog of COP1, PPS (PETER PAN SYNDROME), affects flowering in this species (35). In field tests of both short-day and long-day conditions with the Taichung 65 cultivar of *O. sativa*, *pps-1* mutant plants flowered 3 weeks earlier than WT plants, showing that PPS suppresses the transition from vegetative to reproductive growth (35). PPS substrates in rice have not yet been identified.

In *Arabidopsis*, CO protein abundance after dawn is regulated by another RING-type E3 ligase called HOS1 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1). Nuclear extracts harvested after dawn from *hos1* mutant plants contained more CO protein than nuclear extracts from WT plants, and a CO-LUC fusion protein was more abundant after dawn when overexpressed in *hos1* mutant plants than when overexpressed in WT plants. *hos1* mutants flower earlier than WT plants (36), which could be explained by a lack of appropriate CO degradation. HOS1-dependent degradation of CO protein occurs in a red light–dependent manner requiring the red light photoreceptor phytochrome B (phyB) (37). When CO protein is overexpressed in WT and *hos1* mutant plants, after 24 h under red light, CO protein accumulates more in the *hos1* background than in WT (37).

In long-day afternoons, CO protein is stabilized by the F-box protein FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) (38). FKF1 is an adapter protein in an SCF E3 ligase complex and acts as a blue light receptor. FKF1 interacts with COP1 in a blue light–dependent manner, inhibits COP1 homodimerization, and prevents COP1 ubiquitination of CO and hence CO degradation (39).

In rice, the CO ortholog Hd1 (heading date 1) affects the developmental timing and emergence of the inflorescence. In rice and many monocots, the process of inflorescence emergence from the vegetative leaves that surround them is called heading. The inflorescence consists of all the flowers and the stems that subtend them, and for these crops, the timing of heading and subsequent seed development have a direct impact on quality and quantity of grain produced. The rice RING-type E3 ligase HAF1 (heading date–associated factor 1) ubiquitinates Hd1 *in vitro* and controls Hd1 accumulation in a heterologous transient expression system. *haf1* mutants flower later than WT

plants under both long- and short-day conditions (40). Rice HAF1 also negatively regulates EARLY FLOWERING 3 (ELF3), a transcription factor that promotes flowering under long days (41). HAF1 ubiquitinates ELF3 *in vitro* and promotes its degradation in rice lysates (41).

The ubiquitin-specific proteases UBP12 and UBP13 affect *CO* transcription (42). *CO* transcription is elevated earlier in the day in *ubp12* single mutants and *ubp12 ubp13* double mutants, and they flower earlier than WT plants. *CO* transcripts start to accumulate between 4 and 8 h after dawn in the mutants and between 8 and 12 h after dawn in WT plants (42).

These studies reveal that the ubiquitin system can modulate the levels of key players in flowering at multiple levels, through direct ubiquitination and subsequent degradation by the proteasome, but also through affecting the stability of transcription factors that control synthesis of their mRNAs. Further studies may reveal novel regulatory aspects of the ubiquitin system described in yeast and mammal systems, such as the role of ubiquitination in the nonproteolytic regulation of transcription factor activity (43, 44).

Regulation of other genes or proteins that affect the circadian clock

The ubiquitin system regulates the abundance of many other proteins that affect flowering time, especially proteins involved in regulating the circadian clock and those involved in hormone synthesis and signaling that modulate flowering, especially gibberellins (45). The role of the ubiquitin system in regulating photoperiodic flowering has been reviewed (46). Other reviews highlight flowering control in crop plants (23, 47–49).

The ubiquitin system affects seed size

Seeds accumulate starch, triacylglycerides in lipid bodies, and proteins to sustain seedlings until they can produce their own energy through photosynthesis. In addition to providing energy for seedlings, seeds are a major source of nutrition for animals, including humans. Seed size is an important agronomic trait. The involvement of the ubiquitin system in determining seed size was first noted when a quantitative trait locus controlling grain size and weight in rice was characterized and found to encode the RING-type E3 ligase GW2 (GRAIN WIDTH 2). GW2 negatively regulates seed size and yield (50). Subsequent studies have shown that the ubiquitin system regulates multiple other proteins that affect seed size (reviewed in Refs. 51 and 52). The following examples illustrate some recent findings (see Fig. 3).

The E3 ligase UPL3 affects seed size and lipid content

Several species in the *Brassica* genus, including *B. napus* (*Bna*) and *B. rapa* (*Bra*), are important sources of vegetable oil, including the canola oil found in grocery stores. A recent report on global food markets estimated a harvest of more than 70 million metric tons for 2018/2019 (53). Consequently, *B. napus* seed oil content is a key agronomic trait. The HECT E3 ligase UPL3 (UBIQUITIN-PROTEIN LIGASE 3) negatively regulates seed size and lipid content, first noted in an associative transcriptomics study of 94 different commercial oilseed rape (*B. napus*) lines (called accessions here) representing the breadth of genetic diversity in this species (54). Seed size and lipid content were also studied in one of its progenitor species, *B. rapa* (*B. napus* is a tetraploid hybrid of the functional diploids *B. rapa* and *Brassica oleracea*). Genetic variation in the *BnaUPL3.C03* promoter among different *B. napus* accessions contributes to differences in seed size and lipid levels. Accessions with promoters that drive lower *BnaUPL3.C03* expression have larger seeds with a higher lipid content than accessions with promoters that drive higher levels of *BnaUPL3.C03* expression. Similarly, *B. rapa* plants homozygous for an early stop codon in the *BraUPL3* ORF produce larger seeds (54).

Mechanistic insights come from investigations in *Arabidopsis* (a relative in the Brassicaceae family), where seeds lacking UPL3 protein (denoted *upl3*) are ~10% larger and have a 12% higher lipid content than seeds from WT plants, whereas seeds from transgenic lines overexpressing *UPL3* are smaller and have a lower lipid content (54). *upl3* maturing seeds show elevated expression of several genes positively regulated by the transcription factor LEC2 (LEAFY COTYLEDON 2). *upl3* mutants have higher levels of endogenous LEC2 protein, whereas *UPL3* overexpressors have lower levels. Recombinant LEC2 protein degrades more slowly in a *upl3* seedling lysate lacking an active form of recombinant UPL3 than in a lysate containing added WT recombinant UPL3, suggesting that UPL3 is important for LEC2 degradation *in vivo*. LEC2 promotes expression of lipid biosynthetic genes, such as *OLE1* (*OLEOSIN 1*), and the reduction of LEC2 protein abundance by UPL3 provides a link to the increased lipid content of *upl3* mutants (54).

This negative relationship between UPL3 and LEC2 in *Arabidopsis* informed further studies in *B. napus*. *B. napus* LEC2 levels also inversely correlate with *BnaUPL3* expression; endogenous LEC2 protein is higher in accessions with lower *BnaUPL3.C03* expression. The authors suggest that the higher levels of LEC2 protein could contribute to an extended period of seed maturation during which lipids can accumulate. Miller *et al.* (54) noted that the genetic variation in *B. napus BnaUPL3.C03* promoters has yet to be selected for in the breeding of *B. napus* or other oilseed crops, suggesting a new approach to increasing oil content.

The SAP-like F-box proteins affect seed size in Arabidopsis and crop plants

The *Arabidopsis* F-box protein SAP (STERILE APETALA) is part of the SCF^{SAP} SKP1/CULLIN 1/F-box E3 ligase complex that targets the transcription factors PPD1 and PPD2 (PEAPOD 1 and 2) for degradation (55). F-box proteins are substrate receptors in SCF E3 ligase complexes. SAP promotes cell proliferation and organ growth, whereas PPD1 and PPD2 restrict cell proliferation (56). Seeds from *ppd1* mutants weigh 53% more than seeds from WT plants (57).

SAP and PPD orthologs in some crop species also affect seed size and weight. The cucumber (*Cucumis sativus* L.) *SAP* ortholog *LITTLELEAF* (*LL*) plays a similar role as SAP in controlling organ size, and *ll* mutants have smaller seeds than cucumbers from a WT inbred line (58). Down-regulation of the

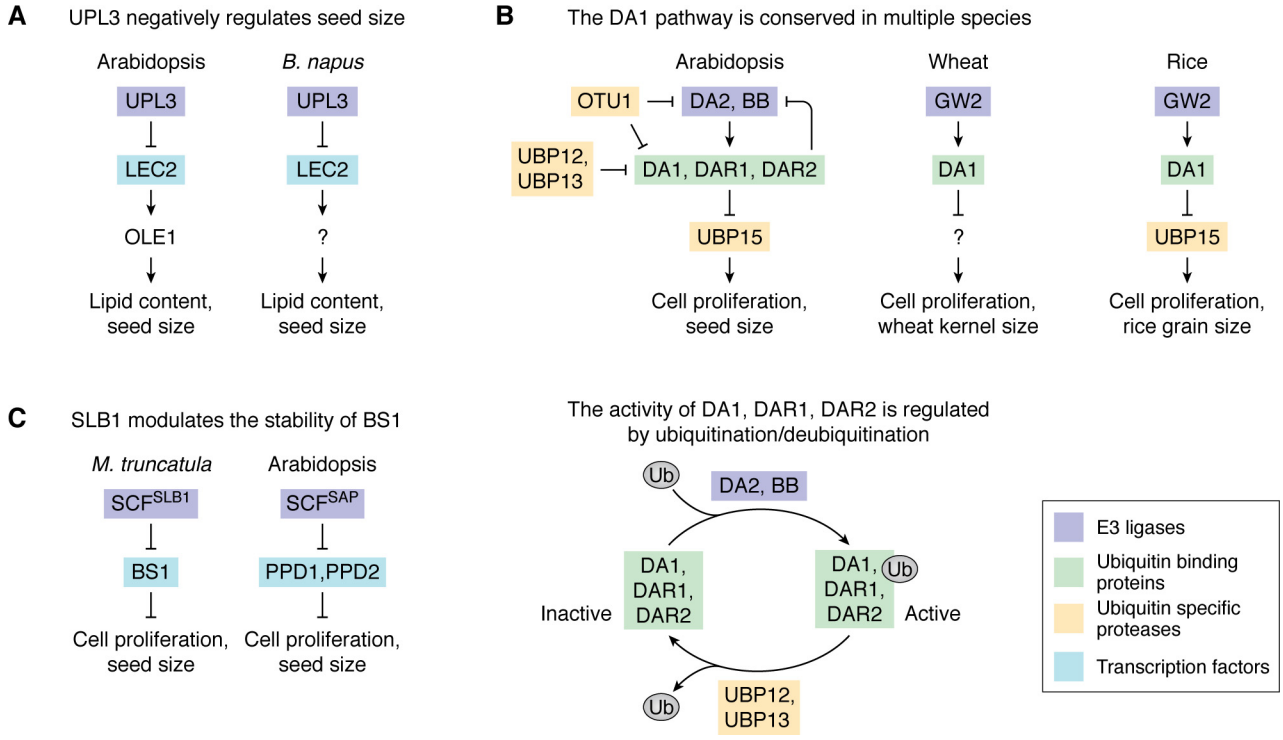

Figure 3. The ubiquitin system affects seed size. Some of the pathways through which the ubiquitin system regulates seed size in *Arabidopsis* and several crop species. *A*, in *Arabidopsis*, the E3 ligase UPL3 negatively regulates seed size and lipid content. It targets the LEC2 protein for degradation. Similarly, in *B. napus*, UPL3 negatively regulates seed size and targets the LEC2 protein for degradation. *B, top*, recent studies in *Arabidopsis* showed that the ubiquitin-specific proteases UBP12 and UBP13 deubiquitinate the ubiquitin receptors DA1, DAR1, and DAR2, reducing their peptidase activity, and that the histone deubiquitinase OTU1 likely decreases expression of *DA1* and the E3 ligase *DA2*. Wheat DA1 restricts cell proliferation and interacts with the E3 ligase GW2, which is orthologous to *Arabidopsis* DA2. A recent study found that the ubiquitin-specific protease OsUBP15 promotes large grain size in rice and interacts with rice DA1. *Bottom*, the effect of ubquitination/deubiquitination on DA1/DAR1/DAR2 activity is depicted. *C*, in the legume *M. truncatula*, SLB1 is a component of an E3 ligase complex that was recently shown to modulate the stability of the transcription factor BS1. Also shown are orthologous proteins in *Arabidopsis*. The F-box protein SAP is an SLB1 ortholog, and the PPD transcription factors are BS1 orthologs.

soybean (*Glycine max*) PPD orthologs *GmBS1* and *GmBS2* results in enlarged seeds, seed pods, and leaves (59).

In the model legume *Medicago truncatula,* the SAP ortholog SMALL LEAF AND BUSHY 1 (SLB1) was recently shown to affect seed size by controlling the stability of the transcription factor BS1 (BIG SEEDS 1) (60). BS1 is a negative regulator of leaf size through suppression of cell divisions, including suppression of cell divisions in the seed leaves, called the cotyledons. SLB1 coimmunoprecipitates with the *M. truncatula* SKP1-like proteins MtASK1 and MtASK2 and the CUL1-like proteins MtCUL1 and MtCUL2 when transiently co-expressed in *Nicotiana benthamiana* leaves, providing direct evidence that SLB1 is likely part of an SCF E3 ligase complex. SLB1 interacts with BS1, BS1 protein levels are lower when *BS1* is transiently co-expressed with *SLB1* in *N. benthamiana*, and BS1 protein degrades more quickly in extracts from *N. benthamiana* leaves when co-expressed with *SLB1*. Overexpression of SLB1 in *M. truncatula* results in seeds that are longer and heavier than WT seeds, and overexpression of *M. truncatula* SLB1 in soybean also results in increased seed size and weight (60).

The DA1 pathway regulates seed size in multiple species

The *Arabidopsis* ubiquitin-binding proteins DA1 (for big in Chinese) and DAR (DA RELATED) are similar proteins that negatively regulate seed size by repressing cell proliferation. In the development of plant organs, a period of cell divisions is followed by cell expansions and, in some plant organs, endoreduplication (DNA replication without cell division). *da1* mutant seeds have increased weight and are larger than WT seeds (61). Developmental timing studies suggest that in *da1* mutants, the period of cell divisions is extended, resulting in additional cells. This result identifies a role for the DA1 pathway in restricting cell divisions and controlling the transition from cell division to cell expansion.

DA1 and DAR1 each contain two ubiquitin-interacting motifs and a peptidase domain (61, 62), which is activated when DA1 is multiubiquitinated by the RING-type E3 ligases DA2 (homologous to the rice E3 ligase GW2) or BB (BIG BROTHER) (62). DA1 interacts with several proteins, including the ubiquitin-specific protease UBP15. DA1 negatively regulates the stability of UBP15; UBP15 acts to promote cell divisions in the cell layers surrounding the developing embryo (63). In addition, DA1 cleaves BB and DA2, increasing their degradation, as part of a negative feedback loop resulting in a transient induction of DA1 activity (62).

DA1 orthologs in maize (64) and *B. napus* (65) have similar effects on seed size, as do DA2 orthologs in rice (50), wheat (66), and maize (67). The role of the DA1 pathway in seed size control has recently been reviewed (52), and the following paragraphs describe subsequent studies on this subject.

New players regulating DA1 have been characterized. The *Arabidopsis* ubiquitin-specific proteases UBP12 and UBP13

interact with and deubiquitinate DA1, DAR1, and DAR2 (another DA1 homolog), reducing their peptidase activity (68). UBP12/13 are not substrates of the cleaving activity of DA1 proteins, indicating that these UBP family members, in contrast to UPB15, act upstream of DA1. The effects of changes in UBP12/13 were investigated in leaves. Mutants with reduced *ubp12* and *-13* expression have smaller leaves with reduced cell number and cell size, and overexpression lines of either UBP12 or UBP13 also have smaller leaves compared with WT plants, from reduced cell size and ploidy. Similar phenotypes were observed previously both in a *da1 dar1 dar2* triple loss-of-function mutant and in *DA1* overexpression lines (69, 70). Thus, WT cell number and size requires appropriate levels of activated DA1 and its homologs, whose activities are balanced by ubiquitination and deubiquitination (68). Too much or too little DA1/DAR1/DAR2 activity disrupts final organ size. Whereas the exact mechanism is still unclear, the DA1 pathway affects both cell division and endoreduplication (affecting ploidy), both of which can affect final leaf area. Investigation of the seed size phenotype in lines with altered UBP12/13 expression is of great interest to determine whether UBP12/13 regulate the DA1 pathway additionally in this organ.

The *Arabidopsis* histone deubiquitinase OTU1 (OVARIAN TUMOR DOMAIN (OTU)-CONTAINING DUB 1) was recently shown to repress *DA1* and *DA2* expression (71). Seeds from *otu1* loss-of-function mutants weigh less and have smaller surface areas than seeds from WT plants. Rosette leaves of *otu1* mutants are also smaller, have larger epidermal cells, and have 22–30% fewer epidermal cells per mm^2 than leaves of WT plants, suggesting that *OTU1* promotes cell expansion and reduces cell proliferation (71). DA1 and DA2 transcripts are elevated in *otu1* mutants, and quantitative ChIP experiments showed that in *otu1* mutants *DA1* and *DA2* chromatin near translation initiation sites has elevated H2B monoubiquitination (associated with active transcription) as well as increased H3K4me3 and H3 acetylation marks. Increased monoubiquitination was not detected in the chromatin of *BIG BROTHER* (*BB*), encoding an E3 ligase that interacts with and ubiquitinates DA1, suggesting that the deubiquitinase activity of OTU1 is somewhat specific. However, direct interaction of OTU1 with *DA1* or *DA2* chromatin was not tested (71).

Recent work has elucidated aspects of the DA1 pathway in crop species. A wheat (*Triticum aestivum*) ortholog of *DA1* affects wheat kernel size and weight (72). There are four *DA1* orthologs in wheat, *TaDA1-A* to *-D*. Similar to *Arabidopsis DA1*, *TaDA1-A* restricts cell proliferation, and TaDA1-A protein interacts with TaGW2-B, a wheat ortholog of *Arabidopsis* DA2. Overexpression of *TaDA1-A* results in kernels with reduced length, width, and weight compared with WT kernels, whereas knockdown of *TaDA1-A* with RNAi results in longer, wider, and heavier kernels. Genetic variation in the *TaDA1* promoter among wheat accessions results in different levels of *TaDA1* expression, and accessions with lower *TaDA1* expression (with the *TaDA1-A* haplotype HapI) have larger and heavier kernels. The HapI haplotype has undergone positive selection in wheat breeding and is the haplotype most frequently found in modern wheat cultivars (72).

The rice (*O. sativa*) UBP15 ortholog OsUBP15 promotes large grain size (73). Seeds from plants with a dominant mutation in *Osubp15* that produces a truncated protein lacking 160 amino acids at the C terminus are larger and weigh 34.5% more than seeds from WT plants. The increased size is mainly due to an increase in the number of cells and to a small increase in cell size. RNAi knockdown of *OsUBP15* results in reduced grain length and thickness and plants with fewer grains per inflorescence but a similar total grain weight per plant compared with WT plants. Plants overexpressing *OsUBP15* have the opposite: longer, thicker grains than WT plants. Like *Arabidopsis* UBP15 and DA1, OsUBP15 has deubiquitinase activity and interacts with OsDA1 (73).

Very recently, a different E3, wheat TaSDIR1-4A (salt and drought–induced RING finger 1) was identified as another negative regulator of seed size through genome-wide association studies and examination of lines with reduced expression or overexpression of *TaSDIR1-4A* (74).This discovery suggests that there may be other currently unknown ubiquitin pathway components that influence seed size, and additional screens in crop plants for such components seem warranted. The conserved role of the DA1 pathway in regulating seed size in diverse plant species suggests that the genes involved could be exploited for breeding purposes in other agronomic crops. Organ-specific alteration of the key ubiquitin system proteins affecting seed size would mitigate their general effects on vegetative growth.

The ubiquitin system affects pathogen responses

Although variable by pathogenic organism and location, it is estimated that in 2010–2014 pests and pathogens reduced potential yields of five major agricultural crops representing over 50% of human caloric intake—wheat, rice, maize, potato, and soybean—by 17–30% overall (75), close to an earlier overall estimate of 34% from 2001 to 2003 for all crops (76). Reducing agricultural losses from pests and pathogens would have a significant impact on overall productivity and food security. Information on mechanisms of plant defenses and their roles in plant growth could inform practices to reduce infections and/or their impact on productivity. Understanding the mechanisms of rapid and specific defense activation and the mechanisms of dampening immune responses in the absence of infection could be significant in reducing negative impacts on plant growth and yield. The ubiquitin system plays important roles in both of these processes.

The first line of plant defense against pathogens is conferred by recognition of extracellular microbe- or pathogen-associated molecular patterns (PAMPs/MAMPs) by plasma membrane (PM)-resident pattern recognition receptors (PRRs). PRR family proteins are transmembrane proteins with either an intracellular kinase domain (called RLK for receptor-like kinase), a glycosylphosphatidylinositol anchor, or a small intracellular sequence with no known functional domain. Pathogen entry and growth are blocked by the initiation of intracellular signaling cascades collectively referred to as PAMP-triggered immunity, triggered after PAMP binding to PRRs. These cascades include reactive oxygen species (ROS) production, calcium

fluxes, and a MAPK–activated kinase cascade. To overcome this resistance mechanism, pathogens insert proteins called effectors inside plant cells to promote disease progression, often through inhibiting defense responses. In a second line of defense called effector-triggered immunity, effectors or their downstream activities are neutralized by intracellularly localized resistance (R) proteins (77).

PRR immune receptors with FLS2 as a paradigm

Studies suggest that the levels of proteins involved in pathogen perception prior to pathogen recognition (referred to as basal levels), as well as the activity and downstream signaling of PRRs and associated proteins after ligand activation, are modulated by the ubiquitin system and intimately linked to initial phosphorylation events (78–80) (Fig. 4). Research focused on the *Arabidopsis* PRR FLAGELLIN SENSING 2 (FLS2), a widely conserved PRR among plants (81, 82) and member of the leucine-rich repeat (LRR) family of receptor-like kinases (83), revealed that its PM abundance is key to defense, and consequently FLS2 levels are under stringent but dynamic control. The FLS2 extracellular domain recognizes a conserved region in bacterial flagellin, mimicked in many studies by a flagellar peptide called flg22 (81, 84). FLS2 protein levels but not mRNAs are affected by flg22 (81). Crop species show similar defense responses; rice plants express an LRR similar to *Arabidopsis* FLS2 that binds flg22, and flg22 elicits an immune response in rice (85). Tomato, pepper, and potato express another PRR, FLS3, that detects a distinct flagellin peptide (86). Consequently, understanding the parameters that control FLS2 PM abundance could guide strategies for engineering enhanced resistance to pathogens that signal specifically through FLS2 and serve as a paradigm for manipulation of PRRs in general. Much of this work utilized the model plant *Arabidopsis thaliana*, and it remains a valid model because many players in *Arabidopsis* are conserved in agricultural species. However, whereas some mechanisms and control points are similar, there can be significant enough differences to warrant studies in each crop species.

FLS2 and several other PRRs require the same PM-localized co-receptor kinase, BRASSINOSTEROID INSENSITIVE-ASSOCIATED RECEPTOR KINASE 1/SOMATIC EMBRYOGENIC RECEPTOR KINASE 3 (BAK1/SERK3, called BAK1 here), for full activation and intracellular signaling (87). BAK1 was first described as a co-receptor for the plant hormone brassinosteriod family, which is recognized by the extracellular domain of BRASSINOSTEROID INSENSITIVE 1 (BRI1), also an LRR receptor kinase (88, 89). Other PRRs interact with BAK1, suggesting shared downstream signaling processes through BAK1 (90). BAK1 is a member of the large LRR RLK family, also with transmembrane and intracellular kinase domains (88, 89). BAK1 interacts with FLS2 in a flg22-dependent manner (91). Interaction in the absence of flg22 may be prevented by BAK1 binding to other RLKs, such as BAK1-INTERACTING RLK 2 (BIR2) and possibly other related BIRs (92). Both FLS2 and BAK1 interact with a second kinase, the cytoplasmic kinase BOTRYTIS-INDUCED KINASE1 (BIK1), a member of the receptor-like cytoplasmic kinase (RLCK) family, in a flg22-independent manner, indicating separate FLS2-BIK1 and BAK1-BIK1 complexes at the PM prior to activation (93).

Distinct mechanisms regulate immune PM proteins prior to and after pathogen perception. In the absence of its ligand flg22, FLS2 cycles between the PM and the early endosome/*trans*-Golgi network in a brefeldin A (BFA)-sensitive process; BFA treatment of plant cells leads to the accumulation of early endosomes/*trans*-Golgi vesicles collectively called BFA bodies, and FLS2-GFP localizes to these vesicles (94). BIK1 levels at the PM are also regulated in the absence of flg22, but here the mechanism involves ubiquitin-dependent proteolysis. Two PUB type E3s, PUB25 and PUB26, bind to and ubiquitinate primarily the basal or underphosphorylated form of BIK1 *in vitro*, and overexpression of PUB25/26 reduces BIK1 levels *in vivo* (95). PUB25/26 loss-of-function mutants hyperaccumulate endogenous BIK1, leaving FLS2 abundance unchanged (95). Control of BIK1 levels is regulated through phosphorylation of PUB25/26 by the calcium-dependent protein kinase CPK28, which stimulates PUB ubiquitination activity, or through PUB25/26 interaction with a G protein complex, which inhibits PUB ubiquitination activity (95). PUB25/26 are also found in complexes with a few other RLCKs in addition to BIK1, suggesting that this mechanism may be more broadly applicable (95).

flg22 binding to the extracellular LRR domain of FLS2 initiates rapid BAK1-FLS2 interaction and *trans*-phosphorylation events among FLS2, BAK1, and BIK1. After flg22 binds to FLS2, FLS2 undergoes greatly enhanced internalization and degradation. Activated FLS2 internalizes via a distinct brefeldin-insensitive pathway; FLS2-GFP is not found in BFA bodies, as observed in the absence of flg22, but rather in late-endosomal vesicles at ~30 min after flg22 interaction, peaking at an hour and then declining. *In vivo* expression of an FLS2 with a substitution at a single conserved threonine ($FLS2^{T867V}$) resulted in plants with reduced disease resistance and impaired FLS2 endocytosis (96), correlating FLS2 competence in conferring disease resistance to its internalization rate. Both internalization and degradation were reduced by MG132 treatment, implicating a proteasome-dependent step in internalization (96).

The closely related PUB-type ubiquitin E3 ligases PUB12 and -13 (in *Arabidopsis*) are implicated in degradation of activated FLS2. Whereas BAK1 stably associates with PUB12/13 in the absence of flg22, after flg22 addition, BAK1 phosphorylates PUB12/13, increasing their interaction with and polyubiquitination of FLS2 (97). FLS2 protein is not increased in *pub12 pub13 Arabidopsis* in the absence of flg22, but the flg22-induced reduction of FLS2 is lost, suggesting that these ligases specialize in the down-regulation of only the activated receptor. What is the disease phenotype in the *pub12 pub13* double mutant? Although showing enhanced defense responses such as increased hydrogen peroxide production after infection and reduced bacterial growth, defense genes are not activated in the absence of flg22, and no growth defects are observed (97). Subsequent studies show enhanced stress-induced leaf senescence and early flowering in the *pub12/13* single- and double-deficient backgrounds (98), but whether this would affect yields in agronomic crops is unknown. Additionally, PUB12/13 are

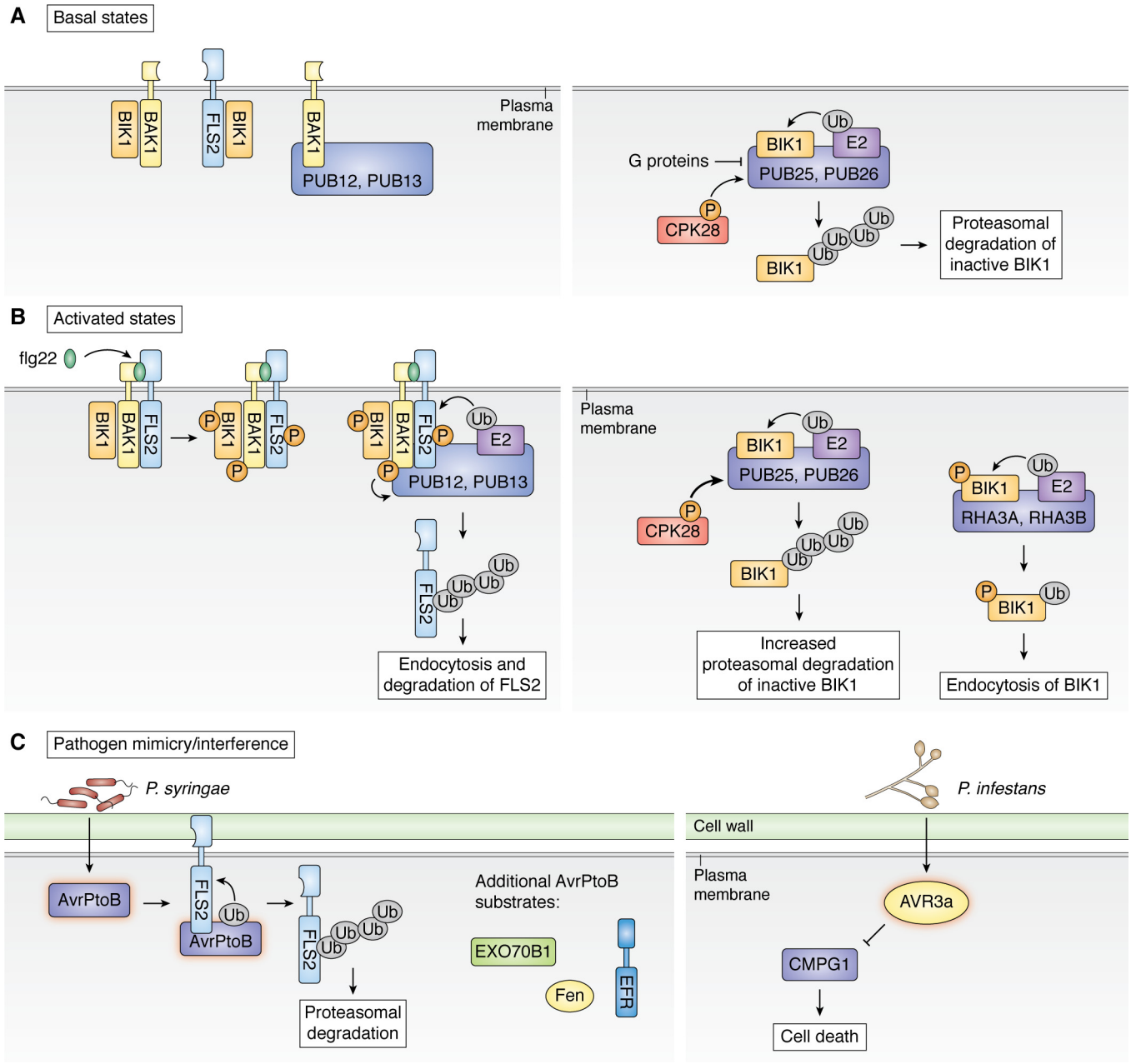

Figure 4. The ubiquitin system regulates the FLS2 receptor complex. *A*, in the absence of the FLS2 ligand, BIK1 interacts with both BAK1 and FLS2. BAK1 stably interacts with PUB12 and PUB13 in the absence of ligand but has reduced activity prior to autophosphorylation, and BAK1 is not a target of PUB25/26. PUB25 and PUB26 target underphosphorylated (inactive) BIK1, and this results in its degradation. *B*, FLS2 extracellular binding to a peptide derived from bacterial flagellin, mimicked in laboratory studies by a conserved 22-amino acid region called flg22, facilitates FLS2 interaction with BAK1 and PUB12/13 and results in multiple phosphorylation events. PUB12/13 activity increases after phosphorylation by BAK1, and PUB12/13 ubiquitinate FLS2, leading to its endocytosis and degradation. Phosphorylation of PUB25 and PUB26 increases their activity, resulting in increased ubiquitination and degradation of inactive BIK1. Phosphorylated (active) BIK1 is monoubiquitinated by RHA3A and RHA3B, resulting in BIK1 endocytosis. *C*, two examples of how pathogen effectors mimic or interfere with the host ubiquitin system. The tomato pathogen *P. syringae* pv. *tomato* DC3000 injects an effector, the E3 ligase AvrPtoB, into the plant cell to suppress the plant defense of programmed cell death. AvrPtoB substrates include FLS2, the related PRR EFR, the exocyst subunit EXO70B1, and the Fen kinase. The pathogen *P. infestans* produces the effector AVR3a, which suppresses cell death defense responses by affecting activity of the endogenous E3 ligase CMPG1.

implicated in other physiological processes, such as regulating PM-endosome cycling for the brassinosteroid receptor BRI1 (99); negatively regulating the abundance of the CERK1 subunit of the RLK-PRR complex of CERK1 and LYK5/CEBIP, which recognizes pathogen-derived chitin (100); and regulating the PP2C-type phosphatase ABA-INSENSITIVE 1 (ABI1) that functions in abscisic acid signaling (101). The possible effects on yield of modulation of immunity via PUB manipulations need to be investigated in field experiments.

After activation by flg22 interaction with FLS2, a distinct mechanism regulates BIK1. A monoubiquitinated form of phospho-BIK1 is observed at the PM, likely catalyzed by the ubiquitin E3 ligase RHA3A (RING-H2 FINGER A3A) and its closest paralog, RHA3B (102). Ubiquitin modification but not

phosphorylation is affected in RHA3A/B reduced function mutants. A single preferred ubiquitination site was not observed, and substitution of nine BIK1 lysine residues (K9R) was required for substantially reduced monoubiquitination (102). Plants expressing the K9R BIK1 form (in the *bik1* background) or the *rha3a/b* reduced function mutant both had reduced defense responses and were more susceptible to a bacterial pathogen. A link between disease resistance and BIK1 ubiquitination is suggested by the reduced dissociation of the K9R BIK1 version from FLS2 and reduced FLS2 endocytosis in the presence of flg22. These results indicate that PM abundance and trafficking of receptors and co-receptors are significant processes in defense signaling.

Activation also stimulates negative regulators of the immune response via the ubiquitin system. Three closely related *Arabidopsis* PUBs, PUB22/23/24, function genetically as negative regulators of immune signaling (103). PUB22 is phosphorylated by a downstream activated MAPK, MPK3, which reduces PUB22 oligomerization, self-ubiquitination, and self-degradation. PUB22 then accumulates, promoting ubiquitination of *trans*-targets (104). An identified PUB22 substrate is Exo70B2, a subunit of the exocyst complex required for immune responses (105). The exocyst was recently demonstrated to play a role in promoting FLS2 at the PM (106). Thus, increased PUB22 protein/activity in response to flg22 acts to reduce subsequent perception of flg22.

Additional negative regulators of immune responses characterized in *Arabidopsis* are the ubiquitin E3s PROTEOLYSIS 1 and 6 (PRT1 and PRT6), which were initially identified as E3s that recognize plant N-terminal hydrophobic or basic residues, respectively (reviewed in Ref. 107). Plants with a *prt6* loss-of-function allele exhibit increased resistance to bacterial pathogens in both barley and *Arabidopsis* (108). Similarly, a *prt1* loss-of-function allele shows enhanced bacterial resistance in *Arabidopsis* (109). Loss of either E3 results in a constitutive increase in defense proteins, creation of a "primed" defense state normally only observed in WT leaves after infection of other leaves, and increased synthesis of other defense metabolites (108, 109). PRT6 modulates the stability of a family of transcription factors that regulate the hypoxic responses to pathogens that induce undifferentiated cell growth, such as cysts or galls (110). The pleiotropic effects seen in *prt1/6* lines suggest that PRT1/6 have multiple substrates that affect cellular metabolism and subsequently pathogen sensing.

Regulation of R proteins by the ubiquitin system

The second line of biotic defense in response to the intracellular presence of bacterial proteins (effectors), called effector-triggered immunity, is mediated by members of a large family of intracellular, soluble, R proteins. Strict proteolytic control of a few R proteins has been demonstrated, suggesting that the ubiquitin system is also important for regulating this step of defense signaling. The largest class of R proteins are nucleotide-binding, leucine-rich repeat (NLR)-containing proteins that also have either a Toll-interleukin-1 receptor or a coiled-coil domain at their N termini. R proteins are present prior to pathogen effector introduction but exist in an inactivated state, likely as a result of autoinhibition. Activation of an R protein after detection of an effector protein typically includes homo- or heterooligomerization and release of inhibited domains (111). Diverse outcomes occur downstream of R protein activation. Depending on the R protein, these events can include interactions with transcription factors, ROS production, kinase activations, increases in the plant hormone salicylic acid and GTP-dependent signaling (111), and production of new metabolites, such as cleavage products of NAD^+ (112) that stimulate defenses similar to those observed after PRR binding to their extracellular ligand. Further downstream responses include localized cell death, which can limit the spread of pathogens that require living cells for propagation.

Activation of R proteins in the absence of pathogens, termed autoimmunity, appears to have negative effects on plant growth, suggesting that the control of R protein activity and abundance is critical in balancing the opposing actions of growth and defense (113, 114). A single amino acid substitution in the *Arabidopsis* R protein SNC1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1), the *snc1-1* allele (115, 116), appears to be a gain-of-function variant. *snc1-1* plants are smaller than WT plants, partly due to overproduction of the defense hormone salicylic acid (116). Several studies link regulation of SNC1 abundance to the ubiquitin system. SNC1 is more abundant and downstream defense gene activation is increased in a partial loss-of-function allele of *CUL1*, the scaffold of SCF-type ubiquitin ligases (117). Plants with reduced or loss-of-function mutations in the gene encoding the F-box protein CPR1 (CONSTITUTIVE PR GENE EXPRESSION 1, also referred to as CPR30), a substrate specificity subunit of CUL1-based E3s, also have constitutively increased downstream defense gene activation (117, 118) and increased SNC1 levels. E3 ligases containing CPR1 likely also ubiquitinate a subset of other R proteins (117). An R protein that interacts with SNC1, SIKIC2 (SIDEKICK SNC1 2), is negatively regulated by two related and functionally redundant E3 ligases, MUSE1 and MUSE2 (MUTANT, SNC1-ENHANCING), distinct from the $CUL1^{CPR1}$ ligase (119). A double loss-of-function *muse1 muse2* plant exhibits constitutive defense transcriptional responses, enhanced resistance, and severely stunted growth (119). A strong autoimmune response is detected in a SIKIC2 overexpression line in the *muse1 muse2* background. No effect on SNC1 protein levels was observed, indicating that despite ~75% amino acid similarity between SNC1 and SIKIC2, their abundance is regulated by distinct E3s, $CUL1^{CRP1}$ and MUSE1/2, respectively.

Analogous R protein regulation by E3 ligases is observed in crop plants as well. The barley R protein MLA (mildew locus A) is negatively regulated by an E3 called MIR1 (MLA-interacting RING 1) (120). Results from *in vivo* overexpression or reduced expression of MIR1 indicate that MIR1 plays an important role in modulating MLA-dependent disease resistance (120). In tobacco, an NLR protein called N confers resistance to tobacco mosaic virus. The tobacco E3 UBR7 (UBIQUITIN RING 7), with the highest identity to mammalian UBR7, interacts with N, and UBR7 overexpression in tobacco reduces N abundance (121). N protein recognition of the viral effector p50 interferes with UBR7-N interaction, stabilizing N and promoting defense (121).

Recently, two E3s in *Arabidopsis*, SNIPER1 and SNIPER2 (for SNC1-INFLUENCING PLANT E3 LIGASE REVERSE), were identified as more global R protein regulators. Overexpression of SNIPER1 suppressed the constitutive activity of the snc1-1 protein (see above) and also reduced the levels of other R proteins. *In vitro* ubiquitination of three diverse R proteins suggests that SNIPER proteins have broad specificity (122).

There are other links between the ubiquitin system and R proteins, specifically with the best-studied R protein in this context, SNC1. An enhancer screen to identify other proteins regulating SNC1 led to CDC48A (123), the subunit of a conserved homohexameric AAA-ATPase complex known to have multiple roles in cells, including unfolding ubiquitinated proteins out of membranes or out of multiprotein complexes to ferry proteins to the proteasome for degradation (124). Increased SNC1 protein is observed in loss-of-function mutants in MUSE3, a protein related to the yeast E4 enzyme UFD2 (124, 125), and in *ptre1* mutants (for PROTEASOME REGULATOR 1), a regulator of proteasome activity (126). Altogether, these studies indicate that inactivation of R gene function alone is insufficient to block their negative effects on plant growth in the absence of pathogens. Their intracellular abundance must also be controlled, and this control appears to be mediated by the ubiquitin system. A major challenge is to elucidate the interactions that distinguish highly specific from more general E3-R protein interactions. Another challenge is to dissect protein regulatory control of each key R protein mediating defense of major pathogens. The reader is referred to several reviews that discuss the intersection of the ubiquitin system and immunity in depth (111, 127–130).

Downstream ubiquitin-dependent defense responses

Responses downstream of the receptor complex are also modulated by the ubiquitin system. ROS both serve a signaling role and function in biotic defense. The main producer of extracellular superoxide is a PM-localized NADPH oxidase enzyme called RBOHD (RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D). After pathogen perception, RBOHD is phosphorylated, ubiquitinated at the PM, and targeted for vacuolar degradation (131). The E3 identified, PIRE (PLB13-interacting RING domain E3), is also phosphorylated. Further downstream, lignin deposition in the cell wall blocks invasion of the leaf and sheath blight pathogen. Lignin deposition slows pathogen invasion, and its synthesis is controlled in part by activity of the biosynthetic enzyme cinnamoyl alcohol dehydrogenase (CAD). Enhanced resistance is observed in a maize line with a missense mutation in an ORF of FBL41, a member of the F-box family of proteins. The maize F-box variant fails to interact with CAD, and protein lysates from this line fail to degrade recombinant CAD *in vitro*, correlating with increased lignin and resistance observed *in vivo* (132). In apple (*Malus domestica*), one of the family of substrate specificity subunits in the CULLIN4-type E3 ligase, POZ/BTB-CONTAINING PROTEIN 1 (POB1), interacts with and negatively regulates another apple E3, a PUB-type E3, PUB29 (133). This interaction affects susceptibility to apple ring rot, a parasitic fungal disease, late in fruit development and during storage. PUB29 acts as a positive element in defense responses (134). Md*PUB29* is induced after pathogen infection, and transgenic apple calli and *Arabidopsis* plants overexpressing PUB29 have enhanced resistance and higher levels of the stress hormone salicylic acid (134). Identification of PUB29 targets will facilitate understanding and manipulation of this defense response.

Pathogenesis and ubiquitin system interplay

There are multiple examples of pathogen-encoded gene products mimicking or interfering with host ubiquitin-dependent processes to enhance virulence. A bacterial effector from the tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000, AvrPtoB, injected into the plant cell to suppress programmed cell death defense, is itself a ubiquitin E3 despite its prokaryotic origin (135, 136). Identified AvrPtoB substrates are proteins involved in defense response, including FLS2 a related PRR, EFR (EF-TU RECEPTOR) (137), EXO70B1, a subunit of the exocyst responsible for shuttling defense components to the cell surface (106, 138), and in tomato, Fen kinase, a Ser/Thr kinase initially discovered for its role in conferring sensitivity to the insecticide fenthion, but Fen kinase is also important in defense responses to *P. syringae* DC3000 (139). Whereas AvrPtoB resembles the characterized U-box type of ubiquitin E3s, the effector XopK from the rice bacterial leaf blight pathogen *Xanathomonas oryzae* pv. *oryzae* is a novel bacterial ubiquitin E3 ligase. Upon injection into rice cells, XopK ubiquitinates OsSERK2, an RLCK member (140), but not rice PRRs, such as the rice FLS2 homolog. Although XopK does not target the receptor *per se*, diminished co-receptor abundance affects virulence as an *X. oryzae* strain with a ligase-inactive XopK version has reduced virulence (140).

In turn, host ubiquitin components are affected by introduced effectors/proteins. The potato cyst nematode *Globeodera pallida* effector GpRbp-1 interacts with a host nuclear-localized HECT-type ubiquitin E3, UPL3. Whereas root infection by this nematode reprograms development for cyst production, the reprogramming is disrupted in the *Arabidopsis upl3* mutant, indicating a role for the GpRbp-1-UPL3 interaction in infection and cyst development (141). The late blight pathogen *Phytophora infestans*, an oomycte, is a serious threat in tomato and potato production. One of its effector proteins, AVR3a, suppresses a cell death defense response through affecting the activity of an endogenous PUB E3, CMPG1 (named for a conserved Cys, Met, Pro, and Gly motif) required for cell death (142, 143). CMPG1 was previously identified as an mRNA rapidly up-regulated after infection in parsley and other species (144, 145). Finally, viral infection also modulates the endogenous ubiquitin system. The rice black-streaked dwarf virus (RBSDV) encodes a protein, P5-1, that inhibits host CSN5a. CSN5a is the active subunit of the signalosome that cleaves the covalently bound protein RELATED TO UBIQUITIN (RUB, Nedd8 in animals) from the cullin subunit of all CUL-based E3 ligases. Cycles of rubylation and derubylation promote CUL-based ligase activity. Given the many roles for CUL-based ligases, likely many processes are impacted. One process relevant to viral infection that was investigated is degradation of a repressor of jasmonate signaling, JAZ8 (146). JA-dependent loss of JAZ8 protein was impaired after hetero-

logous expression when the RBSDV P5-1 protein was also co-expressed.

Recounted above are highlights of a few of the recently described defense responses that the ubiquitin system influences, and is influenced by, to modulate plant defense. The list of pathogen components mimicking or modulating the host system and of host proteins sensing and responding to pathogen proteins will only grow in the future (147). Further dissection of plant ubiquitin system enzymes' target specificities, activities during infection, and whether they are modulated by pathogens will provide rich resources for development of enhanced resistance.

Summary and future directions

The ubiquitin system consists of many proteins that participate in the attachment, recognition, and removal of ubiquitin. Studies of this system in plants have revealed its conserved role in fundamental processes such as the cell cycle and protein quality control and also, somewhat surprisingly, its central role in regulating the synthesis of and responses to all characterized plant hormones. As a consequence, the ubiquitin system plays key roles in developmental processes and environmental responses in plants. This review focused on the roles of ubiquitin in regulating several agronomic traits; it discussed the ubiquitin system's effect on genes and proteins that control flowering, highlighted some recent developments in regulation of seed size by the ubiquitin system, and reviewed some of the ways ubiquitin affects plant pathogen defenses. Work needs to continue on two fronts: on model species whose tools facilitate genetic and mechanistic analyses and on specific crop plants to confirm paradigms and more significantly to identify differences and understand nuances that affect the breeding, selection, and genome modification approaches in these species.

Given the large number of genes involved in the ubiquitin system (1) and in defense responses (148–150), dissecting specific interactions and the consequences of these interactions remains a major challenge. The specific functions of most R proteins, pathogen effectors, and ubiquitin E3s are uncharacterized. A major goal could be to characterize R protein regulation through identifying interactors, such as E3s or ubiquitin adaptor/binding proteins, in directed screens with proteins expressed *in planta* to capture possible post-translational modifications that affect interactions. Identification of effector functions could also reveal new players that disrupt the ubiquitin system as part of their pathogenesis; structure/function studies combined with *in vivo* assays will be required. We are just now understanding the conformational changes in R protein activation (112, 151–153) and downstream consequences, and the understanding of ubiquitin's role in these processes is currently rudimentary. Clarifying ubiquitin's impact on quantitative traits such as flowering and seed size is also challenging, given the modest effect of individual genes/proteins on these phenotypes. However some proteins for manipulation have been identified; a protein's stability or intracellular trafficking are potential sites of modification to improve crop productivity, through changes in the protein or in interacting components of the ubiquitin system. Further research may also identify new, currently unknown players as additional targets for modification or selection.

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Abbreviations—The abbreviations used are: PUB, plant U-box; H3K4me3, histone Lys-4 trimethylation; H3K36me3, histone Lys-36 trimethylation; PM, plasma membrane; PRR, pattern recognition receptor; RLK, receptor-like kinase; R protein, resistance protein; LRR, leucine-rich repeat; RLCK, receptor-like cytoplasmic kinase; BFA, brefeldin A; MAPK, mitogen-activated protein kinase; NLR, nucleotide-binding, leucine-rich repeat; ROS, reactive oxygen species; CAD, cinnamoyl alcohol dehydrogenase; RBSDV, rice black-streaked dwarf virus; DUB, deubiquitinating enzyme; PAMP, pathogen-associated molecular pattern.

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