A role for the ubiquitin–26S-proteasome pathway in gibberellin signaling

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The gibberellin (GA) signaling pathway, like auxin and jasmonate signaling, uses the ubiquitin–proteasome pathway to control expression through protein degradation. A conserved F-box protein of an SCF E3 ubiquitin ligase is a positive regulator of GA signaling in *Arabidopsis* and rice. GA apparently stimulates stem elongation by causing this SCF complex to regulate negatively a family of negative regulators of GA response (the DELLA family of putative transcription factors). The DELLA family members AtRGA or (Repressor of *ga1-3*) and OsSLR1 (SLENDER RICE1) proteins both appear to be subject to GA-induced proteolysis. The need to have the F-box genes *AtSLY1* and *OsGID2* for this proteolysis suggests that GA causes proteolysis of AtRGA/OsSLR1 via the $SCF^{AtSLY1/OsGID2}$ ubiquitin ligase.

Plants are sessile organisms that rely on hormones to induce changes in growth and development in response to a wide range of environmental stimuli [1]. Gibberellin (GA) was originally identified as the substance secreted by the fungus that caused diseased 'bakanae' (or foolish) rice seedlings to grow too long and spindly [2]. GAs are tetracyclic diterpenoid hormones that induce a wide range of plant growth responses including seed germination, hypocotyl elongation, stem elongation, leaf expansion, pollen maturation and induction of flowering [3]. Although much is known about GA biosynthesis and metabolism [4], much remains to be learned about the mechanism of GA signaling. GA is probably perceived by a receptor in the plasma membrane that remains unidentified [5,6]. Genes in the GA signaling pathway have been identified by screening for increased and decreased response to GA [3]. The DELLA subfamily of the GRAS family of putative transcription factors plays an important role in the negative control of GA signaling. Here, we discuss recent evidence that this gene family is subject to control via the ubiquitin–proteasome pathway and that this mechanism is highly conserved between monocots and dicots.

Response to hormones can be exquisitely regulated through control of protein accumulation by the ubiquitin–proteasome pathway [7], which controls response to auxin [8–10], jasmonic acid [11–13] and now GA [14,15]. Recent evidence also suggests a role for the ubiquitin–proteasome pathway in cytokinin, brassinosteroid and abscisic acid signaling [7,16–18]. Ubiquitin, a conserved protein of 76 amino acids, is added to proteins via a multistep pathway. Formation of a polyubiquitin chain on the substrate protein targets it for degradation by the 26S proteasome (Figure 1). The 26S proteasome is a large protease complex composed of a 20S catalytic and a 19S regulatory complex [7]. More recently, the observation that transcriptional activation domain (TADs) and degradation signals (degrons) tend to colocalize [19] led to research showing that ubiquitylation can activate transcription factors by signaling for proteolytic processing by the proteasome or by activation of transcriptional activation domains [20].

SCF complexes are one type of E3 ubiquitin ligase that has been structurally and functionally characterized in animals, yeast and plants [21]. The complex is named for the first three subunits identified in yeast and mammals: Skp1, cullin (or Cdc53) and F-box protein. Later, the subunit containing the RING-H2 motif (Rbx1/Hrt1/Roc1) was identified as an essential SCF component [22]. Our knowledge of the architecture of the SCF complex is derived from protein–protein interaction studies and from the crystal structure of the mammalian SCF^{Skp2} complex [22–24]. The F-box subunit interacts directly with a

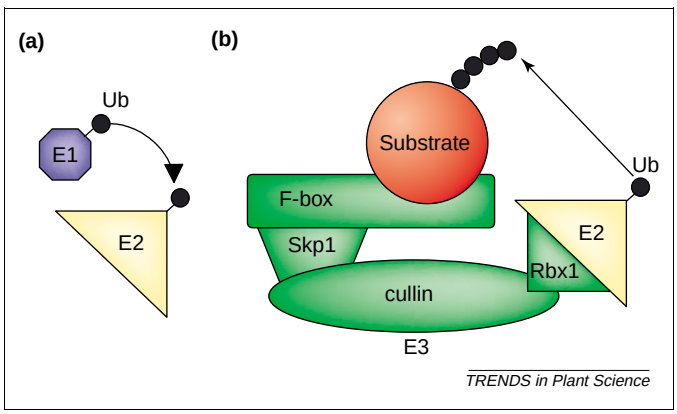

Figure 1. Key steps in the pathway of polyubiquitylation by SCF E3 ligase, which targets substrate protein and leads to degradation by the 26S proteasome. **(a)** Ubiquitin (Ub) is linked via a thioester bond to the ubiquitin-activating enzyme (E1). Ubiquitin is transferred from E1 to the cysteine of the ubiquitin-conjugating enzyme (E2). **(b)** The SCF E3 ubiquitin ligase (Skp1, cullin, F-box and Rbx1) catalyses the transfer of ubiquitin from E2 to a lysine residue on the substrate protein. Formation of a polyubiquitin chain on the substrate protein targets it for degradation by the 26S proteasome.

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specific target for ubiquitylation via a C-terminal protein–protein interaction domain. The N-terminal F-box domain binds the Skp1 subunit of the SCF. Skp1 tethers the F-box protein to the N-terminus of cullin, the backbone of the complex. Rbx1 binds to C-terminus of cullin and to the E2 ubiquitin conjugating enzyme. The SCF E3 ubiquitin ligase catalyses the transfer of ubiquitin from E2 to the substrate protein. Formation of a polyubiquitin chain on the substrate targets it for destruction by the 26S proteasome. The *Arabidopsis* genome contains 694 putative F-box proteins [25,26], at least 21 Skp1 homologs, 11 cullin homologs and 2 Rbx1 homologs [27–29]. The rice genome encodes at least 14 Skp1-like proteins [14].

An SCF E3 ligase positively regulates the gibberellin response in rice and *Arabidopsis*

The *AtSLY1* and *OsGID2* genes function as positive regulators of GA signaling in *Arabidopsis* and rice. Cloning of *AtSLY1* and *OsGID2* revealed that they encode homologous proteins containing a putative F-box motif [14,15]. F-box proteins contain an F-box domain, generally at the N-terminus, and a defined protein–protein interaction domain at the C-terminus, such as leucine-rich repeats, WD40 repeats or Kelch repeats [30]. Although AtSLY1 and OsGID2 both have N-terminal F-box domains, they do not contain a known C-terminal protein–protein interaction domain. Nevertheless, the C-terminal domain of the protein is clearly important for function because it contains a high degree of homology between rice and *Arabidopsis*, and because mutations in this region lead to a loss of function (Figure 2). In yeast two-hybrid assays, OsGID2 interacted with homologous protein to Skp1 in rice [14]. This result, together with the presence of an F-box domain, strongly suggests that OsGID2 is part of an SCF complex that regulates GA response.

The $SCF^{AtSLY1/OsGID2}$ complex is one of a growing family of SCF complexes involved in plant hormone signaling. The SCF^{TIR1} and SCF^{COI1} complexes of *Arabidopsis* function in auxin and jasmonic acid signaling, respectively [8,11]. The TIR1 and COI1 proteins are 34% identical and contain an F-box domain at the N-terminus and a LRR domain at the C-terminus. AtSLY1 has no homology to these proteins outside the F-box domain [15]. The SCF^{TIR1} complex is the first SCF complex whose target has been identified in plants. SCF^{TIR1} controls the stability of AUX/IAA proteins acting as a negative regulator for auxin signaling [8]. The AUX/IAA genes encode a transcriptional regulator isolated based on dominant mutations that give an auxin insensitive phenotype. Interestingly, the dominant mutations always occurred within the highly conserved domain II of AUX/IAA proteins. Biochemical analyses have shown that the domain II is the site of the degron in AUX/IAA proteins [8,31].

Several lines of evidence indicate that *AtSLY1* and *OsGID2* are orthologous genes. First, mutations in both *Arabidopsis SLY1* and rice *GID2* result in a recessive, GA-insensitive phenotype [14,32]. Second, the predicted AtSLY1 and OsGID2 amino acid sequences are 36.8% identical and 56% similar to one another (Figure 2). There are two alleles of *SLY1* and five alleles of *GID2* known to date [14,15]. These alleles affect the coding region downstream of the F-box domain (Figure 2). The high levels of homology and correspondence of function between dicot and monocot species indicate that the role of the $SCF^{AtSLY1/OsGID2}$ complex is highly conserved in the plant kingdom. Indeed, tBLASTn search of plant EST collections detected close homologs of AtSLY1 and OsGID2 in a wide range of plant species. A phylogenetic tree was created (using MEGA2.1 software) based on a ClustalW1.8 alignment (http://searchlauncher.bcm.tmc.edu/) of predicted amino acid sequences using the neighbor joining method to examine the number of amino acid differences (Figure 3). The OsGID2 sequence of rice is most closely related to homologs in the monocot species barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). The AtSLY1 sequence clusters with other dicot species and is most closely related to the sunflower (*Helianthus annuus*) homolog. The nearest relative of SLY1 in *Arabidopsis* (designated MIF21.6 or At5g48170) does not cluster with other dicot homologs of SLY1. The predicted MIF21.6 protein is 22.9% identical to OsGID2 and 23.7% identical to AtSLY1.

Although AtSLY1 and OsGID2 appear to have highly conserved functions in GA signaling, there are some

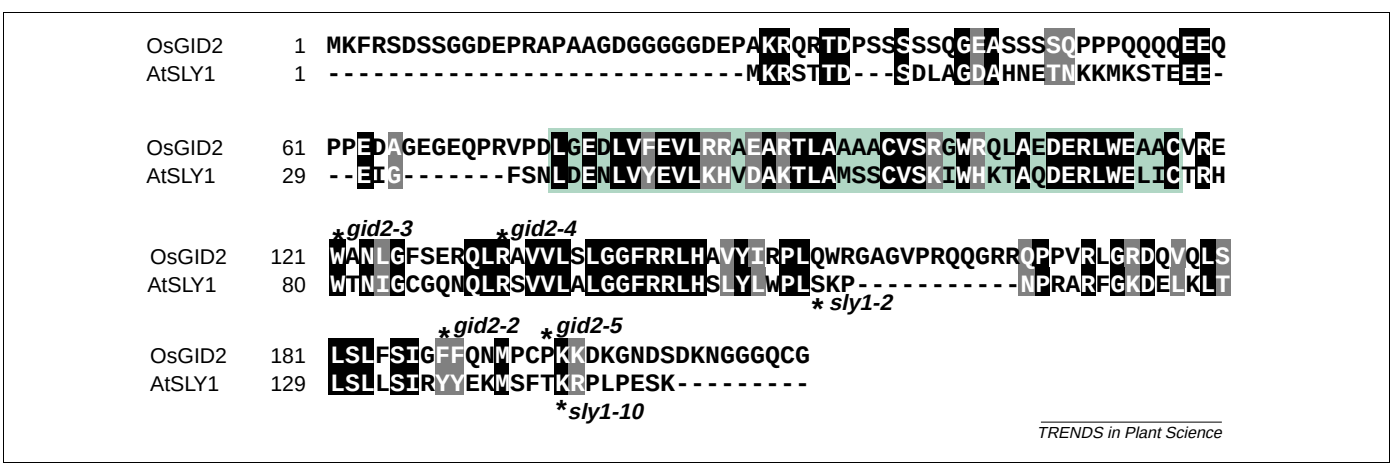

Figure 2. Alignment of SLY1 and GID2 amino acid sequence and position of known alleles. A ClustalW1.8 alignment of the predicted AtSLY1 and OsGID2 amino acids is shown (http://searchlauncher.bcm.tmc.edu/; http://www.ch.embnet.org/software/BOX_form.html). The position of the F-box domain is indicated by a green box. Asterisks indicate the mutation sites in *sly1* and *gid2* alleles: *sly1-2* and *gid2-4* cause deletions resulting in a frame shift; *sly1-10* is a complex rearrangement that causes the loss of the last eight amino acids; *gid2-3* is a nonsense mutation; and *gid2-5* is a 15-base insertion generating a novel stop codon.

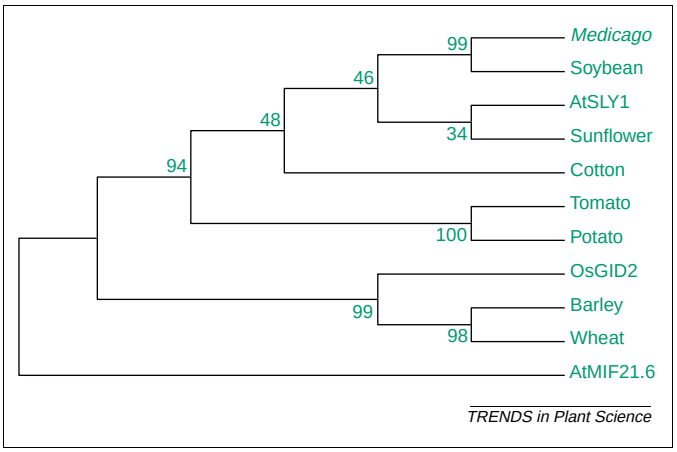

Figure 3. A phylogenetic tree of the *SLY1* and *GID2* gene family. A phylogenetic tree was constructed using the neighbor-joining method to look at the number of amino acid differences in a ClustalW alignment. Numbers shown indicate the percentage of 500 bootstrap repetitions that gave each branch point. Data used were predicted amino acid sequences based of EST clones of *Medicago trucatula* (GenBank BI265104), *Glycine max* (soybean) (BI785351), *Arabidopsis SLY1* (At4 g24210, NM118554), *Helianthus annuus* (sunflower) (AJ412362), *Gossypium arboretum* (cotton) (BE052748), *Lycopersicon esculentum* (tomato) (BG643250), *Solanum tuberosum* (potato) (BF459911), *Oryza sativa* (rice) *GID2* (AB100246), *Hordeum vulgare* (barley) (BF622212), *Triticum aestivum* (wheat) (BQ239225) and the *Arabidopsis* homolog of *SLY1, MIF21.6* or At5g48170 (AB023039).

interesting differences between their mutant phenotypes (Table 1). Mutations in the *AtSLY1* gene were identified in two screens for mutants with increased seed dormancy [32,33]. However, the *gid2* mutants were identified based on a severe dwarf phenotype [14]. Both *sly1* and *gid2* mutants are dark green dwarves. However, whereas the *gid2* mutants are fully infertile, the *sly1* mutants are only partly infertile. The *sly1* mutants show a strong increase in seed dormancy. By contrast, the *gid2* mutants show no apparent increase in grain dormancy. However, induction of α-amylase, a GA-induced gene associated with germination, is greatly reduced in *gid2* mutants. The hypothesis that the *SLY1*-like gene *MIF21.6* of *Arabidopsis* might be somewhat redundant in function with *SLY1* needs to be tested by determining whether a double mutant will cause stronger seed dormancy and infertility phenotypes.

DELLA proteins: to grow or not to grow, that is the question

The GRAS family of putative transcription factors was originally defined by the presence of the conserved domains VHIID and RVER in GAI, RGA and Scarecrow [34]. Members of the DELLA subfamily of the GRAS family contain the unique DELLA domain and are highly conserved negative regulators of GA response in plants [34,35]. Orthologs in this gene family include rice *SLENDER RICE1* (*OsSLR1*), barley *SLENDER1* (*HvSLN1*), maize (*Zea mays*) *d8* and wheat *Reduced Height* genes (*Rht-B1* and *Rht-D1*) [35–37]. Gain-of-function mutations in this gene family result in dwarfism and reduced GA response, whereas loss of function results in increased internode length and resistance to GA biosynthesis inhibitors [3]. Interestingly, although there is but a single DELLA gene in rice and barley [36,37], *Arabidopsis* has five homologs in this gene family, including *GAI* (GA-insensitive), *RGA* (Repressor of *ga1-3*), *RGL1*, *RGL2* and *RGL3* (RGA-like) [38–41]. *GAI* and *RGA* are highly homologous to one another and act redundantly to negatively regulate stem elongation, leaf expansion and transition to flowering [42,43]. *RGL2* is a negative regulator of seed germination whose transcript levels increase transiently during imbibition of dormant seeds [44]. *RGL1* is a negative regulator of germination, stem elongation, leaf expansion, flowering and flower development [41]. Thus, it appears that, in *Arabidopsis*, each DELLA gene might regulate a subset of the GA responses.

Evidence suggests that DELLA proteins AtRGA, OsSLR1 and HvSLN1 are all subject to GA-stimulated proteolysis via the ubiquitin–proteasome pathway. Accumulation of the AtRGA protein in the *Arabidopsis* nucleus is regulated by GA [39,45]. RGA protein accumulates at high levels in nuclei of the GA biosynthesis mutant *ga1-3*, whereas, in the presence of GA, steady-state levels of AtRGA protein are greatly reduced. It has similarly been shown that OsSLR1 [46] and HvSLN1 [47] accumulation is negatively regulated by GA. This raised the possibility that these proteins are regulated by GA-stimulated proteolysis. HvSLN1 protein levels increase in the presence of inhibitors of the 26S proteasome, suggesting that the DELLA proteins could be regulated by 26S-proteasome-mediated proteolysis [48]. This hypothesis is greatly substantiated by the cloning of F-box genes *AtSLY1* and *OsGID2* [14,15].

Several lines of evidence support the notion that SCF^{AtSLY1} and SCF^{OsGID2}, target the DELLA proteins AtRGA and OsSLR1 for degradation in a GA-dependent manner. First, mutations in *AtSLY1* and *OsGID2* result in greatly increased accumulation of AtRGA and OsSLR1 protein, respectively, even in the presence of GA [14,15]. The overaccumulation of RGA in *sly1* mutants is fivefold greater than that seen in the GA biosynthesis mutant *ga1-3*. This suggests that AtSLY1 and OsGID2 are the F-box subunits of a conserved SCF E3 ubiquitin ligase that targets AtRGA and OsSLR1 for degradation by the 26S

Table 1. Comparison of *sly1* and *gid2* phenotypes

Gibberellin response	*Arabidopsis sly1* mutants	Rice *gid2* mutants
Germination	Increased seed dormancy Increased sensitivity to abscisic acid	No apparent change in grain dormancy Abscisic acid sensitivity untested Reduced induction of germination-associated protein α-amylase
Stem elongation	Strong dwarf phenotype	Strong dwarf phenotype
Fertility	Reduced male fertility Short siliques	Fully infertile

proteasome. Thus, AtSLY1 and OsGID2 are positive regulators of GA response because they are negative regulators of the negative regulators of GA response, AtRGA and OsSLR1. Second, genetic analyses using the double mutants showed that the *sly1-10* and *gid2-1* dwarf phenotype was suppressed by the *rga-24* and *slr1-1* knockout mutations, respectively [14,15]. This suggests that the dwarf phenotype of the *sly1-10* and *gid2-1* mutants depends upon overaccumulation of AtRGA and OsSLR1, respectively. Finally, polyubiquitylated OsSLR1 protein is detected in wild-type plants but not in the *gid2* mutant [14]. This result suggests that ubiquitylation of OsSLR1 requires OsGID2. Together, these results strongly suggest that the $SCF^{AtSLY1/OsGID2}$ acts as an E3 ubiquitin ligase for GA-induced degradation of DELLA proteins.

If AtRGA, OsSLR1 and HvSLN1 proteins are all regulated by the ubiquitin–proteasome pathway, can we extrapolate to say that all DELLA proteins are regulated in this way? Perhaps not. The accumulation of fusions of AtGAI or AtRGL1 with green fluorescent protein (GFP) does not appear to be GA regulated [41,49]. Until these data are confirmed by western-blot analysis, they must be interpreted with caution because GFP is known to stabilize proteins [50]. Nevertheless, this does raise the possibility that AtGAI and AtRGL1 protein activity is regulated by another mechanism. One possibility is that these proteins might be regulated by the 26S proteasome by a non-proteolytic pathway.

Gibberellin and DELLA protein phosphorylation: a means to an end

What is the signal that targets DELLA proteins for destruction? Generally, post-translational modification of the substrate protein is required for ubiquitylation by an E3 ubiquitin ligase. Such post-translational modifications include proline hydroxylation, glycosylation and phosphorylation [51–53]. Phosphorylation is the most common type of substrate modification and could serve as a prerequisite for substrate interaction with the F-box subunit of an SCF complex. It is common for gain-of-function mutations in SCF-regulated proteins to result from deletion of the domain that is phosphorylated or that interacts with the F-box protein [52]. For example, phosphorylation targets the yeast protein Sic1 for SCF^{CDC4}-mediated ubiquitylation and degradation in a cell cycle-dependent manner [54]. Sic1 protein is stabilized by mutations in CDK phosphorylation sites [55]. In another example from mammals, the nuclear factor NF-κB is held in the cytoplasm by interaction with IκBα. During inflammation, phosphorylation targets IκBα for destruction via $SCF^{\beta\text{-}TrCP}$-mediated ubiquitylation [56]. Mutations in the phosphorylation sites of the IκBα protein block its interaction with the F-box protein β-TrCP [57].

It appears that OsSLR1 might also be targeted for SCF^{OsGID2}-mediated ubiquitylation by phosphorylation. In the *gid2* mutant, accumulation of a phosphorylated OsSLR1 protein was observed in addition to the nascent form [14]. Furthermore, the level of the phosphorylated OsSLR1 was increased by the GA treatment. The GA-induced phosphorylation of OsSLR1 observed in the *gid2* mutant might indicate that OsSLR1 protein is phosphorylated in a GA-signal-dependent manner and that the phosphorylated OsSLR1 becomes an available target for the SCF^{OsGID2}-mediated ubiquitin–proteasome pathway. There are currently no data indicating phosphorylation of AtRGA. However, it will be important to determine whether AtRGA is also subject to GA-regulated phosphorylation. Mutations in the N-terminal DELLA and TVHYNP regions stabilize DELLA proteins and lead to a dominant gain-of-function GA-insensitive phenotype [46,47,58,59]. It is possible that these domains represent the destruction domain and the mutations obstruct either phosphorylation or interaction with the AtSLY1 or OsGID2 F-box proteins.

The observation that OsSLR1 is subject to GA-regulated phosphorylation raises the intriguing notion that the GA signaling pathway might include a kinase cascade. Although the kinase responsible for OsSLR1 has not yet been identified, several other lines of evidence suggest the involvement of protein phosphorylation in GA signaling. In barley aleurone protoplasts, syntide-2 [a specific substrate for mammalian calmodulin (CaM) kinase II] selectively inhibits GA-induction of *α-Amy2*::*GUS* expression (a fusion of Amy2 with β-glucuronidase), suggesting Ca^{2+}-activated CaM-like-domain protein kinase is involved in GA induction of α-amylase production [60]. More recently, a tyrosine-kinase was suggested (based on pharmacological analysis) to be involved in the GA-induced destabilization of HvSLN1 in barley [48].

Conclusion

The discovery of GA-insensitive *Arabidopsis sly1* and the rice *gid2* mutants, and the cloning of the corresponding F-box genes gives us new insight into the role of the ubiquitin–proteasome pathway in the GA-dependent degradation of DELLA proteins and subsequent derepression of stem elongation. The predicted amino acid sequences of AtSLY1/OsGID2 and DELLA proteins are conserved between dicots and monocots, suggesting that their functions in GA signaling are probably highly conserved in the plant kingdom. A working model for the GA-regulated repression of AtRGA/OsSLR1 is illustrated in Figure 4. In the absence of GA, the DELLA proteins AtRGA and OsSLR1 inhibit GA responses such as stem elongation. GA triggers phosphorylation of AtRGA/OsSLR1 via an unidentified protein kinase. The phosphorylated DELLA protein is then polyubiquitylated by $SCF^{AtSLY1/OsGID2}$ complex causing degradation by the 26S proteasome. Degradation of AtRGA/OsSLR1 relieves inhibition of stem elongation leading to plant growth. It should be realized that this is a working hypothesis and, because phosphorylation of AtRGA has not been demonstrated, it is possible that AtRGA is regulated by a different post-translational modification than OsSLR1.

The new connection between GA signaling and the ubiquitin–proteasome pathway opens up many avenues for future research. The mechanism by which the DELLA proteins AtRGA and OsSLR1 inhibit stem elongation remains unknown. Homology of the VHIID domain to transcription factors suggests that DELLA proteins might be transcription factors [34]. However, no targets of

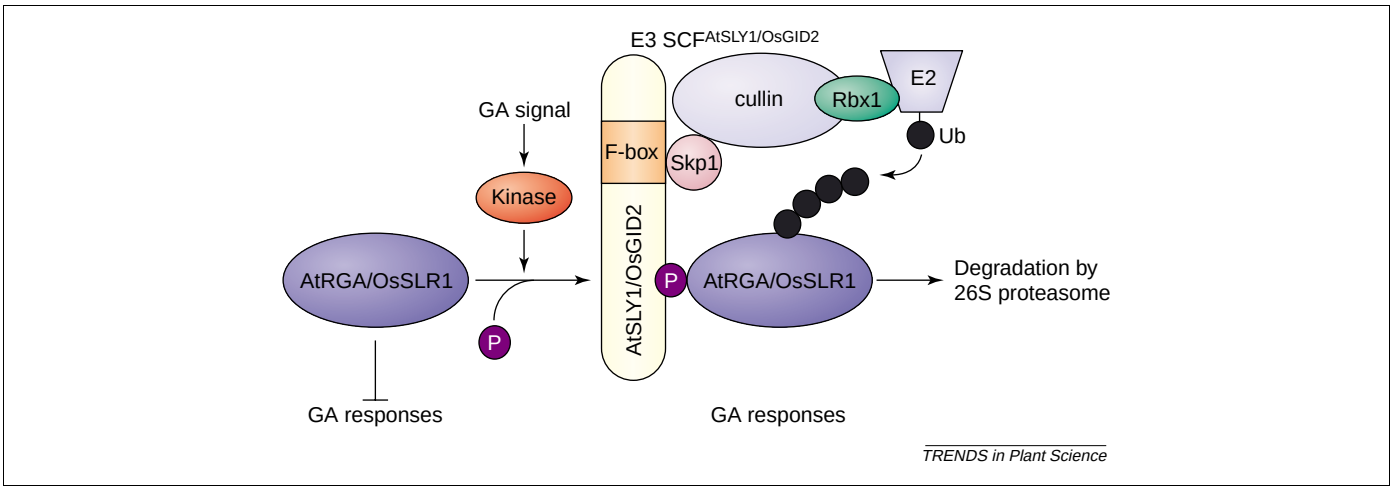

Figure 4. New model of gibberellin (GA) signaling. GA regulates the phosphorylation of AtRGA/OsSLR1 proteins triggering SCF-mediated degradation by the proteasome. Target protein (AtRGA/OsSLR1) is recruited to the E3 $SCF^{AtSLY1/OsGID2}$ ligase by the F-box AtSLY1/OsGID2 proteins. GA-regulated phosphorylation of AtRGA/OsSLR1 is likely to be required before recognition by the F-box protein. Phosphorylated target protein was then ubiquitinated (via an E2–E3 interaction) and finally degraded by the 26S proteasome. Inhibition of the GA response is abolished by this GA-dependent degradation of AtRGA/OsSLR1 proteins.

DELLA transcriptional control have been identified. In addition, it will be important to identify the degron and phosphorylated domains in the AtRGA/OsSLR1 DELLA proteins. The GA-regulated kinase and the mechanism by which the GA signal is transmitted to the kinase is a fertile area for future research.

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