Regulation of DELLA Proteins by Post-translational Modifications

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DELLA proteins are the negative regulators of the gibberellin (GA) signaling pathway. GAs have a pervasive effect on plant physiology, influencing processes that span the entire life cycle of the plant. All the information encoded by GAs, either environmental or developmental in origin, is canalized through DELLAs, which modulate the activity of many transcription factors and transcriptional regulators. GAs unlock the signaling pathway by triggering DELLA polyubiquitination and degradation by the 26S proteasome. Recent reports indicate, however, that there are other pathways that trigger DELLA polyubiquitination and degradation independently of GAs. Moreover, results gathered during recent years indicate that other post-translational modifications (PTMs), namely phosphorylation, SUMOylation and glycosylation, modulate DELLA function. The convergence of several PTMs in DELLA therefore highlights the strict regulation to which these proteins are subject. In this review, we summarize these discoveries and discuss DELLA PTMs from an evolutionary perspective and examine the possibilities these and other post-translational regulations offer to improve DELLA-dependent agronomic traits.

Keywords: DELLA • O-GlcNAcylation • O-Fucosylation • Phosphorylation • SUMOylation • Ubiquitination.

Introduction

DELLAs are land plant-specific proteins that belong to the GRAS family of transcriptional regulators (Hernández-García et al. 2020). The interest in DELLA proteins arose due to their participation as master negative elements in the gibberellin (GA) signaling pathway. This role was confirmed after the isolation of loss-of-function mutants in *DELLA* genes in Arabidopsis and in rice, which rendered constitutive GA responses like enhanced growth (Dill and Sun 2001, Ikeda et al. 2001, King et al. 2001, Feng et al. 2008). Currently, we have a good understanding of the mechanism through which the GA signal is transduced in angiosperms and the role of DELLAs therein. They are polyubiquitinated and degraded by the 26S proteasome after active GAs are perceived by the soluble receptor GA-INSENSITIVE DWARF 1 (GID1), as described below (Ueguchi-Tanaka et al. 2005, Murase et al. 2008, Shimada et al. 2008). Consequently, DELLAs are only allowed to accumulate in the nucleus when GA levels are low. Nonetheless, it seems that DELLAs have not always been subject to GA regulation during evolution. While DELLAs are present in early-divergent plants, suggesting that they were present in the common ancestor of land plants (Hernández-García et al. 2019), GID1 proteins, which likely evolved from carboxylesterases (Yoshida et al. 2018), appeared later coinciding with the origin of lycophytes (Yasumura et al. 2007).

DELLAs modulate the activity of >300 transcription factors (TFs) and transcriptional regulators through protein–protein interactions (Marín-de la Rosa et al. 2014, Van De Velde et al. 2017, Lantzouni et al. 2020). There are different modes of DELLA action (Thomas et al. 2016). They can inactivate TFs and transcriptional regulators by a sequestration mechanism, preventing binding either to DNA or to the TFs they regulate, respectively. The interaction with other TFs, on the contrary, occurs in the vicinity of their target promoters, where DELLAs usually act as transcriptional activators. Although the role of DELLAs in early-divergent plants has not yet been determined, it has been proposed that they could act as transcriptional activators in these species as well (Hernández-García et al. 2019).

Thanks to the ability of DELLAs to interact with other proteins and to the high sensitivity of the GA metabolism to environmental changes (Sun 2011), it is thought that DELLAs act as signaling hubs, connecting the transcriptional pathways regulating growth and defense with the environment (Claeys et al. 2014). Indeed, in silico analyses of DELLA-associated gene coexpression networks suggest that DELLAs coordinate transcriptional programs (Briones-Moreno et al. 2017). The signaling hubs are usually tightly regulated to prevent unwanted regulation of downstream pathways. For example, the stability and activity of p53 and c-Myc, two well-known hub proteins in animals, are controlled by multiple post-translational modifications (PTMs) (Hann 2006, Kruse and Gu 2009). The post-translational control to which DELLA levels are subject by polyubiquitination in response to GAs would be in line with the close regulation expected for a signaling hub. Therefore, the

identification of different PTMs of DELLAs, including GA-independent DELLA polyubiquitination and destabilization pathways, adds novel layers of regulation to the control of DELLA activity (**Figs 1, 2**). In this review, we aim to critically summarize the studies leading to these discoveries and to discuss the evolutionary and biotechnological implications of these post-translational regulatory mechanisms of DELLAs.

Ubiquitination: New Pathways Emerge to Regulate DELLA Stability

The covalent binding of the small protein ubiquitin to other proteins represents a major PTM in eukaryotes, one that determines protein activity or fate, including stability (Sadanandom et al. 2012). Ubiquitin is attached to one or more lysine (K) residue of the target protein. Inter-ubiquitin chains can then be formed by the linkage of additional moieties to specific K residues at the first ubiquitin. In particular, polyubiquitin chains of at least four moieties formed by linkage at K48 lead the substrate protein to degradation by the 26S proteasome. The attachment of ubiquitin is achieved by the consecutive action of three enzymes, E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase, which is the one that recognizes the protein substrate. Among the seven types of E3 ubiquitin ligases, the RING and Skp1-Cullin-F-box (SCF) types are relevant for DELLA ubiquitination.

The first indications that DELLAs were the targets of ubiquitin-mediated degradation by the 26S proteasome came early in DELLA research. Key was the observation that their presence in the nucleus is reduced by GAs (Silverstone et al. 2001, Itoh et al. 2002) and that the reduction in DELLA levels is prevented by treatment with 26S proteasome inhibitors (Fu et al. 2002). A pivotal player to understand the mechanism of DELLA destabilization was identified by various forward genetics strategies conducted in Arabidopsis and in rice (Wilson and Somerville 1995, Steber et al. 1998, Sasaki et al. 2003). Arabidopsis *SLEEPY 1* (*SLY1*) and rice *GID2* encode orthologous, plant-specific F-box containing proteins (McGinnis et al. 2003, Sasaki et al. 2003). F-box proteins are part of the SCF-type E3 ubiquitin ligases where they function as substrate adaptors (Lechner et al. 2006). The GA insensitivity and DELLA overaccumulation found in *gid2* and *sly1* loss-of-function mutants (McGinnis et al. 2003, Sasaki et al. 2003), together with the presence of the F-box in SLY1/GID2 proteins, strongly suggested that they target DELLAs for polyubiquitination prior to degradation by the 26S proteasome. In fact, polyubiquitination of the only DELLA protein in rice, SLENDER RICE 1 (SLR1), in response to GAs was impaired in *gid2* mutant plants (Sasaki et al. 2003), while the GA insensitivity of *gid2* and *sly1* loss-of-function mutants was due to overaccumulation of DELLAs, as their dwarf phenotype was alleviated when combined with *della* loss-of-function alleles (Sasaki et al. 2003, Dill et al. 2004, Fu et al. 2004). As bona fide F-box proteins, SLY1 and GID2 assemble in a SCF$^{SLY1/GID2}$ complex in vivo, anchored through the F-box to the Skp1 subunit (Fu et al. 2004, Gomi et al. 2004), and interact with the substrate DELLA proteins (Dill et al. 2004,

Fu et al. 2004, Gomi et al. 2004). SLY1/GID2 is, thereby, instrumental for DELLA polyubiquitination in response to GAs.

The mechanism used by GAs to promote the polyubiquitination and degradation of DELLAs, however, was still unknown after identifying SLY1/GID2. The last piece of the puzzle was the identification of the GA receptor, GID1 (Ueguchi-Tanaka et al. 2005). GID1 is a soluble protein with homology to hormone-sensitive lipases that binds active GAs (Ueguchi-Tanaka et al. 2005, Nakajima et al. 2006) and that interacts with DELLA proteins in a GA-dependent manner (Ueguchi-Tanaka et al. 2005, Griffiths et al. 2006, Nakajima et al. 2006, Ueguchi-Tanaka et al. 2007, Willige et al. 2007). The formation of the GID1–GA–DELLA complex involves the DELLA/TVHYNP motifs of DELLAs and residues around the GA-binding pocket and lid of GID1 (Murase et al. 2008). More importantly, it enhances the interaction between the DELLA protein and SLY1/GID2, thus favoring its recruitment to the SCF$^{SLY1/GID2}$ complex for polyubiquitination and degradation (**Fig. 1**) (Griffiths et al. 2006, Hirano et al. 2010). This model is consistent with the GA resistance of DELLA mutants lacking the DELLA and/or TVHYNP motifs. In line with these results, in vitro assays showed that DELLAs promote GA binding to GID1 (Nakajima et al. 2006) and they stabilize it as well (Ueguchi-Tanaka et al. 2007). All together, these findings indicate that DELLAs contribute to their degradation in the presence of GAs through a feed-forward biochemical mechanism that facilitates their assembly into a protein complex suitable for their polyubiquitination and degradation by the 26S proteasome.

Recent studies have demonstrated, however, that GA-independent pathways exist for DELLA polyubiquitination and degradation. A crucial observation was that protein levels for the GA-resistant version of the Arabidopsis DELLA REPRESSOR OF *ga1-3* (RGA), rga-*Δ*17, were reduced by the 26S proteasome when seedlings were placed in shaded or warm environments (Blanco-Touriñán et al. 2020). The RING-type E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Lau and Deng 2012) was considered as a prime candidate to define this new pathway for two reasons: RGA accumulates in plants with impaired COP1 function (Cagnola et al. 2018) and, like GAs, COP1 promotes growth in shade or in warm conditions (Djakovic-Petrovic et al. 2007, Stavang et al. 2009, Pacín et al. 2013, Delker et al. 2014, Park et al. 2017). The reduction in rga-*Δ*17 levels in hypocotyls in response to shade or warm temperatures was abolished in *cop1-4* mutant seedlings, indicating that it is COP1 dependent and independent of GAs (Blanco-Touriñán et al. 2020). COP1, aided by its functional partner SUPPRESSOR of *phyA-105* 1 (SPA1) (Hoecker 2017), interacts with DELLAs RGA and GAI in nuclear bodies, leading to their polyubiquitination and degradation by the 26S proteasome, as demonstrated through in vitro and in vivo assays (**Fig. 1**) (Blanco-Touriñán et al. 2020). What is the physiological relevance of the COP1 pathway compared to the canonical one? Do these two pathways act independently? Blanco-Touriñán et al. (2020) proposed that, although both are required to support hypocotyl growth under these environmental conditions, the role of each pathway is different. COP1 leads to DELLA degradation, triggering it soon
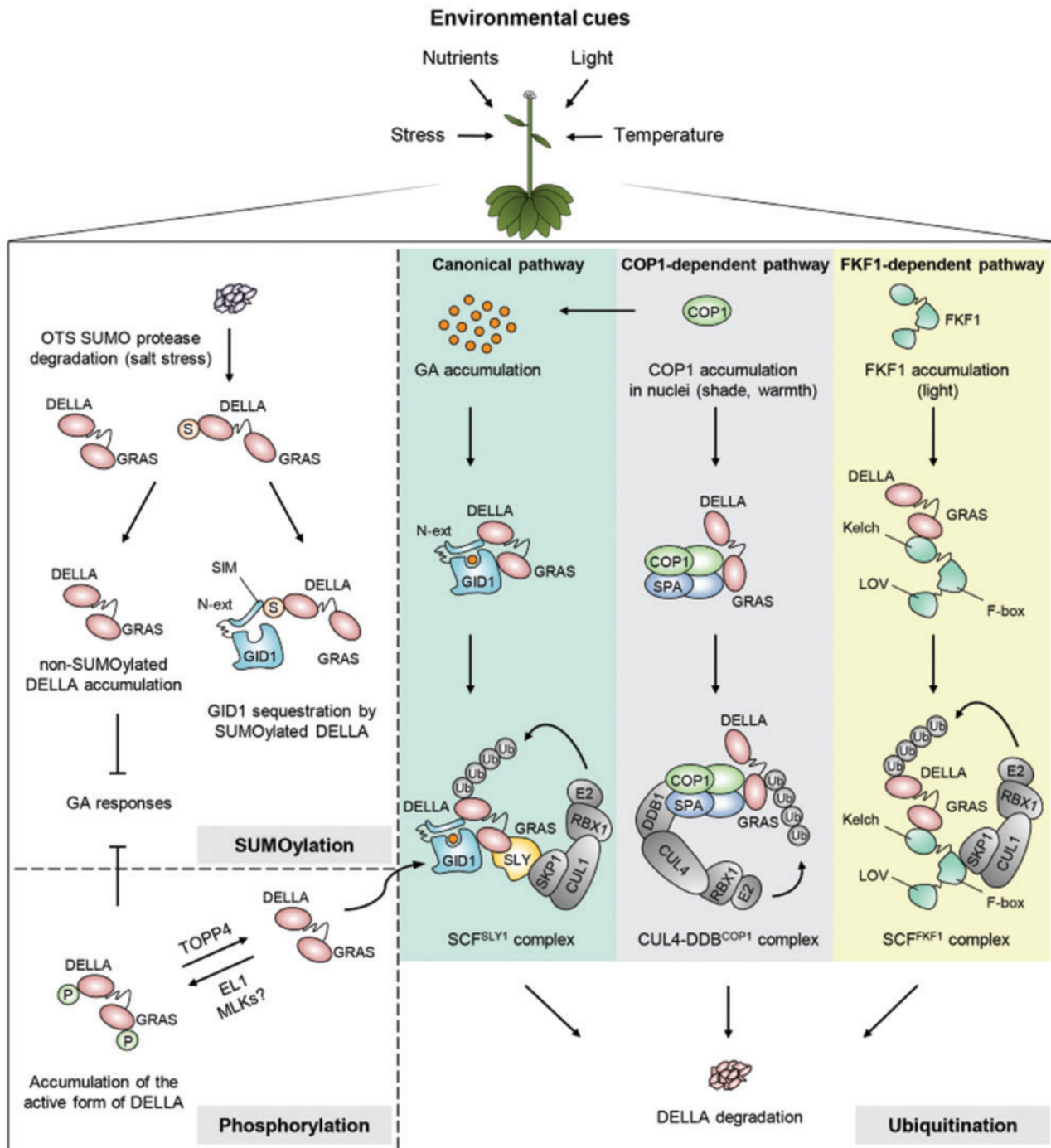
Fig. 1 Regulatory mechanisms of DELLA stability by PTMs. Ubiquitination: (1) canonical pathway: high GA levels allow for the formation of the GA–GID1–DELLA ternary complex and the subsequent recruitment of the SCF$^{SLY1/GID2}$ E3 ubiquitin complex for DELLA ubiquitination (Ub) and degradation. (2) COP1 pathway: under shade or warm temperature, COP1 is quickly re-accumulated in the nucleus to target DELLAs for 26S proteasome-mediated degradation by the CUL4–DDB^{COP1} complex. Likewise, COP1 enhances GA levels, thereby promoting DELLA destabilization through the canonical pathway. (3) FKF1 pathway: under long-day photoperiods, FKF1 interacts directly with DELLAs to promote their poly-ubiquitination by the SCF^{FKF1} complex and proteolytic degradation. SUMOylation: salt stress induces OTS SUMO protease degradation and consequently leads to the accumulation of SUMOylated (S) DELLAs. SUMO-conjugated DELLAs are able to bind to the SIM site of GID1 independently of GAs. Thus, GID1 is sequestered, which allows for the accumulation of non-SUMOylated DELLAs to trigger defense responses. Phosphorylation: phosphorylation (P) mediated by EL1 (and probably by MLKs and/or other kinases) stabilizes DELLAs, whereas TOPP4-mediated dephosphorylation leads to GA/GID1-dependent DELLA degradation.
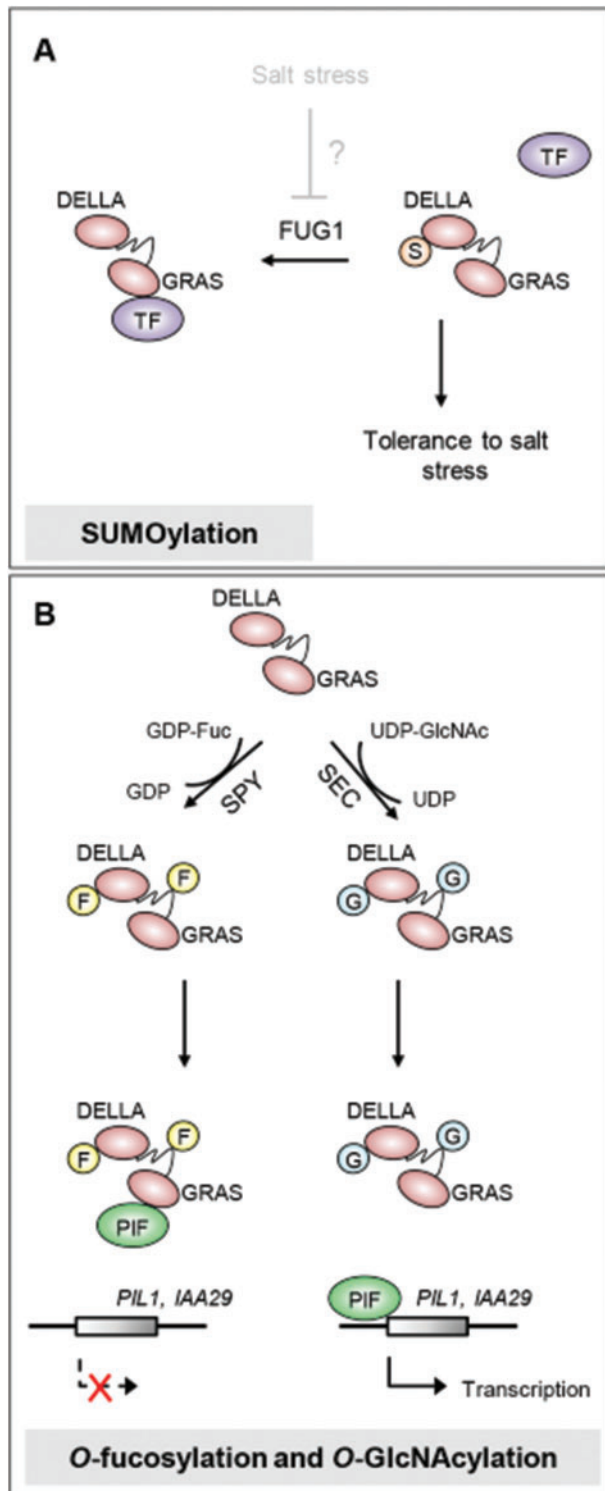
Fig. 2 Proposed mode of action of PTMs that regulate DELLA interaction with transcription factors. (A) SUMOylation: environmental stress may promote the degradation of FUG1 SUMO protease and hence the accumulation of SUMOylated (S) SLR1 in rice. SUMO–SLR1 loses the capacity to interact with certain TFs, which results in improved tolerance to salt stress. (B) Glycosylation: SPY and SEC compete with each other to attach O-fucose (F) and O-GlcNAc (G) sugars, respectively, at the DELLA and poly S/T/V regions of RGA. O-Fucosylation promotes RGA binding to PIF and hence blocks PIF transcriptional activity. Conversely, O-GlcNAcylation impedes RGA binding to PIF, which is then allowed to activate the transcription of target genes.

after the environmental change is perceived and, at the same time, it enhances GA biosynthesis (**Fig. 1**). On the contrary, the GA pathway sets the range of DELLA levels in the hypocotyl sensitive to COP1. The mechanism to connect this pathway with environmental changes might involve the red and far-red light photoreceptor phytochrome B (phyB), a known negative regulator of COP1 (Lu et al. 2015, Sheerin et al. 2015). The rapid inactivation of the phyB in response to temperature increases (Jung et al. 2016, Legris et al. 2016) or to shade (Casal 2013) would activate the pool of COP1 that is already in the nucleus previous to the environmental change (Park et al. 2017), being thus available to derepress the growth restraints imposed by DELLAs. This initial growth derepression might represent an adaptive advantage for seedlings, which need to respond quickly to environmental challenges.

One recent study focused on a different physiological context, the regulation of flowering time by photoperiod, and led to the identification of another pathway triggering DELLA polyubiquitination and degradation by the 26S proteasome (Yan et al. 2020). The Arabidopsis FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) forms an SCF^{FKF1} E3 ubiquitin ligase (Song et al. 2014), similar to SCF$^{SLY1/GID2}$, which promotes flowering when the photoperiod lengthens (Imaizumi et al. 2003). Notably, the work by Yan et al. (2020) shows that the *fkf1* mutant is less sensitive to GAs than the wild type regarding the promotion of flowering or the induction of target genes, while the opposite is observed in *FKF1*-overexpressing plants, this indicating a positive role for FKF1 in GA signaling (Yan et al. 2020). In agreement with this finding, RGA protein levels overaccumulate in *fkf1* mutants, while they are reduced in the overexpressing lines (Yan et al. 2020). However, changes in RGA levels in plants with impaired FKF1 activity are independent of GAs, as demonstrated, for instance, by the additive effects of mutations *ga1-3*, which causes GA deficiency, and *fkf1*. FKF1, through its Kelch domain, is able to interact with the GRAS domain of DELLAs, leading to their polyubiquitination and degradation (**Fig. 1**) (Yan et al. 2020). These results set FKF1 as a new GA-independent pathway triggering DELLA degradation by the 26S proteasome. Interestingly, despite the fact that FKF1 may act as a blue light photoreceptor (Imaizumi et al. 2003), its role in DELLA degradation appears to be independent of the light quality (Yan et al. 2020). Notably, *FKF1* expression is positively correlated with DELLA activity, suggesting that it forms part of the feedback regulatory circuit controlling homeostasis of the GA pathway (Yan et al. 2020).

Phosphorylation: Protecting DELLAs from Degradation

Phosphorylation of serine (S), threonine (T) and tyrosine (Y) residues is a common PTM of proteins that has a pervasive effect in cell physiology (Ubersax and Ferrell 2007). Phosphorylation of DELLAs has been studied for several years, although there is controversy over the effect of this PTM on their function. Early studies suggested that phosphorylation was a prerequisite for DELLA polyubiquitination and

degradation, as demonstrated for other proteins. This hypothesis was supported by several observations: (i) treatment with Y kinase inhibitors prevented the GA-stimulated degradation of barley and Arabidopsis DELLAs (Fu et al. 2002, Hussain et al. 2007); (ii) GA treatments promoted the accumulation of phosphorylated SLR1 in *gid2* mutants but not in wild-type plants (Sasaki et al. 2003); and (iii) GID2 and SLY1 preferably interacted with phosphorylated forms of SLR1 and GAI in vitro (Gomi et al. 2004) and in semi-in vivo assays (Fu et al. 2004), respectively.

Results from other studies, however, provided contradicting information. Phosphorylation of SLR1 occurred in the wild type and also in GA-deficient rice callus (Itoh et al. 2005). Versions of Arabidopsis RGL2 and RGA in which conserved S and T residues were changed either to the negatively charged aspartic/glutamic acid (phosphomimic) or to the conservative cysteine/alanine (de-phosphomimic) resulted in reduced or enhanced degradation in response to GA, respectively (Hussain et al. 2005, Wang et al. 2014). Accordingly, S/T phosphatase inhibitors blocked GA-induced DELLA degradation, whereas S/T kinase inhibitors did not have any obvious effect (Hussain et al. 2005, Wang et al. 2009). DELLA phosphorylation also seemed to affect DELLA activity. The overexpression of the phosphomimic RGA resulted in a GA-deficient phenotype, in agreement with the enhanced accumulation of this protein version, while it lacked the ability to activate the expression of GA biosynthesis genes (Wang et al. 2014). Taken together, these observations suggest that phosphorylation promotes DELLA stability and also affects its activity. Nonetheless, these results should be interpreted with caution. We cannot rule out that phosphatase and/or kinase protein inhibitors affect the phosphorylation status of other regulatory elements involved in GA-induced degradation of DELLAs rather than phosphorylation of the DELLAs themselves. And, more importantly, it remains to be proven whether mutated sites in RGL2 and RGA are actually phosphorylated in vivo. To date, only RGA S18 and S21 have been shown to be phosphorylated (**Fig. 3A**) (Wang et al. 2013) and they were not studied by Wang et al. (2014). The identification of phosphorylated residues seems to be, therefore, a crucial step toward understanding the relevance of this modification for DELLA stability and activity.

Genetic approaches in rice and Arabidopsis provided key advances in the field and led to the identification of protein kinases and phosphatases that contribute to define the phosphorylation status of DELLAs (**Fig. 1**). The characterization of the rice mutant *early flowering 1* (*el1*) revealed that EL1/Hd16 (EL1 onwards), a rice ortholog of the S/T protein kinase Casein Kinase I (CKI), acts as a negative regulator of GA signaling (Dai and Xue 2010). The *el1* loss-of-function mutant displayed enhanced GA responses and partially suppressed the dwarf phenotype caused by SLR1 overexpression. Dai and Xue (2010) showed that both the N- and the C-terminal domains of SLR1 were phosphorylated by EL1 in vitro, coinciding with the presence of CKI-predicted phosphorylation sites in each region (S196 and S510; **Fig. 3A**), and that the GA-dependent degradation of SLR1 was enhanced in the *el1* mutant. Studies with phosphomimic and de-phosphomimic versions of SLR1 led these researchers to propose that EL1-mediated

phosphorylation on the C-terminal domain sustains SLR1 activity, whereas the N-terminal phosphorylated form blocks the GA-mediated degradation (**Fig. 1**) (Dai and Xue 2010). The effect of EL1 on SLR1 phosphorylation levels in vivo remains to be determined. Four EL1-like proteins, also known as MUT9p-LIKE KINASEs (MLKs), have been identified in Arabidopsis, two of them direct interactors of RGA, although it is currently unknown whether these proteins phosphorylate DELLAs (Zheng et al. 2018).

On the other hand, isolating the dwarf and GA-insensitive mutant *topp4-1* in Arabidopsis allowed for the identification of TYPE-ONE PROTEIN PHOSPHATASE 4 (TOPP4), an ortholog of the animal protein phosphatase 1, as a phosphatase that dephosphorylates DELLAs (Qin et al. 2014). The dwarf architecture of *topp4-1* mutant plants was partially due to overaccumulated RGA and GAI, as demonstrated by the milder phenotype of *topp4 rga gai* mutants. Qin et al. (2014) showed that the TOPP4 protein accumulates in response to GAs and that it dephosphorylates GAI and RGA upon interaction, this being required to destabilize both DELLAs. The TOPP4-mediated destabilization is likely dependent on the formation of the ternary GID1–GA–DELLA complex (**Fig. 1**) since TOPP4 overexpression could not suppress the dwarf phenotype of the GA-insensitive *gai-1* mutant. TOPP4 belongs to a family of nine, highly similar members. The original *topp4-1* mutation exerted a dominant negative effect that impaired GAI and RGA dephosphorylation, although its role as a positive element in GA signaling was confirmed by a knockdown line (Qin et al. 2014). TOPP4 seems to represent a highly specific mechanism that operates only on certain DELLAs, GAI and RGA, and at certain developmental stages (Qin et al. 2014, Yue et al. 2016). Therefore, the set of protein phosphatases that controls the stability of the different DELLAs in specific developmental or physiological contexts must still be explored.

SUMOylation: Control of Both DELLA Stability and Activity

Protein modification by the SUMO (Small Ubiquitin-like Modifier) attachment is involved in many aspects of plant development and defense by altering the activity, localization or stability of proteins (Augustine and Vierstra 2018). SUMOylation provides the substrate with a new interaction interface that facilitates binding to proteins that contain a SUMO-interacting motif (SIM). SUMO is conjugated to a single or multiple K residues of the substrate, either as monomer or as poly-SUMO chain, following an enzymatic cascade similar to ubiquitination. In Arabidopsis, around 60% of SUMOylated proteins are modified at the consensus motif ψKxD/E (where ψ is a hydrophobic amino acid and x can be any) (Augustine and Vierstra 2018). SUMOylation is a reversible modification that may be cleaved by SUMO proteases (Morrell and Sadanandom 2019). The SUMOylation machinery is controlled by developmental and environmental signals, making SUMOylation a highly dynamic PTM that transmits information from these cues to hundreds of protein substrates. Notably, SUMO
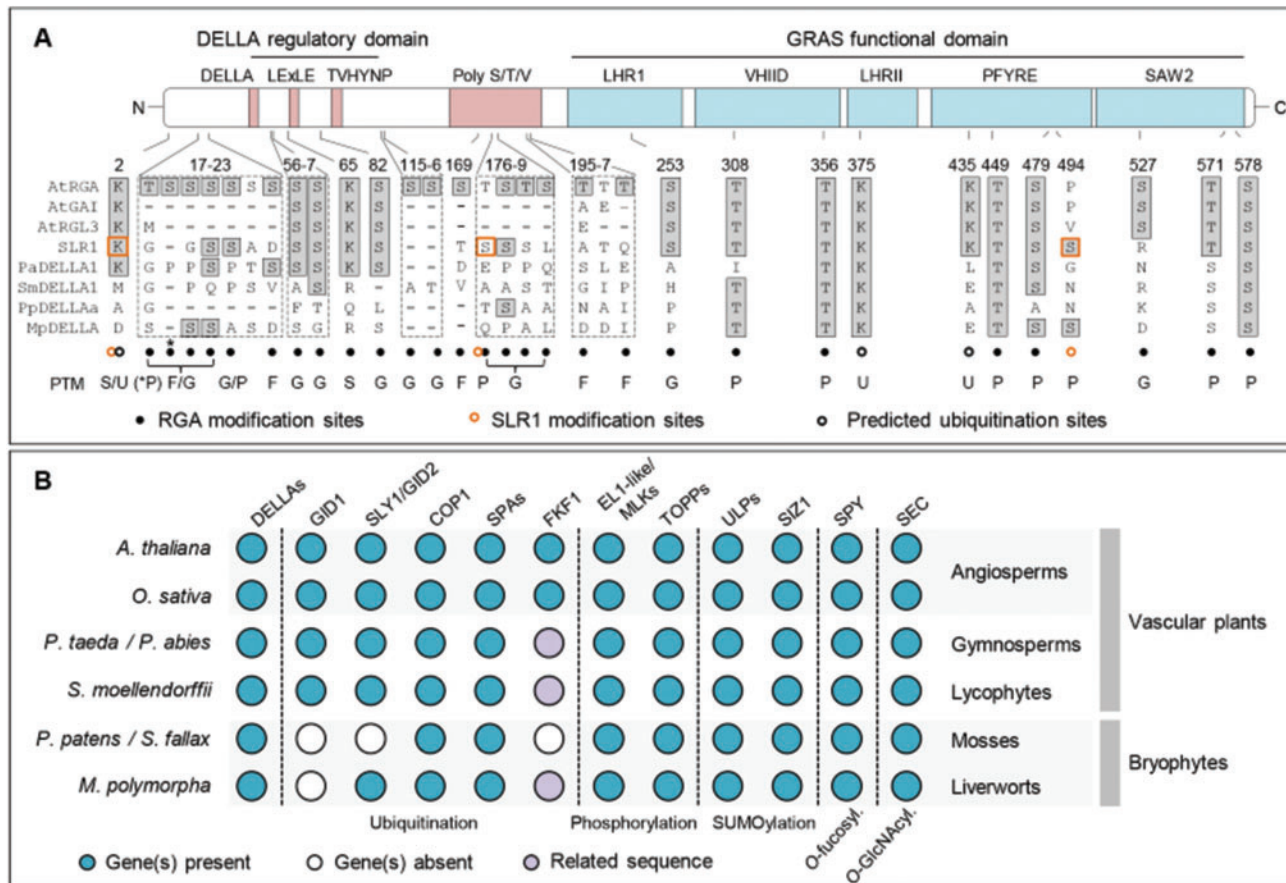
Fig. 3 Evolutionary analysis of DELLA regulation by PTMs. (A) Position of DELLA amino acid residues that are post-translationally modified or susceptible to be. DELLA protein domains and motifs are shown above. The RGA sequence was used as a reference. Residue conservation was determined by multiple protein sequence alignment and highlighted with gray squares. All residues were experimentally analyzed in RGA (black dots) with the exception of K2, S176 and S494 (orange circles), which were identified in SLR1 (K2, S196 and S510 in the SLR1 sequence). The asterisk indicates that RGA S18 can be alternatively phosphorylated. RGA potential ubiquitination sites (black circles) were determined in silico using the MusiteDeep webtool (**https://www.musite.net**). (B) Presence of DELLA post-translational regulators, or their homologs, in land plants. *Arabidopsis thaliana*, *Oryza sativa*, *Pinus taeda* or *Picea abies*, *Selaginella moellendorffii*, *P. patens* or *S. fallax* and *M. polymorpha* were included in the analysis as representatives of the different land plant lineages. The presence/absence of DELLA, GID1, SLY1/GID2, COP1, SPAs, FKF1, EL1-like/MLKs and OTSs/FUG1 orthologs in most of these species was studied in previous works (Kubota et al. 2014, Castro et al. 2018, Liu et al. 2018, Han et al. 2019, Hernández-García et al. 2019, Kang and Wang 2020). Other homologs were identified by a BLASTP search and confirmed by phylogenetic analysis. Sequences were retrieved from the Phytozome (**https://phytozome.jgi.doe.gov/pz/portal.html**) database and from both PLAZA (**https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza**) and ConGenIE databases (**http://congenie.org**) for gymnosperms. Blue circles denote the presence of gene, purple circles denote the presence of a related sequence and white circles denote the absence of gene.

conjugates increase in response to different types of abiotic stress, such as cold, high salinity or drought (Lois and Benlloch 2018).

Several recent studies have identified a role for SUMO in the regulation of DELLA stability or activity (Conti et al. 2014, Campanaro et al. 2016, Gonçalves et al. 2020). For example, salt stress induces the degradation of Arabidopsis OVERLY TOLERANT TO SALT 1 (OTS1) and OTS2 SUMO proteases (Conti et al. 2008). The reduced root growth observed when the *ots1 ots2* mutant is grown in high salt conditions led researchers to investigate if this response was caused by altered levels of DELLAs (Conti et al. 2014). In effect, RGA accumulated to higher levels in the *ots1 ots2* mutant than in the wild type, without significant changes in *RGA* gene expression or in GA levels, highlighting post-translational regulation by

SUMOylation. Biochemical analyses proved that RGA and GAI are indeed SUMOylated at a K in the L/QKLE motif (K65 for RGA and K49 for GAI; **Fig. 3A**) and deSUMOylated by OTS1 (**Fig. 1**) and that a K49R amino acid substitution destabilizes GAI (Conti et al. 2014). But how does SUMOylation affect DELLA stability? The GID1 receptor contains a conserved SIM site at the N terminus overlapping with the DELLA-interacting surface. This motif mediates interaction with SUMOylated RGA independently of GA, while the binding of non-SUMOylated RGA was strictly GA-dependent (Conti et al. 2014, Nelis et al. 2015). These observations led to a model in which salt stress promotes DELLA SUMOylation by destroying OTS1/2 proteases. SUMOylated DELLAs titrate GID1 receptors, allowing for the accumulation of non-SUMOylated DELLAs that inhibit growth and trigger defense responses (**Fig. 1**). A similar

mechanism may operate to restrict plant organ growth under nonstress conditions. Thus, the *ots1 ots2* mutant shows decreased fertility with shorter anther filaments, a phenotype that is fully restored to the wild type in the *ots1 ots2 rga* triple mutant (Campanaro et al. 2016). Conversely, SUMOylation can also promote growth through DELLA destruction. For example, SUMO conjugation stabilizes SLY1 to reduce DELLA levels (Kim et al. 2015) and it also increases COP1 activity (Lin et al. 2016), although the relationship between COP1 SUMOylation and DELLA degradation has yet to be characterized.

SUMOylation of DELLAs also seems to modulate their interaction with other proteins. A recent study revealed that the rice SLR1 was SUMOylated at K2 but not at K60 (the position equivalent to K65 in RGA), suggesting that DELLA SUMOylation operates differently in monocots and eudicots. In turn, SLR1 can be deSUMOyated by the FUG1 SUMO protease (Gonçalves et al. 2020). The fusion of SUMO to the K2 of SLR1 (SUMO–SLR1) disrupted SLR1 interaction with certain TFs but not with others (**Fig. 2A**). Rice plants overexpressing SUMO–SLR1 displayed improved tolerance to salt stress and altered expression of genes involved in the defense response and in GA metabolism (Gonçalves et al. 2020).

Glycosylation: Sugars Adjust DELLA Activity

O-GlcNAcylation consists in the attachment of a single *N*-acetylglucosamine monosaccharide to the hydroxyl group of S or T residues by the *O*-GlcNAc transferase (OGT), which can be reversed by an *O*-GlcNAcase (OGA) (Yang and Qian 2017). *O*-GlcNAcylation serves as a nutrient and stress sensor in animals to regulate a wide variety of cellular processes, many of them through crosstalking with other PTMs (Yang and Qian 2017). A recent study has identified 262 *O*-GlcNAcylated proteins in Arabidopsis, indicating that this PTM is widespread in plants as well (Xu et al. 2017).

O-GlcNAcylation regulates GA signaling by modifying DELLA proteins in Arabidopsis (Zentella et al. 2016). Two potential OGTs were initially described in this species, SPINDLY (SPY) and SECRET AGENT (SEC) (Jacobsen et al. 1996, Hartweck et al. 2002), while no orthologs for OGA have been identified to date. In contrast to SEC, the role of SPY in GA signaling has been known for some time. *spy* loss-of-function mutants exhibit a GA-overdose phenotype and are able to germinate in the presence of paclobutrazol, an inhibitor of GA biosynthesis, pointing to a negative role in GA signaling (Jacobsen and Olszewski 1993). Accordingly, strong *spy* alleles are largely epistatic to GA-insensitive or deficient mutants (Jacobsen and Olszewski 1993, Wilson and Somerville 1995, Silverstone et al. 1997, Peng et al. 1999b, Silverstone et al. 2007). Similar to Arabidopsis, knockdown of rice *OsSPY* increased internode elongation and partially rescued the dwarfism of GA-insensitive or GA-deficient mutants (Shimada et al. 2006). Most of the current knowledge about how SPY and SEC modulate DELLA activity comes from two recent reports in Arabidopsis (Zentella et al. 2016, Zentella et al. 2017). For the first time in plants, Zentella et al (2016) showed the

O-GlcNAcylation of a DELLA protein, RGA (**Fig. 3A** highlights modified peptides detected by mass spectrometry in the aforementioned study). Although both SEC and SPY interacted with RGA in co-immunoprecipitation assays, biochemical analyses unambiguously showed that only SEC *O*-GlcNAcylates RGA. *sec* mutants displayed a semidwarf architecture and slightly reduced fertility, phenotypes that are consistent with diminished GA signaling. *RGA* gene expression, protein accumulation and localization were not affected in genetic backgrounds with altered SEC activity. Pull-down assays showed that *O*-GlcNAcylation prevents RGA interaction with the TFs BRASSINAZOLE RESISTANT 1 (BZR1), PHYTOCHROME INTERACTING FACTOR 3 (PIF3) and PIF4 that otherwise would be inactivated, i.e. not able to bind to target genes (Zentella et al. 2016). Accordingly, genes normally upregulated by BZR1 or by PIF3/4 showed reduced expression in *sec* mutants. Therefore, SEC promotes GA signaling by inactivating DELLAs by *O*-GlcNAcylation (**Fig. 2B**) (Zentella et al. 2016).

But what about SPY? Studies from the same group of researchers shed light on this, expanding the repertoire of DELLA regulation by glycosylation (Zentella et al. 2017). Mass spectrometry analyses revealed a novel PTM of RGA, mono-*O*-fucosylation, consisting in the attachment of *O*-fucose to specific S or T residues. RGA *O*-fucosylation levels were decreased in *spy* but unaffected in *sec* mutants, and highly *O*-fucosylated RGA was recovered after the coexpression of SPY and RGA in tobacco leaves. These observations plus in vitro enzymatic assays demonstrated that SPY is an *O*-fucosyltransferase, which modifies RGA. *O*-fucosylated RGA displayed enhanced binding to BZR1, PIF3 and PIF4 and, accordingly, BZR1 or PIFs downstream targets were upregulated in *spy* mutants (Zentella et al. 2017). Moreover, *O*-GlcNAcylation and *O*-fucosylation sites largely overlap in the DELLA and poly S/T/V regions of RGA (Zentella et al. 2016, Zentella et al. 2017). Transient expression assays in tobacco elegantly showed that SEC and SPY compete against each other to modify RGA (Zentella et al. 2017). Overall, these observations support a model where *O*-GlcNAcylation by SEC and *O*-fucosylation by SPY antagonistically modulate DELLA function. Thus, *O*-GlcNAcylation decreases DELLA-binding affinity to TFs, which are now free to regulate gene expression (**Fig. 2B**). By contrast, *O*-fucosylation promotes DELLA sequestration of TFs and hence blocks downstream gene regulation (Zentella et al. 2017). Likewise, it would be interesting to determine how glycosylation affects DELLA capacity to bind to TFs, e.g. members of the INDETERMINATE DOMAIN (IDD) family, which allow transcriptional coactivation of target genes.

Evolutionary Insights into DELLA Post-translational Regulation

Both the origin of the regulatory proteins and the conservation of the target residues are aspects to be considered in the evolution of PTMs in DELLAs. The proteins that add or remove DELLA PTMs are present in most land plant lineages (**Fig. 3B**). A relevant exception is the acquisition of the GA/GID1 system for

DELLA ubiquitination, reported to coincide with the origin of lycophytes (**Fig. 3B**) (Yasumura et al. 2007). By contrast, orthologs of COP1 and its functional partner SPA are already present in the genomes of the mosses *Physcomitrella patens* (Rensing et al. 2008) and *Sphagnum fallax* (Leebens-Mack et al. 2019) and of the liverwort *Marchantia polymorpha* (Bowman et al. 2017). Indeed, the phytochrome signaling pathway, which would feed COP1 with environmental information, has also been demonstrated in *Marchantia* (Inoue et al. 2016). The COP1 pathway might represent an ancient regulatory mechanism to establish DELLA levels before the acquisition of the GA/GID1 system. On the other hand, bona fide orthologous genes of *FKF1* have not been found in gymnosperms or in early-divergent land plants (**Fig. 3B**) (Liu et al. 2018), suggesting that DELLA regulation by FKF1 could operate specifically in angiosperms. Nonetheless, only the functional characterization of both the COP1 and FKF1 pathways in these plants will determine if they represent ancient mechanisms for DELLA degradation.

The regulation of DELLA activity by SUMOylation is another interesting mechanism from an evolutionary perspective. Notably, the SUMO attachment sites (K2 of rice SLR1 and K65 of Arabidopsis RGA) are conserved across angiosperms and gymnosperms, but not in early-divergent land plant lineages (**Fig. 3A**). By contrast, the SUMO proteases and E3 ligases are present in all land plants (**Fig. 3B**) and, specifically, the SIM site of GID1 is already present in lycophytes (Yoshida et al. 2018). This suggests that the capacity to SUMOylate DELLAs was acquired in the ancestor of seed plants and then maintained during evolution as a tool to refine DELLA interactions with other proteins, including the GID1 receptor.

DELLA phosphorylation and glycosylation machineries are also conserved throughout the land plants' lineage, making it likely that these modifications occur in early-divergent land plants as well (**Fig. 3B**). If this hypothesis was true, both sorts of modifications would modulate the activity of DELLAs, probably by affecting the interaction with TFs, as demonstrated for glycosylation in Arabidopsis (Zentella et al. 2016, Zentella et al. 2017). Regulation of DELLA stability by phosphorylation might have been recruited, eventually, coinciding with the acquisition of GID1 in lycophytes. On the other hand, the modified residues, either glycosylated or phosphorylated, are unevenly conserved (**Fig. 3A**). Indeed, although similar consensus *O*-GlcAcylation sites have been described for plant and animal proteins, the sequence surrounding the modified S/T is relatively ambiguous (Kao et al. 2015). Therefore, glycosylation seems to be a highly specific protein modification that could depend on 3D structural cues as well.

In summary, the post-translational regulation of DELLA proteins appears to have gained in complexity throughout the evolution of land plants. However, the lack of large-scale studies into protein PTMs in different plants hinders comparative analysis from an evolutionary perspective. This information would pave the way to address fundamental questions on the evolution of DELLA functions.

Open Questions and Perspectives

As seen in the previous sections, our current knowledge about how DELLAs are regulated by PTMs is still quite fragmented. For instance, identifying which residues are ubiquitinated in response to the GID1/GA, COP1 or FKF1 pathways would provide crucial information to better dissect the relative relevance of each one for DELLA regulation. In addition, knowing the physiological context in which a post-translational regulation takes place is vital to clarify the role of DELLAs in particular processes. In this sense, we have a good understanding of the contributions of DELLA polyubiquitination or SUMOylation in response to salt stress or flowering. However, many other contexts under DELLA regulation have not been examined (e.g. response to drought, response to biotic stress, cell division, fertility, etc.). How *O*-GlcNAcylation and *O*-fucosylation are regulated by environmental or developmental cues are also issues that need to be addressed. Does SEC function as a sensor for the cellular nutrient status as OGT does in animals? And eventually, does it feed DELLAs with this information? Likewise, *SPY* expression is induced by drought (Qin et al. 2011), a not surprising observation that should be further explored.

Mounting evidence indicates that crosstalk between different PTMs is crucial to fine tune protein activities (Zhang and Zeng 2020). The titration of GID1 receptors by SUMOylated DELLAs is an example of crosstalk between SUMOylation and ubiquitination (Conti et al. 2014). Similarly, crosstalk between both PTMs at the target K might also be envisioned. The interaction of *O*-GlcNAcylation, and by extension *O*-fucosylation, with other PTMs, such as phosphorylation and ubiquitination that has been extensively studied in animals, should also be addressed in plants (Yang and Qian 2017). Indeed, among the peptides identified to be *O*-GlcNAcylated in Arabidopsis, 35% of them were alternatively phosphorylated (Xu et al. 2017). Interestingly, the downregulation of *OsSPY* was associated with enhanced SLR1 phosphorylation in rice (Shimada et al. 2006). In this regard, it is notable that no OGA orthologs have been identified in plants, raising questions about the reversibility of *O*-GlcNAcylation and the dynamics of interaction with phosphorylation and with other PTMs. The accumulation of residues susceptible to be post-translationally modified at the structurally disordered N-terminal region of DELLA proteins (**Fig. 3A**) also suggests the existence of heretofore unidentified crosstalk mechanisms.

An in-depth understanding of DELLA regulation by post-translational regulatory mechanisms will offer the possibility to rationally design novel DELLA alleles as tools to enhance crop yield. The inadvertent introduction of the 'green revolution' dwarfing DELLA alleles that boosted wheat and maize production in the 1960s (Peng et al. 1999a) is only one example of the power of uncoupling DELLA function from a particular PTM, polyubiquitination in this case. The relevance of other PTMs in an agronomical context is just starting to emerge. Recently, it has been discovered that an *OsSPY* allele with reduced activity was inadvertently selected in the process of rice breeding (Yano et al. 2019). However, the manipulation of post-translational modifiers of DELLA, or the over-stabilization

of DELLAs caused by the dwarfing alleles, may impact other signaling pathways and cause undesired side effects. Therefore, the direct manipulation of DELLA residues targeted by PTMs is likely to be an appropriate strategy not only to improve the 'green revolution' alleles but also to design new ones with minimum side effects. Examples of this type of strategy are 'overgrowth' mutants that contain second-site mutations in the dwarfing *Sln1d* and *Rht-B1* alleles of barley and wheat, respectively (Chandler and Harding 2013). One of these mutants, *Sln1d.5* of barley, specifically separates DELLA effects on inflorescence size and stem elongation (Serrano-Mislata et al. 2017). Another 'overgrowth' allele of wheat, *Rht-B1c.17*, includes an S258F substitution that results in a 30% increase in plant height, probably due to reduced DELLA activity (Chandler and Harding 2013). Of note, this substitution corresponds to S479 in RGA, a conserved residue that might be phosphorylated (**Fig. 3A**) (Wang et al. 2014). These observations emphasize the interest to decode the regulation of DELLA proteins by PTMs to create ad hoc alleles to improve agriculture.

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