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Chapter 6

GIBBERELLIN HORMONE SIGNAL PERCEPTION: DOWN-REGULATING DELLA REPRESSORS OF PLANT GROWTH AND DEVELOPMENT

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Abstract: The gibberellin (GA) hormone signal is perceived by a receptor with homology to hormone-sensitive lipases, GID1 (GA-INSENSITIVE DWARF1). This leads to GA-stimulated responses, including stem elongation, seed germination and the transition to flowering. GA-binding enables GID1 to interact with and block the function of the DELLA repressors of GA responses. DELLA repression can be blocked both by proteolytic and non-proteolytic mechanisms triggered by the formation of a GID1-GA-DELLA complex. DELLA is down-regulated by the SLEEPY1/GID2 F-box proteins via the ubiquitin-proteasome pathway, and can be regulated by other post-translational modifications. This chapter reviews the structural requirements for GA-binding by GID1 and for GID1-GA-DELLA protein complex formation, and reviews the current understanding of the mechanisms regulating DELLA repressors.

Keywords: GID1, DELLA, SLY1, GID2, ubiquitin, proteasome, gibberellin, signalling, EL1, SPY

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6.1 Introduction

Gibberellins (GAs) are tetracyclic diterpenoid plant hormones that stimulate seed germination, stem elongation, the transition to flowering and fertility in diverse plant species (see Chapter 1; reviewed in Sun and Gubler, 2004; Ueguchi-Tanaka *et al.*, 2007b; Yamaguchi, 2008). While 136 GAs have been identified in plants, fungi and bacteria, only a small sub-set of these are biologically active. The predominant bioactive GAs are GA_1 and GA_4. The DELLA (Asp-Glu-Leu-Leu-Ala) domain family of proteins act as repressors of GA responses through effects on gene transcription (see Chapter 7; Silverstone *et al.*, 1998; Itoh *et al.*, 2002; Zentella *et al.*, 2007). This chapter reviews the mechanisms by which the GA hormone signal is perceived and transduced to release DELLA repression of GA responses based mainly on evidence from rice (*Orzya sativa*) and *Arabidopsis thaliana* (*Arabidopsis*). In the 'DELLA destruction' model of GA signalling, GA binding allows the GA receptor GID1 (GA-INSENSITIVE DWARF1) to interact with DELLA repressors, thereby triggering DELLA destruction through the ubiquitin-proteasome pathway (Figure 6.1a, b). Alternative mechanisms for GA signalling in which the GA signal is transduced without DELLA destruction, or in which DELLA repressors are regulated by post-translational modification or in a GA-independent manner are also described. GA signalling mechanisms downstream of DELLA are described in Chapter 7.

6.2 DELLA proteins are repressors of gibberellin responses

DELLA proteins are nuclear-localised negative regulators of GA signalling defined by the presence of an N-terminal DELLA regulatory domain, and a C-terminal GRAS (GAI, RGA, and SCARECROW) functional domain (Figure 6.2; Silverstone *et al.*, 1998; Ikeda *et al.*, 2001; Itoh *et al.*, 2002). Mutations in the GRAS functional domain result in loss of DELLA repressor function, leading to a tall or 'slender' plant growth phenotype. This recessive phenotype is observed in the GRAS domain mutations in *SLN1* (*SLENDER1*) and *SLR1* (*SLENDER RICE1*), the sole DELLA genes in barley (*Hordeum vulgare*) and rice, respectively (Ikeda *et al.*, 2001; Itoh *et al.*, 2002; Chandler *et al.*, 2002). GRAS domain genes are a large family of transcriptional regulators unique to plants, and conserved in mosses, rice and *Arabidopsis* (Engstrom, 2011). The C-terminal GRAS domain contains a nuclear localisation sequence (NLS), two leucine heptad repeat motifs (LHR1 and LHR2) that flank the VHIID amino acid motif, and the PFYRE and SAW motifs (Figure 6.2; Richards *et al.*, 2000; Levy and Darnell, 2002; Bolle, 2004). The C-terminal PFYRE and SAW motifs have some homology to mammalian STAT (Signal Transducers and Activators of Transcription) transcription factors. Thus far, only one GRAS protein has been demonstrated to directly bind to DNA, a legume protein called NSP1 (NODULATION SIGNALING

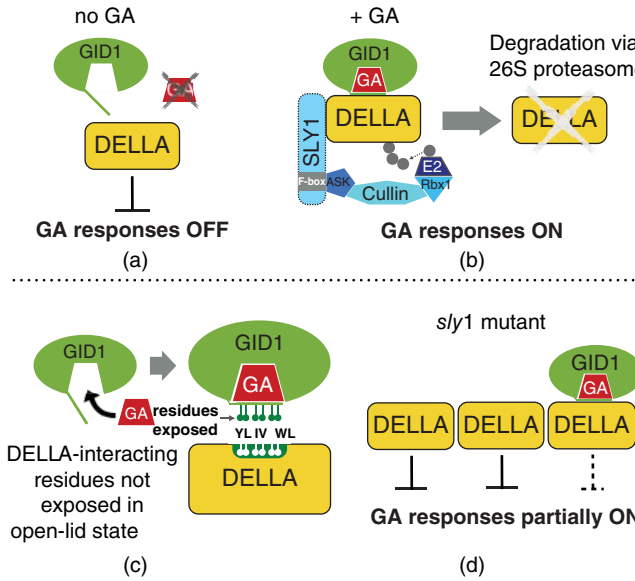


Figure 6.1 Models of proteolysis-dependent and -independent GA signalling. (a, b) The DELLA destruction model: (a) In the absence of GA, DELLA proteins are stable and repress GA responses. (b) GID1 binding to GA allows formation of the GID1-GA-DELLA complex, which in turn allows the SLY1/GID2 F-box protein to bind and polyubiquitinate DELLA, thereby targeting DELLA for destruction by the 26S proteasome. This lifts DELLA repression of GA responses. The SCF^{SLY1} E3 ubiquitin ligase complex consists of the Skp1 homologue ASK1, Cullin, the SLY1 F-box protein and RBX1. The SCF E3 catalyses transfer of ubiquitin (dark grey circles) from E2 to DELLA. (c) GID1 lid closure model: Without GA, the GID1 lid is believed to be open and unable to bind DELLA. When GA is bound, the GID1 lid closes exposing the hydrophobic residues (L, W, V, I, L and Y) needed to interact with DELLA protein. (d) Non-proteolytic DELLA down-regulation: In the *sly1* mutant, DELLA cannot be targeted for degradation, and DELLA over-accumulation represses GA responses. Formation of the GID1-GA-DELLA complex down-regulates some DELLA, partially relieving repression of GA responses.

PATHWAY1), suggesting that most GRAS proteins may indirectly regulate gene transcription (Hirsch *et al.*, 2009).

The N-terminal DELLA regulatory domain contains the DELLA, VHYNP (also called TVHYNP), and poly S/T/V motifs (Figure 6.2). Deletions in these motifs result in increased DELLA repression due to an inability to respond to GA hormone, leading to a semi-dominant semi-dwarf phenotype (Itoh *et al.*, 2002). The first DELLA mutant, *gai-1* (*GA-insensitive-1*), was isolated as a GA-insensitive semi-dominant semi-dwarf in *Arabidopsis* resulting from a 17-amino acid deletion within the DELLA/LExLE motif (Koornneef *et al.*, 1985; Peng *et al.*, 1997). The cloning of the two *Arabidopsis* DELLA genes, *GAI* and *RGA*, led to the cloning of similar GA-insensitive semi-dominant semi-dwarf DELLA mutants in wheat and maize (Silverstone *et al.*, 1997b;

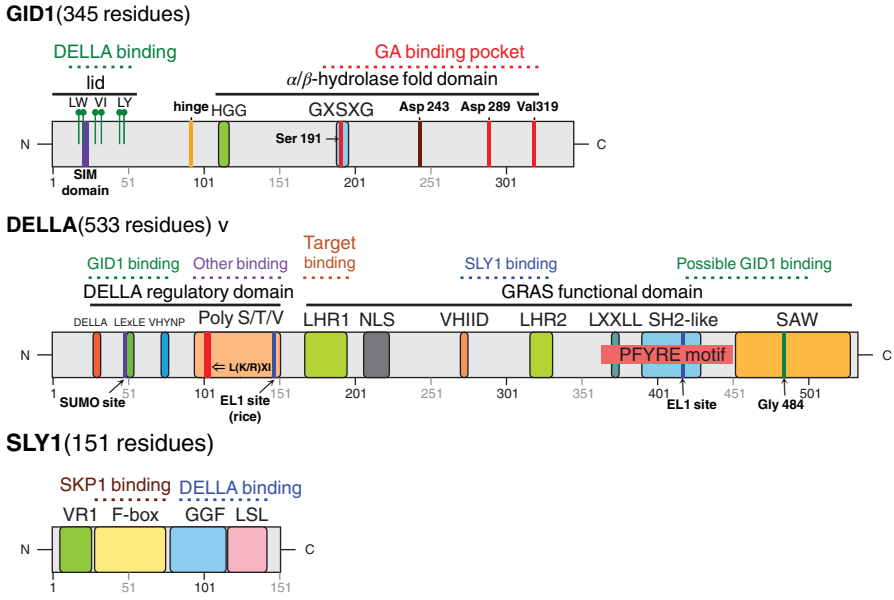


Figure 6.2 Diagrams of the GID1, SLY1 and DELLA domains, motifs and key amino acid residues. Proteins are drawn to scale based on the amino acid sequences of *Arabidopsis* GID1a, DELLA GAI and SLY1. Regions involved in specific protein–protein or protein–ligand interactions are indicated with dotted bars above the diagram. GID1: Two major domains marked by black bars above diagram are the GID1 lid and the α/β hydrolase fold domain including the core GA-binding pocket. Key motifs and significant amino acid residues include: (1) the hinge residue (orange bar for GID1a Pro 92, OsGID1 P99), (2) the catalytic triad (Ser 191, Asp 289, Val 319) involved in GA binding, (3) the six lid hydrophobic residues involved in DELLA-binding (lollipops for L, W, V, I, L, Y), (4) the SUMO-Interaction-Motif (SIM) domain (WVLI) and (5) the HGG and GXSXG motifs characteristic of hormone-sensitive lipases. DELLA: The major DELLA regulatory domain and the GRAS functional domain are marked by black bars above the diagram. Within the DELLA regulatory domain the DELLA, LExLE and VHYNP motifs are involved in GID1 binding (shaded boxes), and the poly S/T/V motif contains the L(K/R)XI motif likely involved in binding an undetermined 'other' GA signalling component. The GRAS functional domain contains: two leucine heptad repeats (LHR1 and LHR2), a nuclear localisation signal (NLS), the VHIID, the PFYRE, LXXLL, SH2-like, and SAW motifs (shaded boxes). Significant residues are marked by full-height bars. SLY1: SLY1 and GID2 contain the F-box domain that binds SKP1, the GGF and LSL motifs involved in DELLA-binding, and a variable region (VR1). (See insert for colour representation of this figure.)

Peng *et al.*, 1997; Silverstone *et al.*, 1998; Peng *et al.*, 1999). The shorter, thicker stems of semi-dwarf DELLA mutants of maize and wheat enabled yield increase that is now called the 'Green Revolution' by allowing farmers to use modern fertilisers to increase yield without causing the plants to fall over or lodge (Allan, 1986).

DELLA proteins appear to act in complex with transcription factors as coactivators or corepressors (reviewed in Chapter 7; Hauvermale

et al., 2012). DELLA proteins have been shown to interact with a wide range of transcriptional regulators including PIF3 (PHYTOCHROME INTERACTING FACTOR3), PIF4, PIF1/PIL5 (for PIF3-LIKE5), PIL2, JAZ1 (JASMONATE ZIM DOMAIN1), ALC (ALCATRAZ), SPT (SPATULA), BZR1 (BRASSINOZALE-RESISTANT1) and the GRAS protein SCL3 (SCARECROW-LIKE3) (Zentella *et al.*, 2007; de Lucas *et al.*, 2008; Feng *et al.*, 2008; Gallego-Bartolomé *et al.*, 2010; Arnaud *et al.*, 2010; Hou *et al.*, 2010; Heo *et al.*, 2011; Zhang *et al.*, 2011; Hirano *et al.*, 2012; Bai *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012). DELLA proteins also interact with the chromatin remodelling factor SWI3C (SWITCH3C) (Sarnowska *et al.*, 2013). JAZ1, PIF4, and BZR1 interact with DELLA proteins via the LHR1 motif (de Lucas *et al.*, 2008; Hou *et al.*, 2010; Gallego-Bartolomé *et al.*, 2012). It has been proposed that DELLA proteins function: (1) as coactivators of genes that negatively regulate GA signalling, (2) as repressors of transcriptional activators by blocking the ability of a transcription factor to bind its promoter and (3) as factors that recruit chromatin remodelling complexes to promoter elements.

6.3 Gibberellin signalling lifts DELLA repression of gibberellin responses

The partly overlapping roles of the five *Arabidopsis* DELLA repressors were defined based on the ability of DELLA loss-of-function alleles to rescue the phenotypes of the strong GA biosynthesis mutant, *ga1-3* (King *et al.*, 2001; Dill and Sun 2001; Cheng *et al.*, 2004; Cao *et al.*, 2005). This 10-kb deletion of the *GA1* gene encoding *ent*-copalyl diphosphate synthase (CPS) results in failure to germinate, extreme dwarfism, inability to transition to flowering under short days, and under-developed flowers (Koornneef and van der Veen 1980; Wilson *et al.*, 1992; Sun and Kamiya 1994; Silverstone *et al.*, 1997a). These phenotypes are rescued by GA hormone application, or by combinations of DELLA loss-of-function mutations. Thus, DELLAs act downstream of *GA1* to repress GA responses. GA stimulates GA responses by lifting DELLA repression. The five *Arabidopsis* DELLA genes encode proteins with 55.2–73.9% amino acid identity, and are named *GAI* (*GA-INSENSITIVE*), *RGA* (*REPRESSOR OF GA*), *RGL1*, *RGL2* and *RGL3* (*RGA-LIKE*) (Figure 6.3). The DELLAs GAI and RGA are the main repressors, and DELLA RGL1 a minor repressor, of stem elongation (King *et al.*, 2001; Dill and Sun, 2001; Wen *et al.*, 2002). However, DELLAs RGA, GAI, RGL1 and RGL2 all repress stem elongation under high temperature stress (Stavang *et al.*, 2009). The DELLA RGL2 is the main repressor of seed germination, since *ga1-3 rgl2-1* is the only *ga1-3 della* double mutant that can germinate without GA application in the light, but not in the dark (Lee *et al.*, 2002; Tyler *et al.*, 2004; Cao *et al.*, 2005). Since the *ga1-3 rgl2-1 gai-t6 rga-t2* mutant can germinate in the dark as well as the light, DELLAs GAI and RGA can also repress seed germination. The DELLA

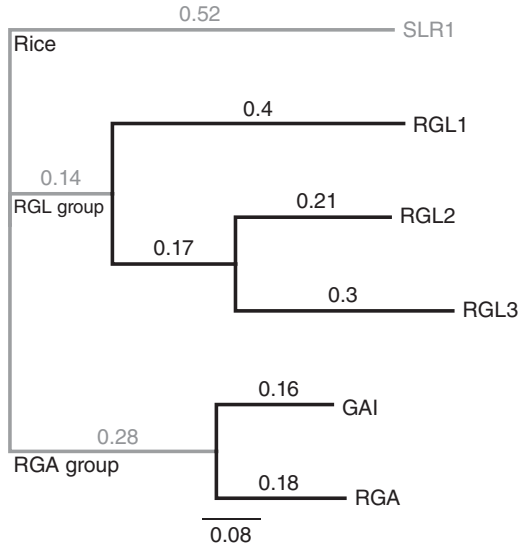


Figure 6.3 Phylogenetic analysis of DELLA protein homologues in *Arabidopsis* and rice based on predicted amino acid sequence analysis using ClustalΩ (Sievers *et al.*, 2011). A maximum likelihood tree was produced based on the JTT model (Jones *et al.*, 1992) and bootstrapping was performed with 1000 bootstrap replicates. Length of horizontal branches are proportional to the estimated number of amino acid substitutions per residue, which is indicated above each branch. The proposed RGL and RGA groups are indicated at their respective branching points.

RGL3 has been implicated in jasmonate (JA) signalling and in endosperm rupture during seed germination (Piskurewicz and Lopez-Molina, 2009; Wild *et al*., 2012). RGL2 and RGA are the main DELLA repressors, and RGL1 a minor repressor, of the transition to flowering (Cheng *et al.*, 2004). GA also stimulates floral development by inducing the expression of floral homeotic transcripts, *APETALA3*, *PISTILLATA* and *AGAMOUS* (Yu *et al.*, 2004; see Chapter 11). RGL1 and RGA are the main DELLA repressors of floral development, whereas RGL2 plays a minor role. The defects in *ga1-3* floral development were partly rescued in *ga1-3 rgl1-1* and *ga1-3 rga-t2* double mutants, and were almost completely rescued in the *ga1-3 rgl1-1 rga-t2 rgl2-1* and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* multiple mutants. The knockout of the four DELLAs *gai-t6 rga-t2 rgl1-1 rgl2-1* in the *ga1-3* background was referred to as 'penta' because it contains five mutations, although it is not a knock-out of all five DELLA genes (Cao *et al.*, 2005). A knockout of all five DELLA genes in the Landsberg *erecta* background was published by Feng *et al*. (2008).

A promoter swap experiment was used to examine whether the functional specialisation of the five DELLA genes was due to gene expression pattern or to differences in protein sequence (Gallego-Bartolomé *et al.*, 2010). DELLA RGL2 normally represses seed germination, but not stem elongation. DELLA

RGA normally represses stem elongation, but has a minor role in seed germination. When *GFP-RGA* and *GFP-RGL2* translational fusions were expressed on a 2-kb *RGA* promoter element, both genes served equally well to partly repress the growth of *ga1-3 gai-t6 rga-24*, and to partly restore feedback regulation of *GA20ox* GA biosynthesis gene expression. Conversely, *GFP-RGA* could function similarly to *GFP-RGL2* in repressing seed germination when expressed on the *RGL2* promoter. This suggests that some of the differences in the roles of *RGA* and *RGL2* are due not to differences in protein functionality, but due to differences in the timing and location of promoter expression. While both DELLA RGA and RGL2 proteins were able to interact with bHLH (basic-Helix-Loop-Helix) transcription factors PIF4 and PIF1/PIL5 in a yeast 2-hybrid assay, we cannot rule out that these DELLAs have different affinities for other DELLA-interacting proteins. For example, different DELLA proteins show different affinities for the three *Arabidopsis* GA receptors (Suzuki *et al.*, 2009), suggesting that differences in DELLA protein structure may lead to differential regulation by the three *Arabidopsis* GA receptors.

6.4 The gibberellin receptor GID1 (GA-INSENSITIVE DWARF1)

DELLA proteins function as negative regulators of GA responses that are down-regulated as a result of GA-stimulated protein–protein interaction with the GA-receptor, GID1 (GA-INSENSITIVE DWARF1) (Ueguchi-Tanaka *et al.*, 2005). The GA receptor was first identified by map-based cloning of the severely dwarfed mutant in rice, *gid1*. Loss of GID1 function in rice causes failure to respond to GA stimulation of leaf and cell elongation, flowering and fertility, and α-amylase expression during seed germination. Furthermore, these mutants accumulate bioactive GA at much higher levels than wild type. This increase in endogenous GA levels is likely due to up-regulation of GA biosynthesis genes as a feedback response to reduced GA signalling. While there is a single *GID1* GA receptor gene in rice, there are three GA receptor genes in *Arabidopsis*, *GID1a*, *GID1b*, and *GID1c* (Nakajima *et al.*, 2006; Yano *et al.*, 2015). The *Arabidopsis gid1* triple mutant exhibits severe GA-insensitive phenotypes including: failure to germinate unless the seed coat is cut, severe dwarfism and complete infertility (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Iuchi *et al.*, 2007). Interestingly, the first *GID1* alleles were identified in barley based on reduced GA sensitivity during leaf elongation (Chandler and Robertson, 1999; Chandler *et al.*, 2008). After the cloning of the rice *GID1* gene, these semi-dwarf *gse1* (*GA sensitivity1*) mutants were found to be missense alleles of barley *GID1*.

The three *Arabidopsis GID1* genes have partially overlapping roles in GA signalling. No single *GID1* T-DNA insertion allele shows a strong GA-insensitive phenotype. However, double and triple mutants show

varying degrees of GA-insensitive phenotypes that provide clues to the specialisation of *GID1* gene function in *Arabidopsis* (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Iuchi *et al.*, 2007). The *gid1a gid1c* mutant has a stronger dwarf phenotype than *gid1a gid1b* or *gid1b gid1c*, suggesting that *GID1a* and *GID1c* play a stronger role in stem elongation. The *gid1a gid1b gid1c* triple mutant is far more severely dwarfed than any *gid1* double mutant, suggesting that *GID1b* also plays a role in stem elongation. Consistent with this, both *GID1a* and *GID1c* mRNAs and GID1-GUS translational fusions are more strongly expressed in inflorescence stems than *GID1b* (Griffiths *et al.*, 2006; Suzuki *et al.*, 2009). The *gid1a gid1b* double mutant had the strongest decrease in silique length and fertility, and the *gid1a gid1c* double mutant showed the most severe reduction in germination efficiency (4% germination) (Griffiths *et al.*, 2006; Voegele *et al.*, 2011). However, a *gid1b* allele in the Nossen ecotype showed a strong decrease in GA sensitivity during seed germination, suggesting that *GID1b* also stimulates seed germination. *GID1a* and *GID1b* stimulate floral bud formation, as the *gid1a gid1b* double mutant has lower fertility associated with shorter stamens (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Iuchi *et al.*, 2007). While one research group published that the *Arabidopsis gid1a gid1b gid1c* triple mutant fails to flower under long day conditions, another published that the same triple mutant flowered under their light conditions (Willige *et al.*, 2007; Plackett *et al.*, 2014). It appears that the requirement for *GID1* genes during *Arabidopsis* flowering may depend on as yet uncharacterised environmental conditions, such as temperature, humidity, light quality or intensity.

The functional specialisation of the three *Arabidopsis GID1* genes may result in part from differences in their ability to regulate different DELLA proteins, given that the five *Arabidopsis* DELLA proteins have partly specialised functions. The strength of the DELLA-GID1 interaction was examined in the presence of GA_4 using both competitive yeast 3-hybrid and *in vitro* QCM (quartz crystal microbalance) assays for each of the three *Arabidopsis* GID1 proteins with each of the five DELLA proteins (Suzuki *et al.*, 2009). Since DELLAs RGA and GAI are the main repressors of stem elongation, we would expect them to interact more strongly with GID1a and GID1c, the main GA receptors regulating stem elongation. Instead, RGA and GAI exhibited the strongest preference for GID1b-binding. DELLA RGL2 and GID1a play strong roles in regulating seed germination. Consistent with this, RGL2 had the highest affinity for GID1a, followed by its affinity for GID1b. RGL1 and RGL3 had the strongest affinity for GID1a and the lowest affinity for GID1b. No DELLA protein had a strong preference for GID1c. Thus, the *Arabidopsis* DELLA proteins can be placed into two groups based on GID1 preference: (1) the RGA group with higher affinity for GID1b including GAI and RGA and (2) the RGL group with higher affinity for GID1a including RGL1, RGL2 and RGL3. While this grouping does not fully explain functional differences, it does coincide with the two phylogenetic groups based on overall DELLA amino acid sequence homology (Figure 6.3; Hirano *et al.*, 2007).

6.5 The structural requirements for gibberellin binding by GID1

The GID1 protein is a soluble GA receptor that localises to both the nucleus and the cytoplasm of rice and *Arabidopsis* cells (Ueguchi-Tanaka *et al.*, 2005; Willige *et al.*, 2007). Early work in barley showed that GA could be perceived both at the cell membrane and in the cytoplasm of barley aleurone cells (Hooley *et al.*, 1991; Gilroy and Jones 1994). While it has been postulated that both membrane-bound and cytosolic GA receptors may exist, no membrane-bound GA receptor has yet been identified (Nakajima *et al.*, 1997; Park *et al.*, 2005; Nakajima *et al.*, 2006). The nuclear localisation of GID1 is consistent with the fact that rice GID1 is the only GA receptor controlling DELLA-regulated gene expression in the nucleus (Yano *et al.*, 2015). GA hormone has also been shown to regulate calcium-dependent protein kinase function, calcium signalling and α-amylase secretion in the cytoplasm of barley aleurone cells (McCubbin *et al.*, 2004). It may be that GID1 also functions in cytoplasmic GA signalling, given that *Arabidopsis* GID1a fused to GFP and a nuclear exclusion signal was able to partially rescue germination and growth phenotypes of the *gid1a gid1c* double mutant (Livne and Weiss, 2014). Future work will need to better characterise GID1 function in cytoplasmic GA signalling.

GID1 is a homologue of the mammalian family of HSLs (hormone sensitive lipases) where the lipid-binding domain has become a GA hormone-binding domain (Østerlund, 2001; Ueguchi-Tanaka *et al.*, 2005; Nakajima *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2007a; Hirano *et al.*, 2008; Murase *et al.*, 2008). GID1 proteins lack hydrolase activity, likely because either Val or Ile replace the His amino acid residue of the Ser-His-Asp catalytic triad (Figures 6.2 and 6.5; Nakajima *et al.*, 2006). Instead, this site forms the binding core for bioactive GAs, including GA_1, GA_3, GA_4 and GA_7 (Murase *et al.*, 2008; Shimada *et al.*, 2008). Both rice and *Arabidopsis* GID1 proteins have the highest affinity for GA_4, although GA_1 is the predominant bioactive GA in vegetative tissues of monocots (Ueguchi-Tanaka *et al.*, 2005; Nakajima *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2007a). Bioactive GAs contain a γ-lactone ring between C-4 and C-10, a carboxyl group at C-6 and are hydroxylated at C-3. The Val/Ile residue of the catalytic triad plays a key role in GA binding through a non-polar interaction with the γ-lactone ring of bioactive GA molecules (Shimada *et al.*, 2008; Ueguchi-Tanaka and Matsuoka, 2010). The crystal structures of the rice OsGID1 (*Oryza sativa* GID1) and the *Arabidopsis* GID1a proteins bound to GA_4 as well as GA_3 have been solved (Murase *et al.*, 2008; Shimada *et al.*, 2008). GID1 resembles hormone-sensitive lipases in that it is composed of a C-terminal core with an N-terminal extension referred to as the lid (Figures 6.2 and 6.4). The core, also referred to as the α/β hydrolase fold domain, is composed of an α/β hydrolase fold surrounded by an eight-stranded β-sheet with α-helices packing the sides. The core contains the conserved HGG and GXSXG motifs characteristic of hormone-sensitive

lipases and other carboxylesterases (Figure 6.5). The catalytic triad within the GID1 core forms a GA-binding pocket. There are six water molecules at the bottom of the binding pocket that form a hydrogen-bonding network with the polar side of GA. GID1 has lower affinity for GA_3 and GA_1 because, unlike GA_4, these GAs contain a 13-hydroxyl group that is inserted close to a negatively charged Asp residue (Asp 243 in GID1a) in the binding pocket (Nakajima *et al.*, 2006; Murase *et al.*, 2008; Shimada *et al.*, 2008). The N-terminal extension of GID1 consists of a loop and three α-helices (αa, αb and αc) that form a flat lid domain that covers both GA and the GA-binding pocket (Figure 6.4). The hydrophobic side of GA interacts with the GID1-lid to induce a stable conformational change. Hydrophobic interactions between the GA molecule and the lid are likely involved in pulling the lid closed. The GA-stimulated folding of the N-terminal lid creates a binding domain for DELLA protein on the outer face of GID1 (Figure 6.1c). There are no direct interactions between GA and DELLA, such that the N-terminal lid serves as 'molecular glue' between the GA-binding core on one face and the DELLA protein on the other face (Murase *et al.*, 2008).

6.6 The structural requirements for the GID1-DELLA protein–protein interaction

The current model of GID1 binding proposes that in its unbound form the exposed surface of the open GID1 lid is hydrophilic, but undergoes a conformational change upon GA binding to expose hydrophobic DELLA-interacting residues (Figure 6.1c; Shimada *et al.*, 2008; Murase *et al.*, 2008; Ueguchi-Tanaka and Matsuoka, 2010). This model is based solely on the structure of the GA-bound form of GID1, since the structure of the unbound form has not been solved. Thus, it is not known whether or not the unbound form is stably 'open' (Hao *et al.*, 2013). GID1 in its GA-bound form has a number of hydrophobic amino acid side chains that protrude from the outer surface of the N-terminal lid, providing a binding domain for DELLA proteins. These hydrophobic residues are Leu-18, Trp-21, Leu- or Val-29, and Ile-33 in α-helix αb, and Leu-45 and Tyr-48 in α-helix αc of OsGID1 and GID1a (Figures 6.2 and 6.5; Shimada *et al.*, 2008; Murase *et al.*, 2008). Alanine scanning has shown that these hydrophobic residues in rice GID1 are required for protein–protein interaction with the DELLA SLR1, but not for GA binding (Shimada *et al.*, 2008). Moreover, the crystal structure of the *Arabidopsis* GID1a-GA-DELLA complex showed that these residues are the major sites of interactions between the closed lid of GID1a and DELLA GAI (Murase *et al.*, 2008). Hydrophobicity is a major force in protein folding, given that native protein structure in an aqueous environment generally does not allow exposure of hydrophobic side chains (Rose *et al.*, 1985; Dill, 1990; Huang *et al.*, 1995). GID1's GA-binding activity is stronger in the presence than in the absence of DELLA (Nakajima *et al.*, 2006; Ueguchi-Tanaka *et al.*,

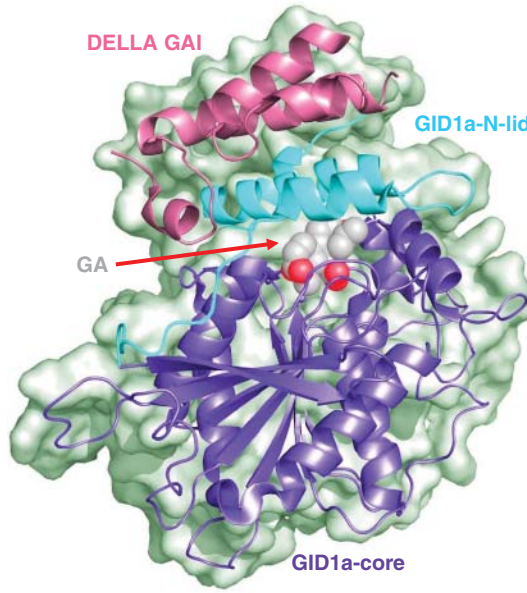
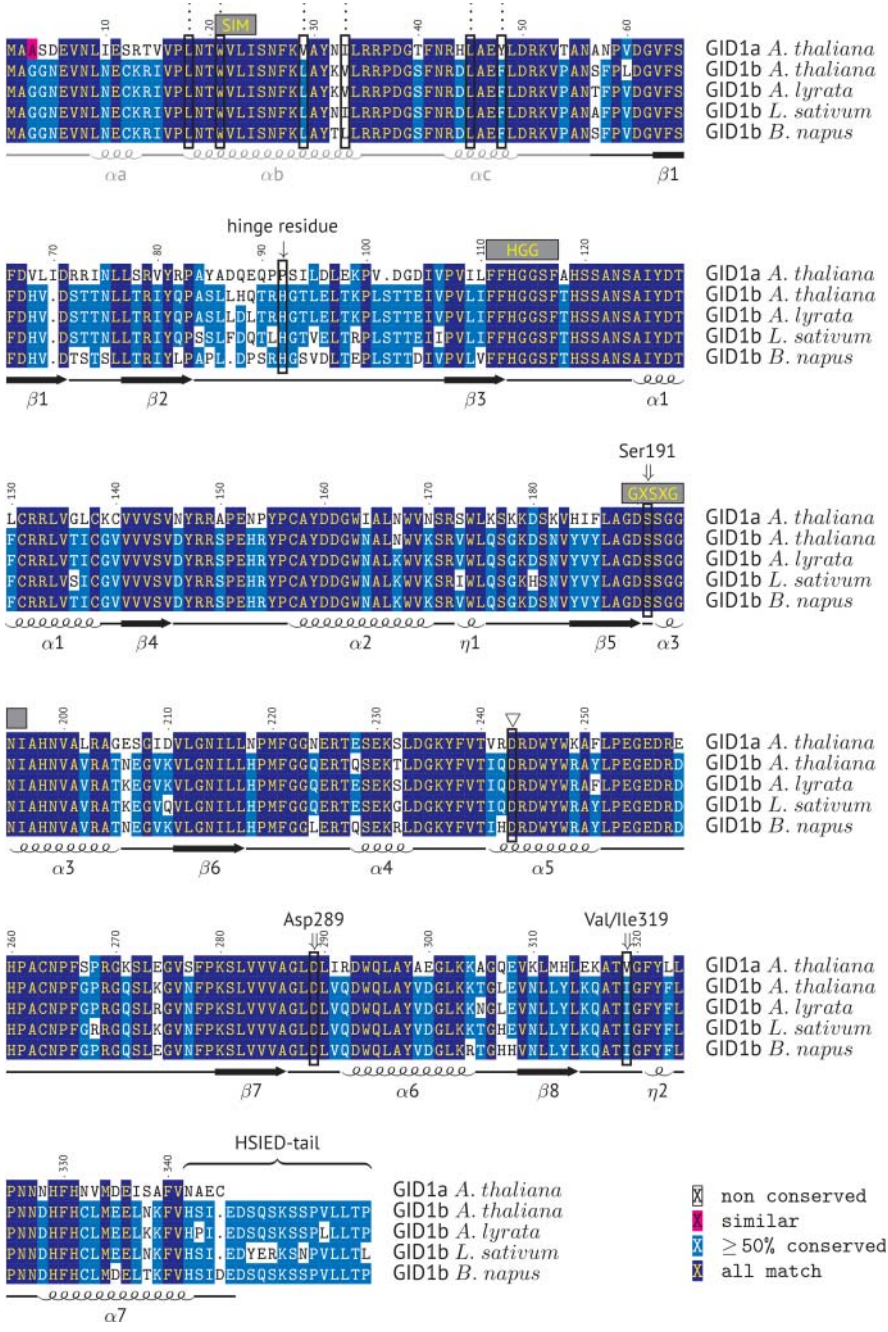


Figure 6.4 The GID1a-GA_3-DELLA complex based on the 1.8 angstrom crystal structure (Murase *et al.*, 2008). Ribbon representation of GID1a in complex with DELLA GAI and GA_3. The N-terminal GAI DELLA domain residues 11–113 (pink) is shown in complex with GID1a residues 1–344. The GID1a N-terminal extension or lid domain (GID1a-N-lid) is shown in blue and the GID1a α/β core domain in purple (GID1a-core). The GA_3 molecule (arrow) is shown in its binding pocket as a space-filling model where carbon is grey and oxygen red. (Figure was kindly provided by Toshio Hakoshima.) (See insert for colour representation of this figure.)

2007a). It may be that the presence of DELLA facilitates the exposure of the hydrophobic GID1 lid residues needed for stable DELLA protein binding.

Major DELLA protein motifs involved in the GID1-DELLA protein–protein interaction are located in the DELLA regulatory domain. The two neighbouring motifs, DELLA and LExLE (sometimes collectively referred to as the DELLA motif), are required for DELLA interaction with GID1 protein (Murase *et al.*, 2008). The crystal structure showed direct GID1 binding to the DELLA motif at the residues DeLLaΦLxYxV and MAxVAxxLExLExΦ, where capitalised residues represent sites of direct interactions, Φ represents a non-polar residue, and 'x' can represent any residue. Mutation analysis demonstrated that the DELLA motif is essential for GID1 binding, whereas mutations in the LExLE motif only resulted in decreased affinity for GA-bound GID1 (Figure 6.2: Peng *et al.*, 1997; Dill and Sun, 2001; Itoh *et al.*, 2002; Murase *et al.*, 2008). The VHYNP motif also plays a role in GID1-binding by stabilising the GID1-DELLA interaction via the residues TVhynPxxLxxWxxxM.


SIM
αa
αb
αc
β1
hinge residue
HGG
β1
β2
β3
α1
Ser191
GXSXG
α1
β4
α2
η1
β5
α3
α3
β6
α4
α5
Asp289
Val/Ile319
β7
α6
β8
η2
HSIED-tail
α7
GID1a A. thaliana
GID1b A. thaliana
GID1b A. lyrata
GID1b L. sativum
GID1b B. napus
non conserved
similar
≥ 50% conserved
all match

The GID1-GA-DELLA complex has a highly ordered structure, but investigation of DELLA in its unbound state revealed that the N-terminal region of DELLA proteins is intrinsically unstructured (Murase *et al.*, 2008; Sun *et al.*, 2010; Sheerin *et al.*, 2011). About 70% of signalling proteins are predicted to be intrinsically unstructured proteins (IUPs), containing long disordered regions believed to play a role in molecular recognition (Dunker *et al.*, 2000; Iakoucheva *et al.*, 2002; Oldfield *et al.*, 2005). Such IUPs can contain short regions of relative order within their unstructured regions called molecular recognition features (or MoRFs) (Oldfield *et al.*, 2005; Mohan *et al.*, 2006). MoRFs undergo a disorder-to-order conformational change upon recognition of their binding partner, and are designated as α, β or ι based on their preferred bound conformational state of α-helix, β-strand, or irregular structure, respectively (Fuxreiter *et al.*, 2004; Mohan *et al.*, 2006). Research suggested that the DELLA N-terminal region is almost entirely disordered, with the exception of the predicted α-MoRFs, DELLA/LExLE and L(K/R)XI, and the ι-MoRF VHYNP (Figure 6.2; Uversky 2002; Sun *et al.*, 2010). The C-terminal GRAS domain was predicted to be mostly ordered. The predicted MoRFs represent the key binding sites for the DELLA interaction with GID1, but for the small L(K/R)KI motif located within the poly S/T/V domain. It is possible that this motif is involved in DELLA binding with another component of the signalling pathway (Figure 6.2).

The ι-MoRF region in the VHYNP motif is an irregular loop (VHYNPSD loop) involved in binding-induced folding of the RGA, but not the RGL group of DELLAs (Figure 6.3; Sun *et al.*, 2010). The VHYNPSD loop of the RGA group, consisting of RGA, GAI and OsSLR1, undergoes a conformational change upon GID1 binding. The RGL group, consisting of RGL1, RGL2 and RGL3, does not appear to undergo a conformational change. One explanation for this difference is that the N-terminal DELLA domain of the RGA group

←

Figure 6.5 Predicted amino acid sequence alignment of *Arabidopsis thaliana* GID1a and GID1b with GID1b-type homologues from *Arabidopsis lyrata*, *Lepidum sativum* and *Brassica napus* showing GID1b-type specific regions of homology using ClustalΩ for alignment and TeXshade package in LaTeX (Beitz 2000; Sievers *et al.*, 2011). Amino acid residue numbers are based on GID1a. Significant residues (boxed) include the DELLA interacting residues in the lid (four dots mark these six residues), the 'hinge residue' in a loop of the lid that differs between GID1ac- and GID1b-type receptors, the catalytic triad involved in binding GA (Ser 191, Asp 289, and Val/Ile 319), and the negatively charged Asp 243 that likely reduces affinity for GA_1 and GA_3 compared to GA_4 (downward facing triangle). Significant motifs are the SUMO-Interaction-Motif (SIM), the HGG motif and the GXSXG motif (grey box). Predicted secondary structures are presented below the aligned sequences as a solid line (loop), spiral (α indicates α-helix, η indicates 3_{10}-helix) and block arrow (β-strand); where the lid containing α-helices αa, αb and αc is grey and the core domain is black. Some regions are conserved only among GID1b-type receptors, such as hinge residue and the C-terminal HSIED-tail (bracket). (See insert for colour representation of this figure.)

may be more structured and less flexible than the DELLA domain of the RGL group. Interestingly, *Arabidopsis* DELLAs can be divided into the same two groups based on amino acid sequence homology and preference for GID1a, GID1b or GID1c as a binding partner (Suzuki *et al.*, 2009). It is possible that the structural difference described above helps to determine the preference for GID1-binding partner.

Some amino acid residues in the GRAS functional domain also appear to participate in GID1 binding (Figure 6.2; Hirano *et al.*, 2010; Sato *et al.*, 2014). The semi-dominant mutation in the rice DELLA, $SLR1^{G576V}$, resulted in reduced GID1 binding in yeast 2-hybrid studies, suggesting that the SAW motif may participate in the GID1-DELLA protein–protein interaction (Hirano *et al.*, 2010). Alanine scanning also detected some decrease in GID1 binding due to changes in the VHIID motif. Another study demonstrated that the SLR1 GRAS domain bound to GID1 with much lower affinity than the DELLA domain, using pull-down assays, NMR spectroscopy and surface plasmon resonance (SPR) analysis (Sato *et al.*, 2014). This suggested that, under physiological conditions, GID1 binding to the GRAS domain likely occurs after GID1 binding to the DELLA domain. Based on mutation analysis, Gly-576 of the SAW motif appears to be a key residue in the GRAS-GID1 interaction. A rice homologue of DELLA SLR1, SLRL1 (SLR1-Like1), was able to function like SLR1 to repress growth when over-expressed (Itoh *et al.*, 2005b). SLRL1 lacks a DELLA domain, but was apparently able to bind to GID1 via the GRAS domain alone. Because it has no DELLA domain, SLRL1 did not undergo GA-stimulated protein destruction. Future work will need to examine whether non-DELLA GRAS proteins can function via protein–protein interaction with GID1. GID1 binding results in a C-terminal conformational change in DELLA that likely promotes binding to the SLEEPY1 (SLY1) F-box protein via the VHIID and LHR2 motifs (Sasaki *et al.*, 2003; Murase *et al.*, 2008; Shimada *et al.*, 2008).

6.7 The DELLA destruction model: negative regulation of DELLA repressors by SLY1/GID2 and the ubiquitin-proteasome pathway

GA signalling down-regulates DELLA repressors of GA responses by targeting them for destruction via the ubiquitin-proteasome pathway (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006; Ariizumi *et al.*, 2008; Wang *et al.*, 2009). The 'DELLA destruction model' for GA signalling originated with the observation that GA rescue of GA biosynthesis mutants was associated with the rapid disappearance of the DELLA protein RGA (Figure 6.1a, b; Silverstone *et al.*, 2001). All of the DELLA proteins of *Arabidopsis* and other plants characterised thus far degrade as quickly as 5 to 60 minutes after GA treatment (Itoh *et al.*, 2002; Fu *et al.*, 2002; 2004; Tyler *et al.*, 2004; Ariizumi and Steber, 2007; Wang

et al., 2009; Zhang *et al.*, 2010). Thus, it is widely accepted that GA lifts DELLA repression of seed germination, stem elongation, and flowering and fertility via DELLA protein proteolysis.

DELLA is ubiquitinated and targeted for destruction by an SCF (Skp1, Cullin, F-box) E3 ubiquitin ligase (Figure 6.1b; Sasaki *et al.*, 2003; McGinnis *et al.*, 2003; Dill *et al.*, 2004; Gomi *et al.*, 2004; Fu *et al.*, 2004; Hussain *et al.*, 2005; Ariizumi *et al.*, 2011). The *Arabidopsis* SLY1 and rice GID2 proteins are the F-box sub-units of the SCF complex that specifically binds to DELLA proteins, leading to their polyubiquitination. Mutations in the F-box genes *Arabidopsis SLY1* (*SLEEPY1*) and rice *GID2* (*GA-INSENSITIVE DWARF2*) block GA-induced DELLA proteolysis, leading to GA-insensitive phenotypes, including dwarfism, infertility and increased seed dormancy in *sly1* (Steber *et al.*, 1998; Sasaki *et al.*, 2003; McGinnis *et al.*, 2003). Thus, DELLA over-accumulation is associated with decreased GA signalling (Figure 6.1d).

Protein ubiquitination occurs via a multi-stage process that concludes with the covalent linkage of the 76-amino-acid ubiquitin peptide to the target protein (reviewed by Smalle and Vierstra, 2004; Wang and Deng, 2011). The E1 ubiquitin activating enzyme catalyses the formation of a thio-ester bond between the C-terminal glycine of ubiquitin and an E1 cysteine residue. The activated ubiquitin is transferred to a cysteine residue of the ubiquitin conjugating enzyme E2 by transesterification. The E2 ubiquitin conjugating enzyme transfers ubiquitin to a lysine residue on the target protein. An E3 ubiquitin ligase like $SCF^{SLY1/GID2}$ can catalyse the transfer of ubiquitin to a specific target by bringing the E2 and the target protein together in a single complex. Addition of a polyubiquitin chain containing four ubiquitin moieties targets a protein for destruction by the 26S proteasome. In a cell-free system, DELLA was polyubiquitinated predominantly by a ubiquitin chain with Lys-29 linkages between ubiquitin moieties, rather than the usual Lys48 linkages (Wang *et al.*, 2009). The Lys residue(s) modified by ubiquitination of DELLA have not yet been identified and may be a good avenue for future investigation.

As shown in Figure 6.1b, the SCF E3 ubiquitin ligase of GA signalling is apparently comprised of: (1) the SLY1/GID2 F-box protein that binds the DELLA target at its C-terminus and binds an ASK (*Arabidopsis* SKP1 homologue) via the F-box motif, (2) the ASK protein that binds CUL1 (CULLIN1), (3) CUL1, the backbone of the complex that binds ASK at its N-terminus and an RBX1 (RING BOX1 protein) at its C-terminus and (4) an RBX1 homologue that binds an E2 ubiquitin conjugating enzyme (Figure 6.1b; Gagne *et al.*, 2002; Gray *et al.*, 2002; Risseeuw *et al.*, 2003; Fu *et al.*, 2004; Wang *et al.*, 2009; Ariizumi *et al.*, 2011). *Arabidopsis* SLY1 and rice GID2 are small proteins of 151 and 212 amino acids, respectively, that show 36.8% amino acid identity and 56% similarity (Sasaki *et al.*, 2003; McGinnis *et al.*, 2003; Itoh *et al.*, 2003). They contain an F-box motif and a C-terminal domain required for interaction with DELLA proteins (Figure 6.2; Dill *et al.*, 2004; Fu *et al.*, 2004; Hirano *et al.*, 2010). The F-box motif, conserved in yeast, mammals and

plants, binds to SKP1 homologues, allowing SCF complex formation (Schulman *et al.*, 2000). SLY1 and GID2 both interact with SKP1 homologues in yeast 2-hybrid assays (Gagne *et al.*, 2002; Sasaki *et al.*, 2003; Fu *et al.*, 2004). SLY1 coimmunoprecipitation with CUL1 depends on the presence of an intact F-box motif, indicating that SLY1 forms an SCF complex *in planta* via the F-box motif (Ariizumi *et al.*, 2011). The conserved GGF and LSL amino acid motifs in the SLY1/GID2 C-terminus are required for interaction with the DELLA protein VHIID and LHR2 motifs (Figure 6.2; Hirano *et al.*, 2010; Ariizumi *et al.*, 2011). Rice *GID2* mutants carrying a 19- or 31-bp deletion in the F-box motif resulted in a GA-insensitive phenotype associated with dwarfism, complete infertility and failure to induce the GA-induced enzyme α-amylase during seed germination (Sasaki *et al.*, 2003). *Arabidopsis sly1* mutants also result in dwarfism associated with partial, rather than complete infertility (Steber *et al.*, 1998; Steber and McCourt, 2001). The *sly1* mutants also show increased seed dormancy, consistent with the role of GA signalling in seed germination (Ariizumi and Steber, 2007). Thus, *SLY1* and *GID2* are required for normal GA responses as well as for DELLA protein destruction.

The formation of the GID1-GA-DELLA complex is the signal that causes $SCF^{SLY1/GID2}$ to polyubiquitinate DELLA, thereby targeting DELLA for destruction by the 26S proteasome (Figure 6.1a, b). While some interaction was initially detected between SLY1 and DELLA protein by yeast 2-hybrid, later work showed the SLY1/GID2 affinity for DELLA is greatly enhanced when DELLA is in the GID1-GA-DELLA complex (Sasaki *et al.*, 2003; Fu *et al.*, 2004; Griffiths *et al.*, 2006; Willige *et al.*, 2007; Ariizumi *et al*. 2011; Hirano *et al.*, 2010). Thus, both the SLY1-DELLA protein–protein interaction and DELLA destruction are stimulated by GA hormone perception. As demonstrated *in vivo* and in cell-free extracts, both *GID1* and *SLY1* are necessary for efficient DELLA proteolysis in response to GA (Sasaki *et al.*, 2003; McGinnis *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005; Willige *et al.*, 2007; Wang *et al.*, 2009). It is widely accepted that the 26S proteasome is responsible for DELLA proteolysis because GA-stimulated DELLA destruction is blocked by 26S proteasome inhibitors, leading to accumulation of ubiquitinated DELLA protein (Fu *et al.*, 2002; Sasaki *et al.*, 2003; Hussain *et al.*, 2005; Wang *et al.*, 2009).

Originally, it was assumed that the *sly1* loss-of-function mutants have a GA-insensitive phenotype because there is only one copy of the *SLY1* gene in *Arabidopsis*. However, the *sly1* mutant phenotypes are not as severe as those of the GA biosynthesis mutant *ga1-3* or the *gid1a gid1b gid1c* triple mutant. A possible explanation for this was a predicted homologue of *SLY*1 in *Arabidopsis* with 23.7% amino acid identity (Itoh *et al.*, 2003). A screen for genes that suppress *sly1* phenotypes when over-expressed on the 35S promoter identified this *SLY1* homologue, referred to as *SNE* (*SNEEZY*) or as *SLY2* in *Arabidopsis* (Fu *et al.*, 2004; Strader *et al.*, 2004). *SNE* over-expression partly rescues *sly1* mutations and results in decreased DELLA protein levels, suggesting that the SNE F-box protein can functionally replace SLY1. An HA:SNE fusion protein coimmunoprecipitated with DELLA RGA, but not with RGL2,

whereas HA:SLY1 coimmunoprecipitated with both RGA and RGL2 (Ariizumi *et al.*, 2011). Thus, it appears that SNE can only down-regulate a sub-set of DELLA repressors. If SNE normally functions in GA signalling, then *sne* mutants should show *sly1*-like GA-insensitive phenotypes. However, T-DNA insertion alleles of *SNE* showed no phenotype (Ariizumi and Steber, 2011). Moreover, the *sly1 sne* double mutant only showed a slight increase in seed dormancy and slight decrease in plant height, suggesting that loss of *SNE* does not eliminate GA signalling in *sly1* mutants. Thus, SLY1 is the major F-box protein directing DELLA degradation. Future work will need to determine whether there are environmental conditions under which the SNE F-box protein plays a stronger role in GA signalling, and determine whether SNE regulates proteins other than DELLA repressors. For example, *SLY1* and *SNE* are expressed in different root cells types, and the *sne-1* mutant exhibited a shortened root phenotype under dry conditions (Cui and Benfey, 2009). This suggests that *SNE* may have a unique function in stimulating root growth.

6.8 Regulation of DELLA by phosphorylation and *O*-GlcNAc modification

The discovery that DELLA proteins can be phosphorylated followed fast on the heels of the discovery that DELLAs are regulated by the ubiquitin-proteasome pathway (Sasaki *et al.*, 2003; Fu *et al.*, 2004). However, the functional significance of DELLA phosphorylation has been elusive. Many proteins regulated by the ubiquitin-proteasome pathway are ubiquitinated and targeted for destruction in response to phosphorylation (reviewed by Willems *et al.*, 1999; Nguyen *et al.*, 2013). For example, phosphorylation of yeast CYCLIN2 stimulates the interaction of the cyclin with the F-box protein GRR1 (GLUCOSE REPRESSION-RESISTANT1), leading to cyclin ubiquitination and destruction by the 26S proteasome. Thus, early models of GA signalling hypothesised that DELLA phosphorylation was the signal for DELLA ubiquitination and destruction (Sasaki *et al.*, 2003; Gomi *et al.*, 2004; Fu *et al.*, 2004). In this model DELLA phosphorylation would stimulate SLY1/GID2 binding to DELLA, thereby targeting DELLA for GA-stimulated destruction. This model was disproved when it was found that the DELLA SLR1 phosphorylation detected in the TVHYNP and poly S/T/V motifs was GA-independent, and that both the phosphorylated and unphosphorylated forms of DELLA SLR1 interacted with the F-box GID2 (Itoh *et al.*, 2005a). Subsequent research proved that the interaction of the F-box protein with DELLA depended, not upon DELLA phosphorylation, but on the formation of the DELLA-GA-GID1 complex (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006; Nakajima *et al.*, 2006). Moreover, protein phosphatase inhibitors appeared to block degradation of barley DELLA SLN1 and *Arabidopsis* DELLAs RGA and RGL2 (Fu *et al.*, 2002; Hussain *et al.*, 2005; Wang *et al.*,

2009). This would suggest that DELLA phosphorylation stabilises, rather than targets DELLA protein for destruction.

Results of genetic studies of the rice Ser/Thr casein kinase I *EL1* (*EARLY FLOWERING1*) are consistent with the idea that phosphorylation positively regulates DELLA repression of GA signalling (Figure 6.6a; Dai and Xue, 2010). The *el1* loss-of-function mutant flowered early and enhanced GA-mediated DELLA degradation. The *el1* mutant has other phenotypes consistent with increased GA sensitivity, including a small increase in stem elongation and an ABA-insensitive increase in α-amylase expression during seed germination. Over-expression of *EL1* resulted in dwarfism. Thus, *EL1* behaves like a negative regulator of GA signalling. Several lines of evidence suggest that EL1 regulates DELLA SLR1 by phosphorylation: (1) EL1 can phosphorylate DELLA SLR1 *in vitro*, (2) loss of the predicted SLR1 phosphorylation sites, in S196A and S510A mutants, leads to reduced accumulation of DELLA activated transcripts and (3) phosphomimic mutations, S196D and S510D, lead to increased accumulation of DELLA-activated transcripts. Moreover, the rice *el1* mutation suppresses the dwarf phenotype associated with SLR1 over-expression, indicating that EL1 is directly or indirectly required for DELLA SLR1 repression of stem elongation. If *EL1* acts primarily through DELLA SLR1 phosphorylation *in planta*, then this suggests that DELLA repressors are positively regulated by EL1-mediated phosphorylation (Figure 6.6a). This is consistent with phosphatase inhibitor studies suggesting that phosphorylation stabilises DELLA repressors in *Arabidopsis* and barley (Fu *et al.*, 2002; Hussain *et al.*, 2005; Wang *et al.*, 2009). Based on an amino acid alignment, the SLR1 Ser-510 residue is conserved in *Arabidopsis* DELLAs RGA, GAI and RGL1, corresponding to Ser-417 in GAI (Figure 6.2). The DELLAs RGL2 and RGL3 have an Ala residue in place of the Ser. The SLR1 Ser-196 residue does not appear to be conserved in *Arabidopsis* DELLAs. Future work will need to examine whether EL1-mediated DELLA phosphorylation occurs *in planta*, is conserved in other plant species and whether there is a direct connection between *el1* phenotypes and DELLA phosphorylation state. It will also be interesting to learn what effects DELLA phosophorylation at Ser-196 in the poly S/T/V motif and/or at Ser-510 in the PFYRE motif may have on DELLA function and protein–protein interactions. This is the first phenotypic evidence suggesting that phosphorylation may stabilise DELLA protein and promote DELLA repression of GA signalling.

Investigation of the *Arabidopsis* protein phosphatase, TOPP4 (TYPE ONE PROTEIN PHOSPHATASE4), provided further evidence that phosphorylation may positively regulate and dephosphorylation negatively regulate DELLA repression of GA signalling (Qin *et al.*, 2014). The *Arabidopsis* TOPP family contains nine members implicated in regulation of plant growth and development (Smith and Walker, 1993; Lin *et al.*, 1998). The dominant negative *topp4-1* mutation results in GA-insensitive phenotypes, including dwarfism, poor fertility, delayed flowering and failure to induce GA-responsive gene expression (Qin *et al.*, 2014). The *topp4-1* phenotypes

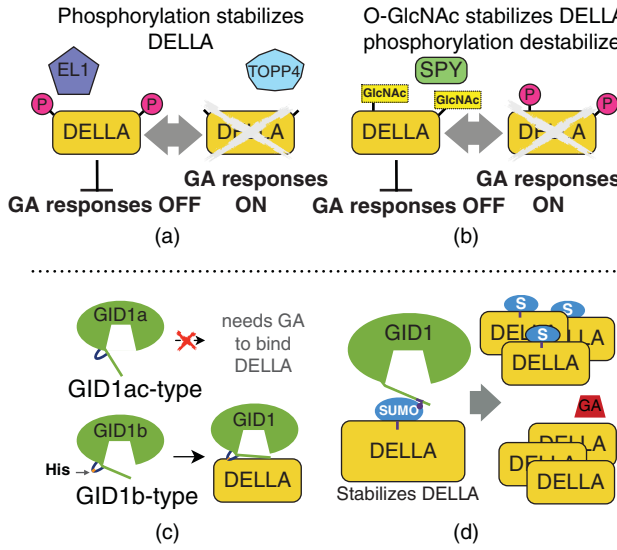


Figure 6.6 Alternative models for DELLA regulation. (a) The EL1/TOPP4 model. EL1-mediated phosphorylation of DELLA proteins stabilises DELLA protein, thereby increasing DELLA repression of GA responses. TOPP4-mediated dephosphorylation destabilises DELLA, thereby stimulating GA responses. (b) The SPY model. Phosphorylation *destabilises* DELLA thereby lifting DELLA repression of GA responses (opposite of EL1 model). SPY directs *O*-GlcNAc modification of DELLA at the same Ser/Thr residues subject to phosphorylation, leading to DELLA stabilisation and repression of GA responses. The effect of phosphorylation on DELLA activity may differ based on the location of the phosphorylation site or changes in binding partners. (c) The GID1b-type lid model. The GID1ac-type receptors can only bind DELLA when GA stimulates lid closure. The GID1b-type hinge has His 91 in place of the Pro in GID1ac-type receptors. This residue causes the lid domain to be partly closed without GA-binding leading to a low level of GA-independent DELLA binding and GA signalling. (d) The SUMO model. DELLA is SUMOylated at a residue directly before the LExLE motif involved in GID1 binding. SUMOylation of DELLA allows GA-independent binding to the SIM domain in the GID1 lid. GID1-binding by SUMOylated DELLA sequesters GID1 away from non-SUMOylated DELLA, thereby preventing DELLA ubiquitination and destruction, resulting in a build-up of both SUMOylated and non-SUMOylated forms of DELLA.

were associated with increased DELLA accumulation and delayed DELLA degradation following GA application. *In vitro* phosphatase assays suggested that wild-type TOPP4, but not topp4-1 protein, can dephosphorylate DELLAs GAI and RGA. Thus, it appears that *TOPP4* is a positive regulator of GA signalling that may negatively regulate DELLA by dephosphorylation. GA treatment stimulated the accumulation of *TOPP4* mRNA and protein. Thus, the proposed model is: (1) phosphorylation by EL1 or other kinases stabilises DELLA proteins, thereby repressing GA responses and (2) GA stimulates TOPP4 accumulation thereby destabilising DELLA via dephosphorylation and proteasomal degradation, thus stimulating GA responses (Figure 6.6a).

Future work will need to establish the *in vivo* effects of DELLA dephosphorylation, examine whether TOPP4 function as a negative regulator of DELLA repressors is conserved in other species, and determine the TOPP4 dephosphorylated amino acid residues in DELLA and whether they correspond to EL1-phosphorylated residues.

Contrary to the EL1/TOPP4 model, evidence from studies of the *O*-GlcNAc transferase, *SPY* (*SPINDLY*) suggest that DELLA phosphorylation can negatively regulate GA signalling (Figure 6.6b). Phosphorylation and *O*-GlcNAc (*O*-linked *N*-acetylglucosamine) modification may compete for modification of serine or threonine residues on DELLA proteins (Shimada *et al.*, 2006; Silverstone *et al.*, 2007). This would be analogous to the competition between phosphorylation and *O*-GlcNAc modification observed in mammals, where *O*-GlcNAc transferases have been found in complex with phosphatases (Wells *et al.*, 2004). The *Arabidopsis* putative *O*-GlcNAc transferase, *SPY* (*SPINDLY*), was identified in genetic screens for increased GA signalling based on the ability to germinate in the presence of a GA biosynthesis inhibitor and suppression of the *ga1-3* biosynthesis mutant (Jacobsen and Olszewski, 1993; Silverstone *et al.*, 1997b). Direct protein–protein interaction between SPY and DELLA has not been observed and specific sites of modification have yet to be proposed. *SPY* is defined as a negative regulator of GA signalling in *Arabidopsis*, barley and rice because loss-of-function results in increased GA signalling associated with increased stem elongation and lack of seed dormancy (Robertson *et al.*, 1998; Swain *et al.*, 2001; Shimada *et al.*, 2006; Filardo *et al.*, 2009). Silencing of rice *SPY* suppresses the GA-insensitive dwarfism of the *gid1* GA receptor and the *gid2* F-box mutants without any change in DELLA protein levels, suggesting that the increase in GA signalling in *spy* requires neither the GA receptor nor DELLA destruction (Shimada *et al.*, 2006). The *Arabidopsis spy* mutant also suppresses the GA-insensitive dwarfism of DELLA gain-of-function mutation *rga*-Δ*17* (Silverstone *et al.*, 2007). The *spy* suppression of *rga*-Δ*17* and *spy* silencing in rice was associated with an apparent increase in DELLA phosphorylation. Thus, the current model is that SPY activates the DELLA repressor via *O*-GlcNAc modification and that increased phosphorylation in *spy* mutants *inactivates* the DELLA repressor leading to increased GA signalling (Figure 6.6b). This disagrees with the *EL1/TOPP4* model in which phosphorylation *activates* DELLA repression of GA signalling. Thus, the role of phosphorylation in controlling DELLA protein function may be more complex than turning DELLA repression on or off. Future work will need to examine whether DELLA phosphorylation at different Ser or Thr residues has different functions, serving either to stimulate or block DELLA repression. One important consideration for future investigations will be to clearly ascertain whether changes in DELLA electrophoretic mobility are due to phosphorylation, *O*-GlcNAc modification or the newly discovered SUMOylation of DELLA protein (Conti *et al.*, 2014).

6.9 Evidence for gibberellin-independent DELLA regulation

Arabidopsis GID1b and some GID1b-type homologues such as soybean GID1b-2 have the ability to interact with DELLA proteins to some degree even in the absence of GA, suggesting that GID1b-type receptors may serve to 'prime the pump' of GA signalling under conditions when GA levels are low (Figure 6.6c; Griffiths *et al.*, 2006; Nakajima *et al*. 2006; Yamamoto *et al.*, 2010). While GA binding stimulates the interaction of GID1b with DELLA, the fact that GID1b can bind DELLA in the absence of GA suggests that GID1b-type receptors can initiate GA-independent GA signalling via DELLA destruction. However, no one has yet demonstrated that a GA-independent GID1b-DELLA protein interaction can stimulate interaction of DELLA with SLY1 leading to DELLA proteolysis. Sequence alignment between *Arabidopsis* GID1 protein sequences shows 85% amino acid identity between GID1a and GID1c, but only 66% and 67% identity of GID1a with GID1b and of GID1c with GID1b, respectively (Figure 6.5). Based on amino acid homology, higher plant GID1 homologues can be divided into two groups: GID1ac-type and GID1b-type receptors (Yamamoto *et al.*, 2010; Voegele *et al.*, 2011). Like the GID1ac-type receptors, monocot GID1 proteins such as OsGID1 show only GA-dependent interaction with DELLA proteins.

Mutation analysis of rice GID1 provided clues to the structural basis for the GA-independent GID1b-DELLA protein–protein interaction, and led to a model to explain this interaction (Yamamoto *et al.*, 2010). A missense mutation causing a P99S amino acid substitution in a loop between the N-terminal lid domain and the body of rice GID1 (in the hinge of the lid) resulted in a GID1b-mimic phenotype, allowing $OsGID1^{P99S}$ to bind DELLA in the absence of GA and suppressing the GA-insensitive phenotype of the *gid1-8* loss-of-function mutation. The same Pro residue is present in the loop region of *Arabidopsis* GID1a at Pro-92 and GID1c at Pro-91, but is replaced by His-91 in GID1b (Figure 6.2 and 6.5). Site-directed mutation analysis showed that P99I, P99V and P99A amino acid substitutions in OsGID1 resulted in GA-independent DELLA-binding, and that a H91P substitution in *Arabidopsis* GID1b reduced DELLA-binding in the absence of GA. In the model proposed, *Arabidopsis* GID1a Pro-92 (OsGID1 Pro-99) is needed to prevent DELLA-binding when GA is not present to 'pull the lid closed' on the GID1 receptor. In this model, having His instead of Pro in the GID1b loop/hinge region causes the GID1b lid domain to remain partly closed, allowing the lid to bind DELLA in the absence of GA (Figure 6.5 and 6.6c). Note that the partially closed lid does not bind DELLA as well as a fully closed lid, so that GA binding greatly increases the affinity of GID1b-type receptors for DELLA. Interestingly, GID1b homologues in *Brassica* and soybean also showed GA-independent DELLA-binding activity, suggesting that multiple plant species have evolved GA-independent DELLA signalling.

In addition to having greater affinity for DELLA in the absence of GA, GID1b-type receptors also have higher affinity for GA. *Arabidopsis* GID1b

has higher affinity for GA_4 (K_d = 4.8 x 10^{-7} M) than either GID1a or GID1c (K_d = *ca.* 2 x 10^{-6} M) (Nakajima *et al.*, 2006). Kinetic studies revealed that GID1b association with GA_4 occurs at about the same rate as GID1a, but that GA_4 dissociation from GID1b is about 17 times slower than from GID1a (Yamamoto *et al.*, 2010). The P99A amino acid substitution in OsGID1 resulted in GA_4 binding kinetics that more closely resembled those of GID1b. GID1b also shows optimal function over a narrower range of pH conditions (optimal pH 6.8) than GID1a or GID1c, which exhibited a consistently high level of binding activity between pH 6.4 and pH 8.3 (Nakajima *et al.*, 2006). Thus, sequence differences between GID1b- and GID1ac-type GA receptors may impact multiple functions. Basal GA signalling by *Arabidopsis* GID1b may explain why the *ga1-3* mutant that produces little or no GA hormone is not as extremely dwarfed as the *gid1a gid1b gid1c* triple mutant (Griffiths *et al.*, 2006).

Comparison of the *Arabidopsis* GID1b predicted amino acid sequence with three other GID1b-type receptors from other eudicot species, *Arabidopsis lyrata*, *Lepidum sativum* and *Brassica napus*, indicated that there is a higher degree of homology between predicted GID1b-type receptors of these four species than between GID1a and GID1b of *Arabidopsis* (Figure 6.5). There are many additional regions with conserved predicted amino acid sequence, in addition to the conserved His91 in the GID1b hinge. For example, there is a region with high homology at the C-terminal end of the four GID1b-type receptors, where the *Arabidopsis* GID1b amino acid sequence is HSIEDSQSKSSPVLLTP. Predicting GID1b structure based on the crystal structure of GID1a, it is possible that this C-terminal HSIED-tail motif of GID1b-type receptors might be oriented such that it could play a role in lid closure or GA binding (Murase *et al.*, 2008; Shimada *et al.*, 2008). Thus, future work will need to examine whether additional amino acid regions participate in the unique properties of GID1b-type receptors. Taken together, this information suggests that the GID1b-type receptors of eudicot plant species may have evolved for a unique and as yet undefined purpose in plant growth and development.

Recent evidence in *Arabidopsis* has suggested another method of GA-independent signalling through an increase in DELLA repression due to SUMO (Small Ubiquitin-like Modifier) modification of DELLAs (Conti *et al.*, 2014). Like ubiquitin, SUMO is a short peptide that can be covalently linked to a protein sequence (reviewed by Vierstra, 2012). SUMOylation of DELLA RGA protein was found within the DELLA regulatory domain at a conserved lysine residue (Lys-65 in RGA, Lys-49 of GAI) immediately before the LExLE motif involved in GID1 binding (Figure 6.2; Murase *et al.*, 2008; Conti *et al.*, 2014). GID1a protein was shown to bind SUMOylated DELLA RGA in the absence of GA via a SUMO-Interaction-Motif (SIM) with the sequence WVLI (residues 21–24 in GAI) (Figure 6.2 and 6.5). This SIM domain includes the Trp-21 residue in the GID1 lid known to directly interact with DELLA protein. A double mutant in the SUMO proteases, *OTS1* and *OTS2* (*OVERLY TOLERANT TO SALT1* and *2*), resulted in increased

accumulation of both SUMOylated and non-SUMOylated forms of DELLA RGA and GAI protein associated with shorter roots, which was further enhanced under salt stress. This short root phenotype was suppressed by a *rga* mutation, suggesting that this phenotype resulted from DELLA RGA repression of root growth. Interestingly, *ots1 ots2* exhibited early flowering, which was enhanced by an *rga* mutation, suggesting that both DELLA and OTS negatively regulate the transition to flowering. RGA was shown to be deSUMOylated by OTS1 *in vitro*, suggesting that DELLAs are direct targets of OTS1. The short root phenotype and enhanced response to the GA biosynthesis inhibitor paclobutrazol during seed germination suggested that the *ots1 ots2* mutant results in decreased GA sensitivity. However, *ots1 ots2* showed no significant change in endogenous GA levels suggesting that these phenotypes result from altered signalling. *OTS1* over-expression suppressed the dwarfism of the partially GA deficient *ga1-5* mutant and resulted in decreased DELLA protein accumulation. Thus, it appears that lack of DELLA deSUMOylation results in increased DELLA repression of plant growth, and increased DELLA deSUMOylation results in decreased DELLA repression of plant growth. The proposed model is that SUMOylation of DELLA, such as in response to salt stress, results in a GA-independent interaction of SUMOylated-DELLA with GID1 protein (Figure 6.6d). The GID1 interaction with SUMOylated-DELLA reduces the amount of GID1 available for GA-dependent interaction with non-SUMOylated DELLA, leading to decreased DELLA ubiquitination/destruction and increased DELLA repressor protein levels. Higher DELLA accumulation under high salt represses root growth, presumably preventing damage due to salt stress. Further validation of this model will require experiments to examine whether SUMOylated-DELLA does one of the following: blocks GA-binding by GID1, blocks the GA-dependent GID1-DELLA protein–protein interaction, or blocks SLY1-binding to DELLA. Future work will need to determine whether SUMOylation of DELLA protein occurs in other plant species or in response to other forms of environmental stress. Increased DELLA protein accumulation and repression of plant growth has been observed in response to stress hormones ABA and ethylene, and in response to environmental stresses including salt, cold and submergence (Achard *et al.*, 2003; 2006; 2008; Fukao and Bailey-Serres, 2008). The notion that stress-induced DELLA SUMOylation leads to increased DELLA repression of plant growth offers an attractive model to explain these observations.

6.10 Evidence for gibberellin signalling without DELLA destruction

The GA receptor GID1 can transmit the GA hormone signal without DELLA proteolysis, referred to as 'non-proteolytic GA signalling'. Based on the DELLA destruction model, the level of GA signalling should

negatively correlate with the level of DELLA repressor protein accumulation (Figure 6.1a, b). In other words, mutants with higher DELLA protein levels should be shorter than mutants with lower DELLA protein levels. Paradoxically, the F-box mutants, *Arabidopsis sly1* and rice *gid2*, accumulate higher levels of DELLA protein than GA biosynthesis mutants or *GID1* null lines, but exhibit less severe GA-insensitive phenotypes (McGinnis *et al.*, 2003; Willige *et al.*, 2007; Ariizumi *et al.*, 2008; Ueguchi-Tanaka *et al.*, 2008). For example, the *Arabidopsis ga1-3* biosynthesis mutant and the *gid1a gid1b gid1c* triple mutants cannot germinate unaided, are severely dwarfed and are completely infertile. The *sly1-2* mutant has dormant seeds that eventually after-ripen, is a semi-dwarf and is only partly infertile. Thus, the *sly1* and *gid2* mutants appear capable of a low level of GA signalling. In fact, these mutants are not completely GA-insensitive, since GA treatment resulted in some increase in stem elongation (Ariizumi *et al.*, 2008; Ueguchi-Tanaka *et al.*, 2008).

The non-proteolytic GA signalling in *sly1* and *gid2* depends on GA and GID1 (Ariizumi *et al.*, 2008; Ueguchi-Tanaka *et al.*, 2008; Ariizumi *et al.*, 2013). For example, the *ga1-3 sly1-10* double mutant is more strongly dwarfed and infertile, and accumulates less DELLA protein than the *sly1-10* single mutant. Moreover, *gid1* mutations exacerbated the GA-insensitive phenotypes of *sly1* and *gid2*, while at the same time reducing DELLA accumulation. Based on the DELLA destruction model, reduced DELLA accumulation should be associated with decreased rather than increased severity of GA-insensitive phenotypes. These results indicate that GA and GID1 are needed both for non-proteolytic GA signalling and for the high level of DELLA protein accumulation observed in *sly1* and *gid2* mutants.

It appears that GID1 can mediate GA signalling without DELLA destruction. *GID1* over-expression partly rescued the GA-insensitive mutant phenotypes of *sly1* and *gid2* mutants without causing a decrease in DELLA protein levels (Ariizumi *et al.*, 2008; Ueguchi-Tanaka *et al.*, 2008; Ariizumi *et al.*, 2013). Thus, GID1 and GA can down-regulate DELLA repressors in F-box mutants that cannot destroy DELLA repressors via the 26S proteasome. Moreover, rescue by *GID1* over-expression was blocked by deletion of the DELLA motif required for GID1-DELLA interaction, suggesting that GID1-GA-DELLA complex formation is required. Higher levels of HA:GID1 protein expression were associated with increased coimmunoprecipation of DELLA and with better rescue of seed germination and stem elongation in *Arabidopsis sly1* (Ariizumi *et al.*, 2013). Thus, the proposed model is that formation of the GID1-GA-DELLA complex decreases the ability of DELLA to repress GA responses leading to increased GA response without DELLA destruction (Figure 6.1d). A final proof of this model would require a direct assay for DELLA function, so that the notion that GID1-GA-DELLA complex formation results in decreased DELLA function could be tested directly. Non-proteolytic DELLA down-regulation is not exclusive to *sly1/gid2* mutants, because loss of *SPY* resulted in increased GA signalling without any apparent decrease in DELLA protein accumulation (Shimada *et al.*,

2006). Thus, future work should examine whether SPY-directed *O*-GlcNAc modification or other DELLA post-translational modifications play a role in non-proteolytic GA signalling. Such work will need to examine whether non-proteolytic GA signalling is important under environmental conditions that reduce DELLA destruction, such as drought and salt stress (Achard *et al.*, 2003; 2006; 2008).

Genetic analysis suggests that the relative roles of the *GID1a*, *GID1b* and *GID1c* genes in non-proteolytic GA signalling in *sly1-2* mutants differed somewhat from their roles in proteolytic GA signalling in the wild-type *SLY1* background (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Ariizumi *et al.*, 2008; 2013; Hauvermale *et al.*, 2014). While *gid1c-2* has an apparently stronger effect on seed germination during proteolytic GA signalling, *gid1a-1* had a stronger effect than *gid1c-2* in the *sly1-2* mutant, interfering with the ability of *sly1-2* seeds to lose dormancy through a long (20 month) period of dry after-ripening (Voegele *et al.*, 2011; Ariizumi *et al.*, 2013). The *sly1-2 gid1b-1* double mutant seed also failed to germinate. *GID1a* appeared to play the strongest role in controlling plant height in both proteolytic and non-proteolytic GA signalling (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Ariizumi *et al.*, 2013; Hauvermale *et al.*, 2014). Whereas *GID1c* had the strongest secondary effect on plant height in proteolytic GA signalling, *GID1b* had the strongest secondary effect in non-proteolytic GA signalling. For fertility, *GID1a* had the primary and *GID1b* the secondary role in proteolytic GA signalling, whereas *GID1b* had the primary and *GID1a* the secondary role in non-proteolytic GA signalling. The *sly1-2 gid1b-1* double mutant had a much stronger infertility phenotype than *sly1-2 gid1a-1* or *sly1-2 gid1c-1*, indicating that *GID1b* plays the major role in stimulating fertility during non-proteolytic GA signalling.

6.11 Concluding remarks

Our understanding of the mechanisms of GA signalling has come a long way since the first mutations in *Arabidopsis* GA biosynthesis genes were identified in 1980 (Figure 6.7; Koornneef and van der Veen, 1980). Genetic studies in rice and in *Arabidopsis* have identified components of and elucidated mechanisms in the GA signalling pathway (Koornneef *et al.*, 1985; Silverstone *et al.*, 1997b; Steber *et al.*, 1998; Steber and McCourt 2001; Griffiths *et al.*, 2006; Nakajima *et al.*, 2006; Willige *et al.*, 2007; Iuchi *et al.*, 2007). The canonical DELLA destruction model was based on: (1) the observation that DELLA repressors disappear after GA treatment and (2) the identification of the SLY1 and GID2 F-box proteins as major positive regulators of GA signalling (Silverstone *et al.*, 2001; Itoh *et al.*, 2002; Sasaki *et al.*, 2003; McGinnis *et al.*, 2003; Gomi *et al.*, 2004; Fu *et al.*, 2004). The cloning of the GA receptor GID1 led to an understanding of how GID1-GA-DELLA complex formation stimulates DELLA destruction (Ueguchi-Tanaka *et al.*, 2005). Biochemical

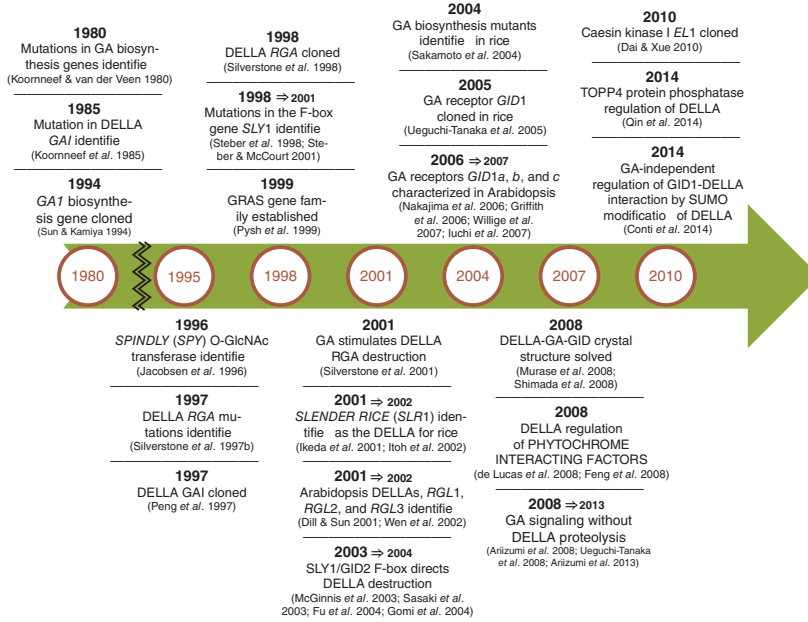


Figure 6.7 A timeline of significant advances in the understanding of GA signalling from 1980 to the present day.

studies and the crystal structure of the OsGID1-GA and GID1a-GA-DELLA has provided a clear understanding of the amino acid motifs involved in this complex interaction (Murase *et al.*, 2008; Shimada *et al.*, 2008). Recent studies have investigated DELLA-targets, post-translational modification of DELLA proteins, and alternative mechanisms of GA signalling in the absence of DELLA-proteolysis or without GID1-GA interaction (de Lucas *et al.*, 2008; Feng *et al.*, 2008; Ariizumi *et al.*, 2008; Ueguchi-Tanaka *et al.*, 2008; Dai and Xue, 2010; Ariizumi *et al.*, 2013; Conti *et al.*, 2014). The new knowledge gained has raised as many interesting new questions as it has answered. Thus, this chapter should be viewed as a starting point rather than as the finished story of GA signalling.

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