Chapter 7

DELLA PROTEINS: MASTER REGULATORS OF GIBBERELLIN-RESPONSIVE GROWTH AND DEVELOPMENT

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Abstract: In flowering plants, DELLA proteins (DELLAs) have emerged as the master transcriptional regulators that are responsible for repressing all aspects of gibberellin (GA)-dependent growth and development. Although we now have a good understanding of how GAs relieve the growth repression exerted by DELLAs, our understanding of the mechanisms through which these proteins act has only started to emerge in the last few years. It has become apparent that DELLAs physically associate with a myriad of proteins, including transcription factors, altering their activities and promoting the transcriptional changes that control GA-responsive growth and development. Fascinatingly, the role of DELLAs does not appear to revolve exclusively around transcriptional control, with a recent report demonstrating that they are directly involved in the reorientation of microtubules in the *Arabidopsis* hypocotyl. These recent observations add a further dimension to a remarkable class of regulatory proteins.

Keywords: DELLA proteins, GA, GAMYB, gibberellin, GRAS proteins, hub proteins, microtubule orientation, protein–protein interactions, transcription factors, transcriptional regulation

7.1 Introduction

Since the discovery that gibberellins (GAs) act as phytohormones controlling diverse aspects of plant growth and development there have been considerable efforts to understand the underlying molecular mechanisms. However, the multitude of cellular processes that are regulated by this phytohormone presented a potential problem for these early investigations, with the likelihood that they are controlled by different mechanisms. The discovery that GAs are synthesized and secreted from the germinating cereal grain embryo and then act specifically on the aleurone cells, to trigger the secretion of hydrolytic enzymes that mobilise endosperm reserves required for seedling establishment, provided an important early experimental system for these studies (reviewed in Sun and Gubler, 2004). These studies have provided some important insights into the GA-mediated transcriptional regulation of genes encoding hydrolytic enzymes, most notably the demonstration that the transcription factor (TF) GAMYB plays a central role in this process. Where the aleurone system has failed to shed further light is in the identification of upstream GA signalling components and the mechanisms through which they regulate these transcriptional responses. It was the use of molecular genetics approaches using the model plant *Arabidopsis* that eventually provided the keys that would unlock this field of research. A pioneering screen by Marten Koornneef and colleagues led to the identification of the *gibberellin-insensitive* (*gai*; later renamed *gai-1*) mutant, which exhibited a classical GA-related dwarf phenotype, but which was insensitive to exogenous application of GA (Koornneef *et al.*, 1985). This phenotype suggested that this dominant gain-of-function mutation affected a gene involved in the GA signal transduction pathway. Several years later, a screen performed by Silverstone and colleagues led to the identification of *rga* (for repressor of *ga1-3*) mutant alleles which rescued the severe phenotypic defects of an *Arabidopsis* GA-deficient mutant, *ga1-3* (Silverstone *et al.*, 1997). Genetic analysis and the subsequent cloning of the *GAI* and *RGA* genes demonstrated that they encoded homologous proteins that are negative regulators of GA signalling (now commonly known as DELLA proteins or DELLAs), acting as potential transcriptional regulators (Peng *et al.*, 1997; Silverstone *et al.*, 1997; Silverstone *et al.*, 1998). Importantly, bioactive GAs were later shown to relieve the repression exerted by *RGA* through its targeted degradation (Silverstone *et al.*, 2001; reviewed in Chapter 6). In this chapter, we discuss these and other early studies that were influential in identifying and characterising the role of DELLAs in regulating GA-responsive growth. However, the main focus of this review is to discuss extensive recent work that has led to an understanding of the mechanisms through which DELLAs control a myriad of physiological processes.

7.2 DELLAs regulate downstream gibberellin signalling

More than 50 years ago, it was proposed that GAs promote plant growth and development by acting as ‘inhibitors of inhibitors’ (Brian, 1957). This hypothesis was based on the observation that the garden pea (*Pisum sativum* L.) mutant *la cry* displays a GA overdose (slender) phenotype that is not dependent on the levels of GAs in the plant (Figure 7.1; Brian, 1957; Potts *et al.*, 1985; Weston *et al.*, 2008). Essentially, this model proposed by Brian (1957) predicted that GAs act to overcome the growth inhibition exerted by the *LA* and *CRY* gene products. The identification of mutants in other plant species that displayed a similar slender phenotype supported a common mechanism for the control of growth by GAs (Foster, 1977; Peng *et al.*, 1997; Silverstone *et al.*, 1997). Whilst the mutations that underlie the slender growth habit are recessive in nature, another class of mutations resulting in a contrasting dwarf phenotype was also identified in several plant species, including maize (Phinney, 1956), wheat (Gale and Marshall, 1973), *Arabidopsis* (Koornneef *et al.*, 1985), barley (Chandler *et al.*, 2002) and rice (Asano *et al.*, 2009). These mutations are partially dominant and give rise to a

Figure 7.1 Shoot and root phenotypes of pea genotypes. The *la cry-s* alleles completely suppress the phenotype of the GA-deficient mutant *na-1*. (Weston *et al.* (2008). Reproduced with permission from American Society of Plant Biologists.)

GA-insensitive dwarf phenotype. It was the *Arabidopsis* mutant belonging to this class, *gai-1*, which led to further supporting evidence for the hypothesis proposed by Brian (1957). Using an elegant genetic approach, Peng and colleagues (1997) demonstrated that transposon-mediated disruption at the *GAI* locus reverted *gai-1* to a phenotype that was almost identical to wild-type plants. Importantly, it also resulted in partially GA-independent growth based on a resistance to the GA biosynthesis inhibitor, paclobutrazol. These observations indicated that *GAI* encodes a GA-inactivated repressor (similar to *LA* and *CRY*) and that the gain-of-function *gai-1* mutation acts to prevent this process, leading to constitutive repression of GA growth responses (Peng *et al.*, 1997; Harberd *et al.*, 1998). Notably, this work led to the cloning of the *GAI* gene, allowing its further functional and genetic analysis. A homologous *Arabidopsis* gene, *RGA*, was then identified as encoding another negative regulator of GA signalling in an elegant screen and cloning strategy employed by Silverstone and colleagues (1997; 1998).

The *GAI* and *RGA* proteins belong to the GRAS family of putative transcriptional regulators (named after the founding members: *GAI*, *RGA* and *SCR*), which in *Arabidopsis* includes 32 members (Pysh *et al.*, 1999; Engstrom, 2011). This family of proteins is characterised by a highly conserved C-terminal GRAS domain that is important for functionality. In the cases of *RGA* and *GAI* they also possess two highly conserved N-terminal domains named after amino acid residues found within them: the DELLA and TVHYNP domains. The DELLA domain has now been adopted as a name for this group of repressors of GA signalling (Wen and Chang, 2002). A potential role for *RGA* and *GAI* in regulating transcriptional responses that control GA-responsive growth was supported by the presence of bipartite nuclear localisation signals in the predicted protein sequences and the subsequent demonstration that a GFP-*RGA* fusion protein was targeted to the nucleus in transgenic plants (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Silverstone *et al.*, 2001). Furthermore, epistasis studies have established the *DELLA* genes as encoding downstream components within the GA signalling cascade (McGinnis *et al.*, 2003; Griffiths *et al.*, 2006). The identification of these genes in *Arabidopsis* quickly led to the cloning of *DELLA* orthologues in other plant species (Peng *et al.*, 1999; Ogawa *et al.*, 2000). In rice, barley and tomato *DELLA* loss-of-function alleles were demonstrated to exhibit a classical GA-insensitive slender phenotype (Ikeda *et al.*, 2001; Chandler *et al.*, 2002; Jasinski *et al.*, 2008). This highlighted a conserved role in repressing GA-responsive growth in monocots and dicots. Importantly, the *LA* and *CRY* genes in the garden pea were also found to encode *DELLA* proteins, identifying them as the growth inhibitors that Brian originally proposed are inhibited by the action of GA (Brian, 1957; Weston *et al.*, 2008).

7.3 Gibberellins relieve DELLA-growth repression by targeting their degradation

The cloning of the *DELLA* genes did not immediately provide any clear insights into how GAs inactivate these growth repressors. However, some clues were provided by the observations that the partially dominant GA-insensitive dwarfing mutations in *Arabidopsis*, wheat, maize, barley and rice affected residues within the N-terminal DELLA and TVHYNP domains (Peng *et al.*, 1997; Peng *et al.*, 1999; Chandler *et al.*, 2002; Asano *et al.*, 2009). The phenotype of these plants suggested that these mutations result in constitutively active DELLA growth repressors that cannot be inactivated by GAs and also highlighted the importance of these domains in controlling this process. The breakthrough in understanding how GAs inactivate the DELLAs was provided by the demonstration that a GFP-RGA fusion protein was rapidly degraded *in planta* following treatment with bioactive GAs (Silverstone *et al.*, 2001). The endogenous DELLA proteins in *Arabidopsis* and other plant species were found to respond in a similar fashion, thus demonstrating that targeted degradation of DELLAs is a common mechanism through which GA relieves growth repression exerted by these proteins (Silverstone *et al.*, 2001; Chandler *et al.*, 2002; Itoh *et al.*, 2002; Dill *et al.*, 2004). In the case of gain-of-function DELLA mutations responsible for conferring GA-insensitivity, it was found that their effect is to abolish or reduce GA-mediated degradation of the encoded proteins (Dill *et al.*, 2001; Chandler *et al.*, 2002; Dill *et al.*, 2004; Asano *et al.*, 2009). This demonstrated that the GA-insensitive phenotype was caused by the presence of mutant DELLAs that constitutively repressed GA responses because they are unable to be inactivated through GA-mediated degradation. It also highlighted the importance of the DELLA and TVHYNP domains in controlling this regulatory process. It is now known that GA-mediated degradation is achieved through the action of a soluble GA receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1), which is localised within the nucleus and cytoplasm (Ueguchi-Tanaka *et al.*, 2005; reviewed in Chapter 6). The binding of bioactive GAs to GID1 promotes the formation of a GA-GID1-DELLA complex, which ultimately results in the targeted degradation of DELLA via an ubiquitin-26S proteasome pathway (see Chapter 6). In a seminal study, Murase and colleagues (2008) resolved the crystal structure of the ternary complex of the *Arabidopsis* GA receptor GID1A bound to bioactive GA and the N-terminus of GAI, thereby defining the DELLA domains that were required for binding to the GID1-GA complex. It highlighted the importance of the DELLA, LEXLE and TVHYNP domains within which conserved residues are in direct contact with GID1. Mutations within these conserved domains cause GA-insensitive dwarfism because they prevent binding of

DELLAs to the GID1-GA complex, thereby blocking GA-mediated degradation and causing constitutive growth repression (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Asano *et al.*, 2009; Pearce *et al.*, 2011). See also Chapter 6 for a more detailed description of the processes controlling GA-mediated DELLA degradation.

7.4 Functional diversification of *DELLA* genes

A major difference between the GA signalling components in monocots and dicots is the level of genetic redundancy. In monocots, including rice and barley, genetic evidence and genome sequence data supports the existence of a single *DELLA* gene in these plants (Ikeda *et al.*, 2001; Chandler *et al.*, 2002). The rice and barley *DELLA* loss-of-function mutants, *slr1* and *sln1*, respectively, display extremely slender phenotypes that mimic constitutively active GA signalling (Foster, 1977; Ikeda *et al.*, 2001; Chandler *et al.*, 2002). These defects are consistent with SLR1 and SLN1 acting to repress all aspects of GA-responsive growth in these cereals. However, interrogation of the rice genome sequence has identified two *SLR1-like* genes encoding SLRL1 and SLRL2 that have high homology to the GRAS domain of SLR1, but which lack the N-terminal DELLA and TVHYNP domains required for GID1-mediated degradation (Itoh *et al.*, 2005). Interestingly, the existence of SLRL-type GRAS proteins appears to be restricted to monocots. When over-expressed in transgenic plants, SLRL1 was able to suppress the slender phenotype of the loss-of-function *slr1* mutant, supporting its role in controlling GA-responsive growth. As SLRL1 lacks the GID1 recognition domains, this raises the prospect that it is able to repress GA-responsive growth in the presence of bioactive GAs. In fact, a recent study of submergence-tolerant rice has implicated SLRL1 and SLR1 in repressing GA-dependent stem elongation following submergence (Fukao and Bailey-Serres, 2008). To confirm this role for SLRL1 it will be important to identify and characterise the submergence response in an *slr1* loss-of-function mutant.

In contrast to rice and barley, the genome of the model dicot *Arabidopsis* contains five *DELLA* genes: *RGA*, *GAI*, *RGA-LIKE1* (*RGL1*), *RGL2* and *RGL3* (Dill and Sun, 2001; Tyler *et al.*, 2004). A phylogenetic analysis of *DELLA* proteins from evolutionarily diverse flowering plants supports their expansion occurring after the monocot-dicot split (Figure 7.2; Dill and Sun, 2001; Gallego-Bartolome *et al.*, 2010). Although two main clades have been identified in dicots, species within the Asterids genera only possess DELLAs belonging to one of these. For example, genetic analysis and interrogation of genome sequence data supports the existence of a single *DELLA* gene in *Solanum lycopersicum* (Jasinski *et al.*, 2008; Fleishon *et al.*, 2011). In contrast, species within the Rosids possess DELLAs that group within both of the two main clades (Gallego-Bartolome *et al.*, 2010). It has therefore been suggested

Figure 7.2 Phylogenetic history of *DELLA* genes. The image depicts the phylogenetic relationships between the multiple *DELLA* genes arisen from successive duplications in dicotyledoneous plants. (Adapted from Gallego-Bartolomé *et al.* 2010. Reproduced with permission from Oxford University Press.) *At*, *Arabidopsis thaliana*; *Bn*, *Brassica napus*; *Gb*, *Gossypium barbadense*; *Ls*, *Lactuca sativa*; *Mh*, *Malus hupehensis*; *Mt*, *Medicago truncatula*; *Os*, *Oryza sativa*; *Ps*, *Pisum sativum*; *Pt*, *Populus trichocarpa*; *Pv*, *Phaseolus vulgaris*; *Sl*, *Solanum lycopersicum*; *Zm*, *Zea mays*.

that this diversification in DELLAs occurred through the duplication of an ancestral gene within the Rosids. Further duplication events have occurred in the Brassicaceae family, which have led to the presence of the five paralogues in *Arabidopsis*. The order of these duplication events is supported by the presence of these genes within syntenic regions of the *Arabidopsis* genome (Dill and Sun, 2001; Gallego-Bartolomé *et al.*, 2010).

Extensive studies to establish the roles of the five *Arabidopsis* *DELLA* genes in controlling GA-responsive growth have been performed (Silverstone *et al.*, 1997; Dill and Sun, 2001; King *et al.*, 2001; Lee *et al.*, 2002; Wen and Chang, 2002; Cheng *et al.*, 2004; Tyler *et al.*, 2004; Wild *et al.*, 2012). In addition to confirming that all of them encode repressors of GA signalling, these studies have demonstrated that they have both distinct and overlapping roles in controlling *Arabidopsis* growth (a more detailed description of these roles controlling seed germination, vegetative and reproductive development and how they

were elucidated are provided in Chapters 9, 10 and 11). These roles have been elucidated through the genetic analysis of DELLA loss-of-function mutations and their effect on suppressing the growth defects of a GA-deficient mutant, *gal-3*, coupled with spatial and temporal expression analysis. These studies have demonstrated that *RGA* and *GAI* have partially overlapping roles in controlling root elongation, leaf expansion, initiation of flowering, stem elongation and floral development (Silverstone *et al.*, 1997; Dill and Sun, 2001; King *et al.*, 2001; Fu and Harberd, 2003; Cheng *et al.*, 2004; Tyler *et al.*, 2004; Cao *et al.*, 2006). However, it appears that *RGA* has a dominant role in the majority of these growth processes. In contrast, *RGL2* is responsible for repressing germination under certain conditions, and also has a role in controlling floral development (Lee *et al.*, 2002; Cheng *et al.*, 2004; Tyler *et al.*, 2004). It is interesting to note that differences in the function of DELLA paralogs are apparent between the widely used Landsberg *erecta* and Columbia-0 ecotypes, specifically in relation to floral development (Tyler *et al.*, 2004; Plackett *et al.*, 2014). These observations highlight the need for care when comparing observations between these two ecotypes.

While the functional roles of *RGA*, *GAI*, *RGL1* and *RGL2* in controlling *Arabidopsis* development have been uncovered relatively easily by genetic analysis, the importance of *RGL3* has proven more difficult to establish. Although there was some evidence that *RGL3* has a minor role in repressing germination (Piskurewicz and Lopez-Molina, 2009), the major breakthrough in our understanding was uncovered by Wild and colleagues (2012). By interrogating publically available microarray data, they established that *RGL3* is an early target of jasmonate (JA) signalling that is induced by this pathway. The impact of elevated *RGL3* levels is to enhance the output of JA signalling through a direct repression of the JASMONATE ZIM-DOMAIN (JAZ) JA response repressors (the mechanisms involved are discussed in more detail in Section 7.7.3; see also Chapter 8). To establish the biological significance of this response, Wild and colleagues (2012) investigated the role of *RGL3* in the JA-dependent resistance to the necrotrophic fungus *Botrytis cinerea*. Consistent with a role in modulating JA signalling, the absence of *RGL3* was found to enhance susceptibility to *Botrytis cinerea*. Furthermore, they also established that JA-mediated susceptibility to the hemibiotroph *Pseudomonas syringae* was reduced in the absence of *RGL3*. Because *RGL3* is degraded in response to GA signalling (Feng *et al.*, 2008; Wild *et al.*, 2012), the status of GAs within the plant will determine the modulation of the JA response. This suggests that the transcriptional regulation of *RGL3* by JA signalling provides a mechanism to integrate growth status of the plant with its ability to respond to pathogens. It is interesting to note that *RGL3* is also regulated by other abiotic stresses, including cold and drought (Achard *et al.*, 2008; Colebrook *et al.*, 2014), suggesting that this is a conserved mechanism for controlling growth of the plant in response to multiple environmental factors.

The presence of multiple *DELLA* genes with functionally divergent roles in controlling GA-responsive growth in dicots raises the important

question as to how this functional specificity is conferred. Characterisation of the tissue-specific expression patterns of the five *DELLA* paralogues in *Arabidopsis* has highlighted clear differences that potentially underlie their functional specificity (Tyler *et al.*, 2004), although this study could not rule out other factors, such as differential activities regulating downstream targets. This question was addressed by Gallego-Bartolomé and colleagues (2010) who used promoter-swapping experiments to investigate whether *RGA* and *RGL2* could complement each other's function when expressed in the respective *rgl2* or *rga* loss-of-function mutant background. These two DELLAs were most suitable for this study because they control distinct developmental processes that could be easily monitored (*RGA* and *RGL2* control vegetative growth and seed germination, respectively). Characterisation of the transgenic lines that were generated in this study demonstrated that *RGA* and *RGL2* were in fact capable of functional complementation. These findings have led to the conclusion that the functional diversification of *Arabidopsis* *DELLA* genes can result from differences in their spatial and temporal expression patterns. In view of the importance of GA signalling in controlling plant growth in response to changes in environmental conditions, it is tempting to speculate that the expansion and functional diversification of *DELLA* genes in some plant species provides a fitness benefit by allowing fine tuning of environmental responses.

7.5 *DELLA* activity invokes rapid changes in the transcriptome

Genomic approaches have shown that GAs induce rapid changes in the transcriptome, likely mediated by *DELLA* activity (Ogawa *et al.*, 2003; Cao *et al.*, 2006; Willige *et al.*, 2007). In agreement with the idea that DELLAs are transcriptional regulators, *GAI* and *RGA* are able to alter gene expression very rapidly (Zentella *et al.*, 2007; Gallego-Bartolomé *et al.*, 2011a). In these two studies, the *Arabidopsis* transcriptome was interrogated in lines expressing the dominant versions *rga*- Δ 17 and *gai*-1 under the control of inducible promoters, and microarray analyses identified 475 and 148 genes whose expression was altered at least 1.5-fold within the first four hours after the induction, respectively. Consistently, more genes were up- than down-regulated in response to *DELLA* activity, whereas the opposite situation is observed following treatment with GA (Willige *et al.*, 2007). From a mechanistic point of view, it is important to note that both DELLAs were able to affect gene expression in a direct manner, as expected for bona fide transcriptional regulators. This was supported by two lines of evidence. First, chromatin immunoprecipitation (ChIP) analyses showed that *in vivo* *RGA* associates with the promoters of some of its target genes (Zentella *et al.*, 2007). This study defined a core of 14 *bona fide* *RGA*- and GA-responsive

genes, of which RGA was associated with the promoters of a sub-set of eight genes, which were all DELLA up-regulated. Nevertheless, RGA also associates with the promoters of genes that are down-regulated by DELLAs (Park *et al.*, 2013), consistent with the finding that the expression of other RGA targets is reduced in response to RGA activity (Zentella *et al.*, 2007). This was a remarkable result, since it indicated that DELLAs can act as *cis*-acting transcriptional regulators on target genes. The lack of a recognizable DNA binding motif within DELLAs suggests that they bind chromatin through an interaction with other *trans*-acting factors (see below). Second, *gai-1* was able to regulate the transcription of target genes in the absence of protein synthesis (Gallego-Bartolomé *et al.*, 2011a, b, c). This was demonstrated by using a transgenic line that expresses a translational fusion between *gai-1* and the receptor domain of the rat glucocorticoid receptor (GR) enabling the chimeric *gai-1*-GR to move from the cytosol, where it accumulates, to the nucleus after treatment with the synthetic steroid, dexamethasone (Gallego-Bartolomé *et al.*, 2011c). This technology allows the activation of *gai-1*-GR, i.e. relocation into the nucleus, without the need for protein synthesis. Thus, the combination of dexamethasone and cycloheximide treatments demonstrated protein-synthesis-independent changes in gene expression for many GAI targets, indicating that GAI can directly regulate gene expression. The regulation of direct targets is compatible with DELLAs associating directly with the promoter of their target genes, but does not exclude alternative mechanisms, such as sequestration of TFs (see below). *In silico* analysis of the promoter regions of DELLA-regulated genes identified several *cis* elements that were over-represented (Gallego-Bartolomé *et al.*, 2011a). Some of these elements provided important clues about the classes of TFs that might bind the promoter of these genes, mediating regulation by DELLAs (see below). For instance, the presence of G (CACGTG) and E (CACATG) boxes, the CARG-like, and the AAAG and NGATT sequences suggests that bHLHs, bZIPs, MADSs, Dofs and type-b response regulators, respectively, are likely involved in regulating these genes (see below).

7.6 DELLA proteins activate transcription

Soon after their discovery, it was apparent that DELLAs were able to activate transcription. This was first demonstrated for the rice DELLA SLR1 in trans-activation assays performed in spinach leaves (Ogawa *et al.*, 2000). SLR1 activated transcription of a reporter gene when fused to the DNA-binding domain of the yeast GAL4 TF. Deletion analyses identified the N-terminal DELLA domain of SLR1 as responsible for this transcriptional activation ability. A more comprehensive study showed that both the DELLA and TVHYNP motifs residing in the N-terminal region are important for this activity (Hirano *et al.*, 2012). Several loss-of-function mutations in DELLA

proteins reside in the GRAS domain, which, however, was dispensable for transactivation activity. The introduction of these mutations in a chimeric protein lacking the N-terminal region, but fused to the strong transcriptional activator VP16 lowered its ability to exert the repressive function in transgenic rice plants, suggesting that the GRAS domain, despite not being necessary for transactivation, is required to manifest the full DELLA activity *in planta* (Hirano *et al.*, 2012). It is becoming apparent that this activity of the GRAS domain is most likely mediated through its interaction with TFs that direct DELLAs to specific target promoters (Fukazawa *et al.*, 2014; Yoshida *et al.*, 2014; see below). Based on the association between the growth phenotype and transcriptional activity of *SLR1* deletion alleles, it appears that DELLAs control plant growth at least partly through transcriptional activation (Hirano *et al.*, 2012). The transcriptional activation ability is manifested in heterologous systems as well, such as yeast (Hirano *et al.*, 2012), indicating that DELLAs might interact with conserved elements of the basal transcriptional machinery. This ability was inhibited upon interaction with GID1, both in rice callus and in yeast (Hirano *et al.*, 2012), which is consistent with the capacity of the receptor to inactivate the DELLA protein through a physical association (Ariizumi *et al.*, 2008; Ueguchi-Tanaka *et al.*, 2008). It is somewhat surprising that no reduced or loss-of-function DELLA mutations affecting residues within the N-terminal transactivation domains have been identified. However, it is possible that the impact of such mutations could be masked by them also affecting GA-mediated degradation of DELLA. The identification of additional components involved in DELLA-mediated transactivation should shed further light on the mechanisms responsible.

7.7 DELLAs regulate transcription by physical interaction with transcriptional regulators

The lines of evidence presented in the previous sections strongly suggest that DELLAs exert their activity, at least in part, by modulating the transcriptional rate of their target genes. In brief: (1) there are motifs in their primary structure typical of transcriptional regulators, although none of them resemble known DNA-binding domains (Gubler *et al.*, 2002), (2) DELLAs possess an intrinsic transcriptional activation ability (Ogawa *et al.*, 2000; Hirano *et al.*, 2012), (3) they are able to rapidly alter transcription of target genes (Zentella *et al.*, 2007; Gallego-Bartolomé *et al.*, 2011a) and (4) they associate with several target gene promoters (Zentella *et al.*, 2007; Lim *et al.*, 2013; Park *et al.*, 2013; Fukazawa *et al.*, 2014; Yamaguchi *et al.*, 2014). Nonetheless, an outstanding question still remained to be answered: how do DELLAs regulate transcription? The key to the answer came from the identification of DELLA protein partners, as demonstrated by pioneering studies from Salome Prat's and Xing-Wang Deng's laboratories (de Lucas *et al.*, 2008; Feng *et al.*, 2008).

Figure 7.3 Molecular mechanisms of DELLA activity. (a) DELLA proteins prevent the interaction between a TF and its target DNA sequence. (b) DELLA proteins facilitate the activity of a TF by releasing negative regulation imposed by a repressor. (c) DELLA proteins promote transcriptional activation of a TF at the target loci.

Subsequently, several classes of TFs and transcriptional regulators have been identified as bona fide DELLA interactors, mainly in *Arabidopsis* (see below). Remarkably, the elucidation of the molecular events that are a consequence of these interactions has defined several different mechanisms by which DELLAs regulate transcription (Figure 7.3). In addition and equally important, the identification of DELLA interactors provides us with the possibility to answer, from a mechanistic point of view, two other relevant questions in the field: (1) how GAs regulate so many different processes during the life cycle of the plant and (2) how the GA pathway interacts with other signalling pathways to exert this regulation.

7.7.1 DELLAs sequester *bona fide* TFs by physical interaction

The first TFs identified as DELLA interactors were PHYTOCHROME INTERACTING FACTORS (PIFs), PIF3 (Feng *et al.*, 2008) and PIF4 (de Lucas *et al.*, 2008). These TFs belong to the bHLH superfamily (Toledo-Ortiz *et al.*, 2003) and were formerly identified as growth-promoting, negative regulators of phytochrome-regulated responses (Huq and Quail, 2002; Kim *et al.*, 2003). Importantly, it was shown by ChIP and transcriptional assays that the ability of these TFs to bind to the promoter of several of their target genes was inhibited upon interaction with the DELLA protein (de Lucas *et al.*, 2008; Feng *et al.*, 2008). Thus, a series of biochemical assays unambiguously showed that DELLAs inhibit PIF3 and PIF4 upon interaction with the conserved bHLH DNA-binding domain, providing the first functional evidence of a mechanism by which DELLAs regulate transcription, i.e. by sequestering TFs (Figure 7.3a).

The next important question was to establish the physiological relevance of these interactions. These authors demonstrated that the interactions are important for regulating hypocotyl elongation in response to two antagonistic signals, GAs and light (de Lucas *et al.*, 2008; Feng *et al.*, 2008). More specifically, light represses hypocotyl growth by negatively acting on PIF3 and PIF4 by two means, on the one hand it induces their rapid phosphorylation and degradation by the 26S proteasome upon interaction with photoactivated phytochromes (Al-Sady *et al.*, 2006; de Lucas *et al.*, 2008), and on the other, it enhances accumulation of DELLAs (Achard *et al.*, 2007) that can interact with and inactivate the remaining pool of PIF proteins. Thus, the identification of the DELLA-PIF3/4 interaction not only provided the first mechanistic understanding of how DELLAs regulate transcription, but also a molecular mechanism for the interaction between GAs and light in controlling photomorphogenic growth in plants.

The activity of these and other related bHLH TFs (mainly PIF1 and PIF5) is required for the correct progression of skotomorphogenesis that takes place just after germination in darkness (Leivar *et al.*, 2008; Shin *et al.*, 2009). The basic features of the skotomorphogenic growth are that hypocotyls grow fast and form an apical hook to protect the shoot apical meristem and cotyledons, which remain small and folded. In addition, the gene expression programmes associated with photomorphogenesis remain off. Accordingly, this developmental program also requires low DELLA levels in order to progress (Alabadí *et al.*, 2004; Achard *et al.*, 2007). Despite their levels being low, DELLAs inactivate PIF5 by physical interaction, and this is relevant to the control of ethylene production and auxin transport during apical hook development (Gallego-Bartolomé *et al.*, 2011b; Willige *et al.*, 2012). Similarly, low DELLA levels seem to be important to guarantee the activity of the PIFs controlling hypocotyl elongation and cotyledon opening (Alabadí *et al.*, 2008), and also to regulate the expression of many genes involved in photosynthesis before and during the greening process triggered upon illumination, either directly, such as *CONDITIONAL CHLORINA (CHLH)*, *NADPH:PROTOCHLOROPHYLLIDE OXIDOREDUCTASE C (PORC)* and *CHLOROPHYLL A OXYGENASE (CAO)* (Cheminant *et al.*, 2011), or indirectly, through the TFs *GATA*, *NITRATE-INDUCIBLE*, *CARBON-METABOLISM INVOLVED (GNC)* and *GNC-LIKE (GNL)* (Richter *et al.*, 2010). The repression of *PORC* in skotomorphogenic seedlings is also exerted by *SCARECROW-LIKE 27 (SCL27)*, a GRAS TF, by binding to GT elements in its promoter (Ma *et al.*, 2014). This repression is alleviated by a dual mechanism upon illumination: (1) accumulating DELLAs interact and sequester *SCL27* and (2) light induces the expression of the *miR171* that targets *SCL27* mRNA for degradation.

The interplay between GAs and light in the control of growth responses might be subject to daily fluctuation, since seedlings are naturally growing under photoperiod. The coordinated activity of light signalling through the phytochromes and the circadian clock leaves a daily window of high PIF

activity towards the end of the night that causes maximum hypocotyl growth rates (Nozue *et al.*, 2007; Nusinow *et al.*, 2011; Soy *et al.*, 2012). Remarkably, the circadian clock gates GA signalling by preventing the accumulation of DELLAs at the end of the night as a way to allow hypocotyl growth (Arana *et al.*, 2011). Therefore, the regulation of PIFs by DELLAs might also be part of the mechanism that generates the daily rhythm in hypocotyl elongation.

The activities of both GAs and PIFs are intimately related in the control of other developmental responses involving elongation growth. For instance, petiole and hypocotyl elongation in response to either shade or increased ambient temperature (Djakovic-Petrovic *et al.*, 2007; Lorrain *et al.*, 2008; Koini *et al.*, 2009; Stavang *et al.*, 2009). Both environmental cues seem to act primarily on the GA pathway by promoting the degradation of DELLA proteins (Djakovic-Petrovic *et al.*, 2007; Stavang *et al.*, 2009; Bou-Torrent *et al.*, 2014). The enhancement of PIF activity occurs at different levels in response to these environmental cues: increasing ambient temperature acts at both the transcriptional and post-transcriptional levels, promoting *PIF4* and *PIF5* expression (Koini *et al.*, 2009; Stavang *et al.*, 2009) and stabilizing the protein (Foreman *et al.*, 2011), while only the latter occurs in response to shade (Lorrain *et al.*, 2008). In both situations, it is reasonable to think that the interaction between DELLA and PIFs performs a regulatory role.

DELLAs also interact with other bHLH TFs belonging to the same clade as PIFs, including ALCATRAZ (ALC), PIF3-LIKE 2 (PIL2) and SPATULA (SPT) (Arnaud *et al.*, 2010; Gallego-Bartolomé *et al.*, 2010). The interaction between DELLAs and the developmental regulator ALC was the first example highlighting how the regulatory module formed by a DELLA-bHLH TF association has been recruited to control processes other than elongation growth and also revealed a previously unidentified role for GAs in fruit patterning (Arnaud *et al.*, 2010). In particular, DELLA accumulation in the valve margins of the *Arabidopsis* fruit inactivates ALC, thus preventing the differentiation of the separation layer needed for fruit opening and seed dispersal. As fruit development progresses, the upstream regulator INDEHISCENT (IND) promotes GA biosynthesis in the valve margins through induction of *AtGA3ox1* gene expression, causing DELLA degradation and the concomitant activation of ALC, which in turn directs the differentiation of the separation layer. However, no role has been identified for the interactions between DELLAs and the other members of the PIF clade, PIL2 and SPT. It is interesting to note that both DELLAs and SPT act as repressors of cotyledon expansion (Josse *et al.*, 2011). They exert this effect as part of a regulatory circuit in which SPT levels, but not activity, are negatively regulated by DELLAs in response to the light quality of the environment as a way to self-regulate the total growth repressive activity of both proteins.

DELLAs are able to interact with and inactivate bHLH TFs that are members of other clades within this family, such as MYC2, GLABRA 3 (GL3) and ENHANCER OF GLABRA 3 (EGL3) (Hong *et al.*, 2012a; Qi *et al.*, 2014). The interaction of DELLAs with MYC2 regulates volatile production in

Arabidopsis flowers, uncovering a role for GAs in this process (Hong *et al.*, 2012a), whereas the interaction with GL3 and EGL3 provides a molecular mechanism that explains the long-standing role for GAs promoting trichome development (Chien and Sussex, 1996; Qi *et al.*, 2014). In the latter case, the DELLA interactions extend to the inhibition of the MYB GLABRA1 (GL1) component of the WD-repeat/bHLH/MYB regulatory complex, which is also needed to trigger trichome development. These DELLA partners are also inactivated by the negative regulators of JA signalling, the JAZ proteins (Chini *et al.*, 2007; Thines *et al.*, 2007; Qi *et al.*, 2011). In both processes, GAs and JA act synergistically to enhance the expression of the genes involved in volatile production or in trichome development through releasing the repression imposed by DELLAs and JAZs. Therefore, MYC2 and GL1/GL3/EGL3 integrate both GA and JA signalling, and the molecular interaction described represents only one of the mechanisms for the cross-regulation between both hormone pathways (see below).

Remarkably, the cell-type determination mechanism represented by the interaction between DELLAs and the epidermis-specific TFs, GL1, GL3 and EGL3 provides clues about how GAs regulate other processes in a tissue-specific manner, such as the regulation of cell elongation and cell division in roots (Úbeda-Tomás *et al.*, 2008; Úbeda-Tomás *et al.*, 2009; Shani *et al.*, 2013), anther development (Plackett *et al.*, 2014), or flowering time (Galvao *et al.*, 2012; Porri *et al.*, 2012), i.e. by DELLA interaction with tissue-specific partners. Indeed, the dual role of DELLAs on the activity of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9), first inactivating it to delay flowering (Yu *et al.*, 2012), and later on acting together as a coactivator to promote *APETALA 1* (*API*) expression and thus flower formation (Yamaguchi *et al.*, 2014), or the inactivation of the HD-ZIP TFs MERISTEM LAYER 1 (ML1) and PROTODERMAL FACTOR 2 (PDF2) upon interaction to regulate seed germination (Rombola-Caldentey *et al.*, 2014), represent other examples of how tissue- or organ-specific TFs provide spatial specificity for certain roles of GAs.

The interaction and inactivation of BRASSINAZOLE-RESISTANT 1 (BZR1)/BRI1-EMS-SUPPRESSOR 1 (BES1) (Bai *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012; Li *et al.*, 2012), ETHYLENE INSENSITIVE 3 (EIN3) (An *et al.*, 2012) and RELATED TO AP2 3 (RAP2.3) (Marin-de la Rosa *et al.*, 2014), belonging to different families of TFs, represent cross-regulatory points between the GA and the brassinosteroid (BR) and ethylene signalling pathways, respectively (see also Chapter 8). The interaction with BZR1/BES1 explains the synergistic effect of GAs and BRs promoting hypocotyl elongation, both in seedlings undergoing skoto- and photomorphogenesis. In contrast, the interaction of DELLA with EIN3 and RAP2.3 is important for regulating apical hook development in etiolated seedlings. In this situation, DELLAs repress the expression of the *HOOKLESS 1* (*HLS1*) gene leading to hook opening through inactivation of the positive regulator EIN3 (An *et al.*, 2012), whereas the inactivation of RAP2.3 affects a discrete transcriptional

branch downstream of EIN3 also leading to hook opening (Marin-de la Rosa *et al.*, 2014). It seems likely therefore that GAs control several aspects of skotomorphogenesis by controlling a transcriptional network through the interaction between DELLAs and several TFs – PIFs, BZR1/BES1, EIN3 and RAP2.3 – that promote various aspects of this developmental program. PIF4 and BZR1 also interact physically, acting together to promote elongation growth both in light grown and in etiolated seedlings and defining a complex transcriptional module negatively regulated by DELLAs that also integrates BR and light information (Bai *et al.*, 2012). Interestingly, a recent report has broadened this module with the addition of two AUXIN RESPONSE FACTORS (ARFs) that mediate transcriptional regulation in response to auxin, ARF6 and ARF8, forming the so-called BAP module (Oh *et al.*, 2014), and providing a molecular mechanism for the observation of the synergism between many BR and auxin-regulated responses (Nemhauser *et al.*, 2004). Oh and colleagues have demonstrated the existence of large sets of genes coregulated by ARF6/8-PIF4 and ARF6/8-BZR1 complexes, in addition to those regulated by BZR1-PIF4 (Bai *et al.*, 2012), and that are relevant to regulate growth by cell elongation, both in seedlings growing in darkness and in the light (Oh *et al.*, 2014). Importantly, DELLAs are able to prevent the activity of all these pairs of TFs through a physical association.

Plant growth and form is a consequence of coordinated cell elongation and cell division. Interestingly, two recent studies report on the role that DELLAs exert in mediating the effects of GAs in promoting cell division, both at the shoot and the root apical meristems (Daviere *et al.*, 2014; Resentini *et al.*, 2015), in addition to their well-established role in regulating cell elongation. Both works identify the class I TCP TFs as targets for inactivation by DELLAs upon interaction. A series of biochemical assays demonstrated that the DNA binding ability of these TFs, and thus their transcriptional activity, was compromised by DELLAs. This effect results in reduced expression of core cell-cycle genes, which lowers the cell division capacity at the shoot apex (Daviere *et al.*, 2014). Similarly, it also provides a likely explanation for the reduced cell division that occurs at the root apex when DELLA levels increase (Achard *et al.*, 2009; Úbeda-Tomás *et al.*, 2009; Resentini *et al.*, 2015).

7.7.2 DELLAs interact with TFs in the context of promoters

All of the DELLA-TF interactions discussed above result in the inactivation of the particular TF by sequestering it away from its target genes. Nonetheless, several reports show that DELLAs can associate with specific target gene promoters (Zentella *et al.*, 2007; Lim *et al.*, 2013; Park *et al.*, 2013; Fukazawa *et al.*, 2014; Yamaguchi *et al.*, 2014; Figure 7.3c). Remarkably, ChIP followed by massive sequencing has demonstrated the binding of RGA to the regulatory regions (2500 bp up- or 500 bp downstream of a gene or within introns or UTRs) of 421 genes, indicating that transcriptional regulation by DELLAs in the context of the chromatin might be a common mechanism as well

(Marín-de la Rosa *et al.*, 2015). In this study, a series of *cis* elements enriched in the peak regions were also identified through an *in silico* approach that led to predict possible TFs responsible for the binding of RGA. The list included sequence elements bound by the INDETERMINATE DOMAIN (IDD) and bZIP families, some of their members being known interactors of DELLA proteins thus demonstrating the strength of the approach (see below; Lim *et al.*, 2013; Fukazawa *et al.*, 2014; Marín-de la Rosa *et al.*, 2014; Yoshida *et al.*, 2014). Interestingly, *cis* elements bound by the GARP-*ARABIDOPSIS* RESPONSE REGULATOR (ARR) family were also found. Type-b GARP-ARRs ARR1, ARR2 and ARR14 are positive regulators within the cytokinin (CK) signal transduction pathway and interact with both GAI and RGA (Marín-de la Rosa *et al.*, 2014, 2015). A series of biochemical, molecular genetics and physiological assays showed that these two DELLAs enhance the activation ability of ARR1 over a variety of target genes and that this effect occurs in the vicinity of their promoters and controls at least two physiological responses known to be antagonistically regulated by GAs and CKs: root meristem size and de-etiolation (Marín-de la Rosa *et al.*, 2015). Furthermore, Lim and colleagues (2013) have demonstrated that DELLAs are targeted to the promoter of *SOMNUS* (*SOM*), a negative regulator of germination, through an interaction with ABSCISIC ACID INSENSITIVE3 (*ABI3*) and the bZIP ABSCISIC ACID INSENSITIVE5 (*ABI5*). This interaction leads to an increase in *SOM* expression when ambient temperatures are high resulting in the inhibition of germination (Lim *et al.*, 2013). Importantly, these two recent studies provide molecular mechanisms for the interaction between the GA and the ABA and CK signalling pathways during the control of these particular processes. However, it is highly probable that these mechanisms of cross-talk could also operate at other stages of development influenced by these two hormones antagonistic to GAs. Similarly, and as mentioned above, DELLAs promote flower formation thanks to their ability to activate the expression of the floral identity gene *AP1* by acting in the context of its promoter through interaction with SPL9 (Yamaguchi *et al.*, 2014). The role that DELLAs perform in these transcriptional complexes has not been explicitly investigated. However, it is tempting to speculate that they are acting as transcriptional coactivators that are targeted to the *SOM* and *AP1* promoters by the TFs, as demonstrated for DELLA-ARR1 (Marín-de la Rosa *et al.*, 2015). This has also been demonstrated for the interaction between DELLAs and several members of the INDETERMINATE DOMAIN (IDD) family in *Arabidopsis* (Fukazawa *et al.*, 2014; Yoshida *et al.*, 2014). These two studies demonstrate that DELLAs participate in a mechanism in which the interchange of transcriptional coactivators (DELLAs) and corepressors (*SCL3* and *TOPELESS-RELATED* (*TPR*)) bound to IDDs activate or repress gene targets in a GA-dependent manner (Figure 7.4a). Despite their similarities, the mechanisms are not identical. For instance, IDD3, 4, 5, 9 and 10 activate transcription of target genes upon interaction with DELLAs (Yoshida *et al.*, 2014), including *SCL3*, which encodes a coactivator of GA signalling (Heo

et al., 2011; Zhang *et al.*, 2011). Previous studies had indicated that *SCL3* promotes GA responses, including promotion of *SCL3* expression itself, by antagonizing DELLA function through a direct protein–protein interaction (Zhang *et al.*, 2011). In light of these findings, Yoshida and colleagues (2014) proposed a regulatory transcriptional feedback loop controlled by *SCL3* and DELLA levels that buffers GA signalling by providing a homeostatic control mechanism for this pathway, the important factor being that *SCL3* is a transcriptional target of both the activator DELLA-IDD and the repressor *SCL3*-IDD complexes. Further insights into the importance of the DELLA-IDD transcriptional module in controlling GA responses have been gained from a recent study with *Arabidopsis* characterising an interaction between GAF1 (GAI-ASSOCIATED FACTOR1)/IDD2 and the DELLA, GAI (Fukazawa *et al.*, 2014). GAF1/IDD2 activates transcription of its target genes when it interacts with DELLAs, but represses transcription when it interacts with the TPR1 and TPR4 corepressors. Importantly, this transcriptional system provides a molecular mechanism for the feedback regulation that operates to control GA levels and perception: GAF1/IDD2 binds to the promoters of the *GA20ox2*, *GA3ox1* and *GID1b* genes to activate their expression through interaction with DELLAs when GA levels are low, but repress their expression through interaction with TPR1 and TPR4 when GA levels are high (see also Chapter 2). In essence, the levels of expression of these GA-feedback regulated genes are controlled by a balance between DELLA and TPR levels, which is determined by the GA concentration. These interactions define two key mechanisms that provide homeostasis to the GA pathway, including biosynthesis, perception and signalling. The closest homologue to GAF1/IDD2 is ENHYDROUS (ENY)/IDD1, which has also been demonstrated to physically interact with DELLAs (Feurtado *et al.*, 2011). ENY/IDD1 appears to have a positive effect on GA responsiveness during seed development, and although the mechanism of action is not known, it is likely that it acts similarly to GAF1/IDD2.

7.7.3 DELLAs interact with other transcriptional regulators

DELLAs not only control transcription by binding to bona fide TFs, they also interact with other transcriptional regulators that function in a similar manner to DELLAs by regulating transcription without binding directly to DNA (Figure 7.3b). This ability likely amplifies the effect of DELLAs on transcription, given that each transcriptional regulator might influence the activity of multiple TFs. One of the best known examples is the interaction between DELLAs and JAZ proteins (Hou *et al.*, 2010; Yang *et al.*, 2012). Hou and colleagues (2010) demonstrated that this interaction mediates the antagonism between GAs and JA in the control of root growth in *Arabidopsis*. In this context, DELLA accumulation inactivates the JAZ1 protein, thus allowing MYC2, and likely other JAZ-regulated TFs, to prevent root growth and enhance the expression of JA-responsive genes. This regulatory module

Figure 7.4 Modes of regulation of DELLA activity. (a) SCL3 and other transcriptional repressors compete with DELLAs to prevent DELLA-mediated transcriptional activation through IDDs. (b) The inhibition of PIF4 by DELLAs is relieved by BBX24 in the context of shade avoidance. (c) BOIs promote DELLA-mediated transcriptional activation.

involves direct competition between DELLAs and MYC2 for binding to the JAZ protein. A similar mechanism operates in other physiological contexts, including coordination of JA-regulated defence and growth responses (Yang *et al.*, 2012). It is competition between JAZ9 and PIF3 for DELLA binding that provides the mechanism for these responses. When defence is a priority, JAZ

proteins are degraded in response to JA and defence responses activated. The lack of JAZ proteins allows DELLAs to bind to and inactivate PIFs, resulting in growth repression. One could envision a very dynamic and responsive mechanism in which environmental signals from light, temperature or the presence of pathogens influences the relative levels of DELLA and/or JAZ through the GA and JA pathways, which in turn regulate the activity of a plethora of interacting TFs to trigger the appropriate physiological response. The set of TFs targeted will likely be different depending on the cell type. This mechanism contrasts with the synergistic inactivation of GL1/GL3/EGL3, or MYC2, by DELLAs and JAZ to regulate volatiles production in flowers, as described above.

The variety of classes of DELLA interactors is also reflected in the different transcriptional regulators that have been identified recently. Besides the JAZ proteins, several other unrelated transcriptional regulators have been identified in *Arabidopsis*. The double B-box BBX24 (Crocco *et al.*, 2015) interacts with DELLAs away from chromatin, whereas the interaction with the RING finger proteins BOTRYTIS SUSCEPTIBLE1 INTERACTORS (BOIs) occurs within the vicinity of target promoters (Park *et al.*, 2013). The interaction between BBX24 and DELLAs appears to be involved in the coordination of growth responses in response to shade avoidance (Figure 7.4b; Crocco *et al.*, 2015). It involves a similar mechanism to the JAZ-DELLA-PIF regulatory module, with BBX24 competing with PIF4 for binding to DELLA. It has been speculated that an early effect of the shade response is to promote the BBX24-DELLA interaction, thereby allowing PIF4 to activate downstream target genes that promote hypocotyl elongation. It is currently unclear how this early response to shade modulates the BBX-DELLA interaction.

In contrast to the situation with BBX24, the activity of the BOIs is required by DELLAs to regulate several GA responses, including germination, and the juvenile-to-adult and floral transitions (Figure 7.4c; Park *et al.*, 2013). Furthermore, BOI has been shown to act as an E3 ubiquitin ligase due to the presence of a RING domain (Luo *et al.*, 2010). This suggests that it may ubiquitinate TFs or other proteins involved in the regulation of GA-responsive genes to suppress their activity. Despite the presence of the RING domain and their functional importance in repressing GA responses, it is still conceivable that the role of BOIs in regulating transcription is unrelated to their E3 ligase activity. For example, DELLAs and BOIs might interact and together recruit the TFs or coregulators of transcription, allowing their association with chromatin and initiating downstream transcriptional events that promote growth responses.

7.7.4 DELLAs regulate chromatin dynamics

The activity of TFs must ultimately allow the assembly of the basal transcriptional machinery to initiate transcription of the target genes. This is usually achieved by targeting chromatin remodelling and/or histone modifying complexes to the promoter through interaction with TFs (Clapier and Cairns,

2009). It is reasonable to think that all DELLA-TF interactions described above result indirectly in modifications of the epigenetic landscape at the target promoters, whether DELLAs sequester the TF or act bound to the chromatin, ultimately changing gene expression. Remarkably, a recent study in *Arabidopsis* demonstrates that DELLAs can directly regulate epigenetic mark deposition through their physical association with PICKLE (PKL), a chromatin remodelling protein of the CHD3 type (Zhang *et al.*, 2014). In this study, it is demonstrated that PKL is targeted to genes promoting cell elongation, such as *IAA19* and *PRE1*, through an interaction with PIF3, BZR1 or both, in etiolated seedlings. This targeting of PKL reduces trimethylation of lysine 27 of histone 3 (H3K27me3) at these promoters, which would normally act as a repressive mark. The effect of the DELLA interaction with PKL is therefore to increase the H3K27me3 mark in the promoters of *IAA19* and *PACLOBUTRAZOL RESISTANCE 1 (PRE1)* and reduce cell elongation. Together with the direct regulation of PIF3 and BZR1 through their association with DELLAs, this mechanism increases the negative effect that DELLAs exert on cell elongation. Thus, the DELLA-PKL interaction represents an extra layer of regulation coupled to the PIF-BZR1 module (Bai *et al.*, 2012). It seems likely that this form of epigenetic regulation will also affect the activity of the BAP module (Oh *et al.*, 2014). Whether the DELLA-PKL interaction occurs in physiological contexts distinct from the regulation of skotomorphogenesis requires further investigation. It has recently been reported that RGL2 and RGL3 interact with the catalytic sub-unit SWI3C of the SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeller, suggesting another potential mechanism for epigenetic regulation by DELLAs (Sarnowska *et al.*, 2013). However, the physiological significance of these interactions has yet to be established.

7.8 A non-genomic response regulated by DELLAs

All DELLA interactions described above are related to their role as transcriptional regulators. However, the identification of prefoldin 5 (PFD5) and PFD3 as partners of DELLA provided hints of a non-genomic role for DELLAs (Locascio *et al.*, 2013). These proteins are part of the heterohexameric PFD complex that is formed by sub-units PFD1 to 6. This complex is conserved from yeast to humans and functions as a cochaperonin in the cytosol, presenting nascent polypeptides of actins and tubulins to the molecular chaperone CCT (Vainberg *et al.*, 1998). The activity of the PFD complex controls the formation of the α/β -tubulin heterodimers that are the forming units of the microtubules (MTs). Indeed, several mutants of *Arabidopsis* affecting sub-units of the complex have defects in MT organization (Gu *et al.*, 2008; Rodriguez-Milla and Salinas, 2009). It is generally accepted that the MT organization determines the cell growth direction, in such a way that transverse MTs allow anisotropic cell elongation, whereas random

or longitudinal MT arrays prevent it (Lloyd, 2011). It has been known for several years that GAs are able to promote the transverse organization of MTs in epidermal cells (Wenzel *et al.*, 2000). The mechanism for this role of GAs has remained, however, unknown. The DELLA-PFD interaction has provided an explanation (Locascio *et al.*, 2013). The PFD localizes to the nucleus upon interaction with DELLA proteins and this has immediate consequences for the cytosolic function of the complex. The amount of properly folded α/β -tubulins heterodimers drops, likely causing the disorganization of MTs that prevents anisotropic growth. Thus, the MT organization is indirectly regulated by GA levels through the DELLA-PFD interaction. The regulation of PFD by DELLAs seems to fluctuate daily, thus contributing to restricting the maximum growth rate of seedlings towards the end of the night (Arana *et al.*, 2011; Locascio *et al.*, 2013). Interestingly, a role for the yeast PFD complex in the nucleus has been recently described, showing that it participates in transcription elongation of long genes (Millán-Zambrano *et al.*, 2013). Given the conservation of the PFD in different organisms, a similar role for the plant counterpart could be envisaged.

7.9 Analysis of DELLA protein structure-function

An understanding of the structure-function relationship for DELLA proteins is slowly emerging through a combination of genetic analysis, protein structure-function studies and the identification of interacting downstream components. As discussed earlier, DELLAs belong to one of ten predicted sub-groups of the GRAS family of plant transcriptional regulators (Sun *et al.*, 2012). Members within these different sub-groups have been demonstrated to have diverse roles in controlling plant growth and development, including root radial patterning and elongation, shoot apical meristem maintenance, formation of nodules in legumes, axillary meristem development and light signalling. Proteins belonging to this family have a highly conserved C-terminal GRAS domain that is important for functionality and suggests a common mode of action. Within the GRAS domain there are five highly conserved motifs: LHR1, VHIID, LHR2, PFYRE and SAW (Figure 7.5). The sub-groups of the GRAS family share common N-terminal motifs that are predicted to occur within intrinsically disordered regions of the protein (Sun *et al.*, 2012). In the case of the DELLA sub-group the common N-terminal motif includes the DELLA, LEXLE and TVHYNP domains that are required for binding to the GID1-GA complex (Murase *et al.*, 2008). Extensive structure-function studies of the GA signalling components SLR1, GID1 and GID2 in rice have further extended our understanding of the mechanisms controlling GA-induced DELLA degradation (Hirano *et al.*, 2010; see also Chapter 6). Based on these studies, a model has emerged in which binding of GID1-GA to the N-terminal domains within SLR1 is predicted to induce a conformational change that allows stabilisation of the SLR1-GID1-GA

Figure 7.5 Conserved domains in DELLA proteins and sites affecting functionality. The position of mutation sites of reduced- and loss-of-function mutants are indicated by arrows. The plant species that are the source of the mutations are indicated on the right. See also Figure 6.2 for information on the regions within DELLAs that are necessary for their post-translational modification. (Adapted from Chandler and Harding, 2013).

complex through an additional interaction between GID1 and the GRAS domain of SLR1. This secondary interaction with GID1 involves the VHIID, PYFRE and SAW domains within the C-terminus of SLR1. The stabilised SLR1-GID1-GA complex is then recognised and bound by the F-Box component, GID2, presumably facilitating ubiquitination through the associated E3 Ubiquitin ligase activity (Hirano *et al.*, 2010). Multiple regions within the GRAS domain are required for SLR1 binding to GID2, but the VHIID and LHR2 domains appear to have a major role. A less well characterised function of the GRAS domain is to facilitate dimerization. Although this has yet to be established *in planta*, self-interaction studies using SLR1 or RGA in yeast have demonstrated that homodimerisation occurs through the LHR1 domain (Itoh *et al.*, 2002; Bai *et al.*, 2012). Given the importance of this domain in facilitating the interaction with downstream components (see below) it will be intriguing to establish whether this represents another mechanism for regulating DELLA activity.

The characterisation of reduced or loss-of-function *della* mutants has provided some important insights into the domains that are required for conferring the ability to repress downstream GA responses. It is apparent that an almost complete GRAS domain is necessary for this functionality, with mutations that lead to very short C-terminal truncations causing complete loss of activity (the *slr1-4* allele encodes a protein lacking six residues from the C-terminus; Ikeda *et al.*, 2001). Whilst much of our knowledge of the GA signalling pathway has come from the study of the model plants rice and *Arabidopsis*, an extremely extensive collection of *della* mutants have recently been identified from more genetically challenging sources. Using suppressor screens of barley and wheat GA biosynthesis and signalling mutants, Chandler and Harding (2013) identified over-growth mutants that exhibited increased leaf and stem elongation. In the majority of alleles, the mutations were demonstrated to occur in the barley and wheat *DELLA* genes, *SLN1* and *Rht-B1*, respectively. A total of 26 independent *della* missense mutations

were identified in these screens (Figure 7.5). Remarkably, in several cases two independent alleles resulting in an identical amino acid substitution were identified in the two plant species, highlighting the critical importance of these residues in conferring functionality. All of the amino acid substitutions occur within the GRAS domain of SLN1 and RHT-B1. More importantly, they are clustered largely in two specific regions of these DELLAs: the LHR1 and PFYRE motifs. In addition, there are also four *Rht-B1* missense mutations that also affect residues within the VHIID motif, although based on the suppression of stem elongation in these mutants their impact on reducing DELLA functionality appears to be less. Other *della* missense mutations in *Arabidopsis* (*rga-2*; Silverstone *et al.*, 1998), pea (*cry-c*; Weston *et al.*, 2008), rice (*slr1-6*; Hirano *et al.*, 2012) and tomato (*procera*; Bassel *et al.*, 2008) have also been demonstrated to affect residues within the LHR1, PFYRE and VHIID motifs. In addition to these domains identified from studying *della* mutants, an alanine-scanning approach in transgenic rice has also implicated the LHR2 motif in controlling the repressive activity of SLR1 (Hirano *et al.*, 2010).

In many of the cases in which DELLAs have been demonstrated to modulate the activity of transcriptional regulators through direct protein–protein contact, these findings have been based on yeast two-hybrid screens that have uncovered these interactions (For examples see: de Lucas *et al.*, 2008; Hou *et al.*, 2010; Gallego-Bartolomé *et al.*, 2012; Fukazawa *et al.*, 2014; Yoshida *et al.*, 2014). These assays have subsequently allowed the DELLA-interaction domains to be interrogated by deletion analysis. In general, these studies have highlighted the importance of the GRAS domain in mediating the interactions. Only in the case of the interaction with JAZ1 is there any evidence that the N-terminus of DELLA has any role (Hou *et al.*, 2010). However, this is not supported by similar interaction studies between JAZ9 and GAI, which identify only the GRAS domain as being required (Yang *et al.*, 2012). Further investigations are necessary to clarify the nature of DELLA-JAZ interaction domains. The finding that the GRAS domain is largely responsible for the interaction with multiple transcriptional regulators is consistent with the genetic evidence that supports its functional role in regulating downstream GA responses. What is not currently clear is whether all of the characterised DELLA interactors bind to a conserved binding site within the GRAS domain. Deletion of the LHR1 domain has been shown to abolish the interaction with BZR1, MYC2, JAZ1 and EIN3 in yeast (Hou *et al.*, 2010; An *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012; Hong *et al.*, 2012a). In addition, C-terminal deletion of the SAW and/or PFYRE domains also abolished interactions with BZR1, MYC2 and EIN3 (An *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012; Hong *et al.*, 2012a). An exception was the interaction between JAZ1 and RGA, which did not appear to require 180 residues of the C-terminal GRAS domain (Hou *et al.*, 2010). Because DELLAs have been shown to interact with IDD proteins to directly activate the expression of GA signalling components to control homeostasis of this

pathway, it raised the prospect that this association might shed some light on the proposed transactivation role of the N-terminus (Fukazawa *et al.*, 2014; Yoshida *et al.*, 2014b). However, interaction studies in yeast have also demonstrated that it is only the GRAS domain, including the LHR1 and PFYRE/SAW domains, that is required for this association. It therefore seems likely that the components and mechanisms responsible for controlling the transactivation activity of the N-terminal domain of DELLAs are yet to be determined. Within the GRAS domain it is clear that multiple regions are important for the interactions with downstream components, but there is still considerable uncertainty about the precise identity of these regions. By investigating how amino acid substitutions that alter DELLA function affect these interactions will further refine the extent of these domains. There is the possibility that different regions of the GRAS domain are important for interaction with specific partners that control different growth responses. At present, the genetic and biochemical evidence for this is limited. However, characterisation of leaf elongation and α -amylase induction in the aleurone in barley and wheat over-growth mutants did provide some evidence that the regions of the GRAS domain controlling these GA responses are different (Chandler and Harding, 2013). The identification of downstream components responsible for controlling these GA responses should provide us with further clues regarding the specificity of the GRAS domain. The ultimate goal will be the elucidation of the 3-D crystal structures of these binary complexes, which will establish the precise structural requirements that specify these interactions. The recent expression and purification of recombinant SLR1 provides an important step towards this goal (Sato *et al.*, 2014).

7.10 GAMYB: A transcriptional regulator of gibberellin responses during cereal grain germination and pollen development

During the germination of a cereal grain, GAs, which are released from the imbibed embryo, act on aleurone cells to promote the secretion of hydrolytic enzymes into the endosperm (Jacobsen *et al.*, 1995). This produces the nutrients and other components required to sustain heterotrophic growth of the germinating seedling until it reaches a stage and environment that allows auxotrophic growth. The cereal aleurone cells provided some important advantages over other systems for studying GA response: (1) these cells do not synthesize GAs that could interfere with response assays, (2) substantial increases in the expression of genes encoding hydrolytic enzymes, including α -amylases, which breakdown starch reserves within the endosperm, are rapidly induced by GA providing convenient reporters for monitoring responses, (3) aleurone cells can be easily separated from other grain tissues in the form of protoplasts or de-embryonated half seeds,

allowing GA response to be monitored in a single cell type and (4) transient transformations can be performed on aleurone cells allowing the dissection of the signalling pathways responsible for the expression of hydrolytic enzymes. These factors have led to a good understanding of the mechanisms controlling GA-regulated expression of α -amylase genes, although there are some important gaps in this knowledge. In light of the extensive research carried out in this area, we are unable to provide a comprehensive review of the literature. The reader is therefore directed to some excellent articles, which provide a more comprehensive review of earlier studies (Jacobsen *et al.*, 1995; Bethke *et al.*, 1997; Sun and Gubler, 2004). In this section we will mainly focus on recent developments in the field.

7.10.1 GAMYB positively regulates gene expression in cereal aleurone cells

In cereal aleurone cells the GA induction of α -amylase activity has been studied extensively over the last 50 years (reviewed in Jacobsen *et al.*, 1995 and Sun and Gubler, 2004). Up-regulation of α -amylase gene expression is observed after around 3 to 6 hours following a GA treatment to barley aleurone cells (Chandler *et al.*, 1984; Gubler *et al.*, 2002). Analysis of the α -Amy1 promoter has identified several *cis*-elements that are required for the transcriptional control by GA and ultimately led to the identification of multiple transcriptional regulators that have a regulatory role (Sun and Gubler, 2004). Most notably, the R2R3 MYB TF GAMYB has been demonstrated to bind to the GA response element (GARE) of the α -amylase promoter and activate expression in barley (Gubler *et al.*, 1995; Gubler *et al.*, 1999). In rice, a role for GAMYB in this process has been confirmed by the absence of GA-induced α -amylase expression in a *gamyb* loss-of-function mutant (Kaneko *et al.*, 2004). Other *cis*-elements that have been identified to have a role in the GA-regulated expression of the α -Amy1 gene include the pyrimidine box and the TATCCA (TA) box (reviewed in Sun and Gubler, 2004). In addition to being regulated by hormonal signals, α -amylase genes within the aleurone are also regulated by sugar and nutrient starvation signals, allowing coordination of endosperm reserve mobilisation to facilitate optimal seedling establishment. The TA box has been demonstrated to be an important element for the induction of α -amylase expression in response to sugar starvation. Activation of α -amylase expression is induced under sugar starvation by another MYB TF, MYBS1. Identified in rice, MYBS1 is sugar repressible and activates α -amylase expression through binding to the TA-box element (Hong *et al.*, 2012b). The importance of the TA box in regulating both GA and sugar starvation responses has highlighted an important mechanism of cross-talk between these signalling pathways (see below).

The mechanisms through which GA signalling activates GAMYB to promote the expression of direct downstream response genes in the aleurone are still not entirely clear. It has been demonstrated in barley and rice that

DELLAs act as upstream repressors of this response (Ikeda *et al.*, 2001; Gubler *et al.*, 2002). In barley aleurone cells, GA-mediated degradation of SLN1 protein is complete within 10 minutes of a GA treatment (Gubler *et al.*, 2002). A subsequent increase in *GAMYB* transcript levels is then observed around 1 to 2 hours later and is thought to be responsible for the induction in α -amylase gene expression. However, recent studies have suggested that GA signalling also causes changes in the sub-cellular localisation of *GAMYB* (Hong *et al.*, 2012b). Based on the study of a *GAMYB*-GFP reporter in transiently transformed barley aleurone cells, a 24-hour treatment with GA was demonstrated to promote *GAMYB*-GFP redistribution from the cytoplasm to the nucleus. If this sub-cellular redistribution is required for the activation of downstream GA-response genes it will be important to establish the kinetics of GA-mediated *GAMYB* nuclear redistribution, to determine whether it correlates with activation of α -amylase gene expression. Interestingly, the same study by Hong and colleagues (2012b) demonstrated that nucleocytoplasmic partitioning of MYBS1 is also important for mediating aleurone transcriptional responses to both nutrient starvation and GA signalling. When nutrients, including glucose, phosphate and nitrogen, are available, MYBS1 is located predominantly in the cytoplasm through active nuclear export. Under nutrient starvation a partial redistribution of MYBS1 to the nucleus occurs, allowing it to bind to the TA-box elements within the promoters of target genes resulting in their activation for resource mobilisation. Acting through *GAMYB*, GAs enhance the activation of MYBS1 target genes in both the presence and absence of nutrients. An increase in *GAMYB* levels in response to GA signalling facilitates the nuclear import of a *GAMYB*-MYBS1 complex. Within the nucleus, binding of *GAMYB* and MYBS1 to the GARE and TA box, respectively, results in the strong activation of target gene expression that is necessary for resource mobilisation and seedling establishment. It has been hypothesized that this cross-talk between nutrient and GA signalling, mediated by these two classes of MYB TFs, is important for the environmental regulation of the transition between the dormant seed and actively growing seedling (Hong *et al.*, 2012b). However, further studies to confirm the physiological importance of this cross-talk mechanism are still required.

A major gap in our knowledge of GA signalling within the aleurone cells is an understanding of how DELLA proteins act to repress *GAMYB*. In barley, the lag between SLN1 degradation and an increase in *GAMYB* transcript levels suggests that the regulation of transcription is unlikely to be direct (Gubler *et al.*, 2002). It is conceivable that SLN1 represses an activator of *GAMYB* expression. Alternatively, it is tempting to speculate that SLN1 may regulate the nucleocytoplasmic distribution of *GAMYB*, by inhibiting its nuclear import. Although there is currently limited evidence for such a form of regulation, if established, it could provide an explanation as to why the presence of *GAMYB* transcripts in the absence of GA signalling does not result in expression of downstream target genes (Gubler *et al.*, 1995;

Gubler *et al.*, 2002). It is likely that DELLAs have multiple targets to regulate different aleurone responses.

The recent demonstration that DELLAs interact with the ABA signalling components ABI3 and ABI5 in *Arabidopsis* (Lim *et al.*, 2013; see above) provides a potential mechanism for the well-characterised GA and ABA cross-talk within this cell type. In cereals, ABI5- and ABI3-related TFs have been identified and demonstrated to regulate aleurone responses (Casaretto and Ho, 2003; Johnson *et al.*, 2008; Harris *et al.*, 2013). Understanding whether these components represent direct activation targets for DELLAs could provide some important insights into the mechanisms controlling ABA and GA cross-talk during this response.

7.10.2 GAMYB regulates gibberellin-dependent anther development

The importance of GAMYB in controlling downstream GA responses is not restricted to the control of endosperm mobilisation in cereal grains. As discussed in more detail in Chapter 11, GAMYB also functions as a TF that is essential for promoting GA-dependent anther development required for the formation of viable pollen in angiosperms (Murray *et al.*, 2003; Kaneko *et al.*, 2004; Millar and Gubler, 2005; Aya *et al.*, 2009). This role, which appears restricted to the tapetum cell layer, involves the promotion of both tapetal programmed cell death (PCD) and pollen wall biogenesis, processes that are essential for pollen development (Aya *et al.*, 2009). Potential genes that control these developmental processes have been identified in rice anthers and demonstrated to be acting downstream of the GA-GID1-DELLA-GAMYB regulatory system. These include genes encoding proteases or protease inhibitors and putative sporopollenin biosynthetic genes that have been implicated in controlling tapetal PCD and pollen exine production, respectively. Notably, many of the genes that are positively regulated by GAMYB contain GAMYB binding motifs within their promoters, suggesting that they are direct transcriptional targets. Aya and colleagues (2009) have provided strong evidence supporting this model by demonstrating that GAMYB directly regulates the sporopollenin biosynthesis genes, CYP703A3 and KAR, through its interaction with these promoter binding sites. In view of the fact that aleurone and tapetal cells undergo similar developmental programmes involving targeted secretion/reserve mobilisation and ultimately PCD in response to GA signalling, it is tempting to speculate that DELLA inhibition of GAMYB occurs through a similar mechanism. Interestingly, recent work suggests that the GA-GID1-DELLA regulatory system evolved in early vascular plants to regulate aspects of reproductive development through its control of GAMYB (Aya *et al.*, 2011). Therefore, understanding how DELLAs regulate GAMYB is of key significance for understanding how the GA signalling pathway evolved to have such an important role controlling plant development.

7.11 Concluding remarks

During the last eight years we have gained a considerable understanding of the molecular mechanisms by which DELLA proteins act to control a multitude of cellular responses. As well as providing important insights into how GAs control plant growth and development, these studies have also uncovered some of the mechanisms responsible for controlling cross-talk with other phytohormone and environmental signalling pathways. It is perhaps not surprising that these recent studies have established that DELLAs control transcription through their association with other transcriptional regulators; however the diversity of protein classes which these include and the different transcriptional regulatory mechanisms employed is truly remarkable (Figure 7.6). DELLAs have therefore emerged as important signalling hubs, true connectors modulating the activity of the interacting transcriptional regulators and therefore the signalling pathways in which they participate. It is likely that the extent of these connections will expand substantially as our knowledge of these growth repressors improves. Therefore, to fully understand the implications on plant growth and development it will be necessary to employ systems-biology-based approaches. Furthermore, because DELLAs are essentially a reporter for the level of bioactive GAs, it will also be important to improve our understanding of

Figure 7.6 DELLA proteins act as hubs. Different environmental cues regulate GA metabolism, eventually affecting DELLA stability. DELLA levels thus integrate environmental conditions and relay this information to prewired transcriptional networks and other cellular processes, facilitating the most appropriate adaptive response.

how developmental and environmental signals control GA accumulation at the cellular level.

Perhaps the most surprising recent finding is that DELLAs have modes of action that are distinct from controlling transcription. In addition to controlling microtubule reorientation, a recent study in rice has provided evidence that SLR1 interacts with the strigolactone receptor, D14, in a strigolactone-dependent manner (Nakamura *et al.*, 2013). At present the physiological significance of this interaction is yet to be established. Nevertheless, it highlights the need to keep an open mind when investigating DELLA function.

Not only have recent insights into DELLA functionality helped to improve our understanding of how GAs control plant growth and development, they also have important implications for studying how other GRAS proteins function. In light of the sequence homology within their functional GRAS domains, it is likely that all members of this family function as hub proteins that interact with a myriad of downstream components. Arguably, our knowledge of how DELLAs act has progressed more rapidly than for other GRAS proteins because we know that they are subjected to receptor-mediated degradation by a process involving their N-terminal domain (see above; see also Chapter 6). As most other GRAS proteins contain N-terminal domains that are predicted to serve as interaction domains (Sun *et al.*, 2011; Sun *et al.*, 2012), it will be fascinating to see whether receptor-mediated degradation is a common mechanism through which they are regulated.

Acknowledgements

We thank Camille Steber for helpful comments on this manuscript and John Ross for kindly providing the image used in Figure 7.1. Dr Stephen Thomas receives support from the 20:20 Wheat[®] Institute Strategic Programme at Rothamsted Research funded by the Biotechnology and Biological Sciences Research Council of the United Kingdom. Work in the laboratory of Drs Miguel A Blázquez and David Alabadí is supported by grants BIO2013-43184-P and RTC-2014-2876-2 from the Spanish Ministerio de Economía y Competitividad, and SIGNAT-644435 from the H2020-MSCA-RISE-2014 program.

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