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REVIEW

Transcriptional regulation of seed storage protein genes in *Arabidopsis* and cereals

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Abstract

Seed storage proteins (SSPs) are synthesized during development and the expression of their genes is under tight tissue-specific and temporal transcriptional regulation. In this review we summarize the current knowledge concerning the regulatory steps controlling SSP synthesis in *Arabidopsis* and cereals, which involves the interaction of *cis* regulatory elements with corresponding *trans*-acting factors. In some cases, the regulation of SSP genes requires the concerted action of multiple transcription factors (TFs). There is an evolutionary conservation between the prolamins (the main group of SSPs in many cereal grains) and a major group of dicot seed albumins; this relates to both the regulatory elements and the TFs that are functionally exchangeable between the monocot and dicot species.

Keywords: *Arabidopsis*, cereal, promoter region, seed storage protein, transcription factors

Introduction

Seed formation is a key step that provides many plants a unique opportunity to suspend their life cycle and withstand adverse environmental conditions in a desiccated state. A dominant process characteristic of seed development is the accumulation of reserve carbohydrates, lipids and proteins that are subsequently used following germination as a source of energy, carbon and nitrogen until the seedling becomes autotrophic. In dicot plants, the cotyledons are frequently the main tissue in which reserves accumulate, although in some a persistent endosperm or perisperm may be the major storage tissue. In *Arabidopsis*, only a single layer of endosperm cells remains in the dry seed, and storage compounds are deposited in the embryo itself (Vicente-Carbajosa and Carbonero, 2005). In monocots, especially members of the grass/cereal families, the endosperm is the prevailing reserve tissue in which starch and seed storage proteins (SSPs) accumulate. Storage proteins in seeds are the products of a number of specific genes whose expression is tightly regulated during development. Thus, SSP genes provide a powerful system to investigate the regulation of their expression. Several lines of evidence indicate that the synthesis of storage proteins is controlled primarily at the transcriptional level. This review will focus on the current knowledge of the transcriptional regulation driving SSP gene expression in *Arabidopsis* and cereals, which involves both *cis* regulatory elements and transcription factors (TFs).

Cis-elements involved in the expression of SSP genes

Extensive analysis of the SSP gene promoters has identified numerous *cis* regulatory elements involved in controlling seed-specific expression. Among them in dicot species is the widely distributed RY motif (5′-CATGCA-3′), which represents the central core of the previously identified 28-bp-long legumin box

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Abbreviations: ABA, abscisic acid; ABI3, ABSCISIC ACID-INSENSITIVE3; bZIP, basic leucine zipper; DOF, DNA binding with one finger; FUS3, FUSCA3; LEC1, LEAFY COTYLEDON1; O2, Opaque2; P box, prolamin box; PBF, prolamin-box binding factor; SSP, seed storage protein; TF, transcription factor; VP1, Viviparous1.

(Baumlein *et al.*, 1992), and its deletion abolishes most of the seed-specific promoter activities. The RY motif was originally discovered in the promoters of dicot seed protein genes, but later was found also to be conserved in many of the studied promoters of cereal SSP genes. This observation supports a putative role for the RY box in endosperm-specific gene expression. Mutational analyses in tobacco demonstrated that the RY repeats of legumin, glycinin and β-conglycinin genes are necessary for positive regulation in seed tissues (Baumlein *et al.*, 1992; Chamberland *et al.*, 1992; Lelievre *et al.*, 1992) and negative regulation in vegetative organs (Baumlein *et al.*, 1992).

In the promoters of genes encoding cereal SSPs, the best characterized *cis*-consensus elements include the prolamin box (P box; 5′-TGT/CAAAG-3′), GCN4 motif (5′-TGAG/CTCA-3′) and AACA motif (5′-AAC-AAAC-3′) (Takaiwa *et al.*, 1996; Vicente-Carbajosa *et al.*, 1997; Wu *et al.*, 2000). Mutations in the GCN4 motif of the *GluB-1* gene change its expression from the outer to the inner starchy endosperm, and the extent of expression is severely reduced (90-fold) (Wu *et al.*, 1998). Multimers of GCN4 fused to a minimal cauliflower mosaic virus (CaMV) 35S core promoter can drive expression in the outer region of the endosperm, whereas a single GCN4 motif cannot (Wu *et al.*, 1998). In many cases, GCN4 and P box are coupled to each other and separated by only a few nucleotides; they are termed a bifactorial endosperm box. The involvement of these two motifs in regulating gene expression has been demonstrated for numerous cereal prolamin genes (Hammond-Kosack *et al.*, 1993; Albani *et al.*, 1997; Marzabal *et al.*, 1998). A third motif, AACA, conserved in rice glutelin genes is also involved in controlling the endosperm-specific expression (Washida *et al.*, 1999). Interestingly, AACA is also closely linked with the GCN4 element in most rice glutelin genes (Qu le *et al.*, 2008) and together these have been shown to confer endosperm-specific enhancement to a truncated −90 CaMV 35S promoter (Yoshihara *et al.*, 1996).

Major TFs

Major TFs involved in regulation of SSP gene expression in Arabidopsis

Seed-specific traits, such as desiccation tolerance, reserve accumulation and entry into quiescence are acquired during seed maturation ((Wobus and Weber, 1999; Vicente-Carbajosa and Carbonero, 2005). In *Arabidopsis*, this phase is genetically controlled by at least four master regulators, namely FUS3, ABI3, LEC1 and LEC2. *FUS3*, *ABI3* and *LEC2* encode TFs containing the conserved B3 DNA binding domain which targets the RY element (Fig. 1) (Giraudat *et al.*, 1992; Luerssen *et al.*, 1998; Stone *et al.*, 2001), whereas *LEC1* encodes a HAP3 subunit of the CCAAT-binding TF (CBF, also known as NF-Y) (Lotan *et al.*, 1998). In addition, two Opaque2-like (O2-like) factors, AtbZIP10 and AtbZIP25, are also involved in SSP gene expression regulation (Lara *et al.*, 2003). Recently, bZIP53 has been reported to play a pivotal and crucial role in quantitative control of seed maturation gene transcription in cooperation with several TFs (Alonso *et al.*, 2009).

FUS3 is a regulator of seed development that is involved in the establishment of dormancy, desiccation tolerance and cotyledon identity, as well as in the synthesis of SSPs and anthocyanin accumulation (Keith *et al.*, 1994; Gazzarrini *et al.*, 2004). ABI3 is a transcription activator required for abscisic acid (ABA) responses in seeds; its importance as a regulator of transcription of seed-specific genes has been demonstrated by the fact that ectopic expression of *ABI3* in vegetative tissues confers on them the ability to accumulate seed-specific transcripts in response to ABA (Parcy *et al.*, 1994; Parcy and Giraudat, 1997). LEC1 is required for normal development during early and late phases of embryogenesis and is sufficient to induce embryonic development in vegetative cells (Lotan *et al.*, 1998). LEC2 is a transcriptional regulator that establishes a cellular environment sufficient to

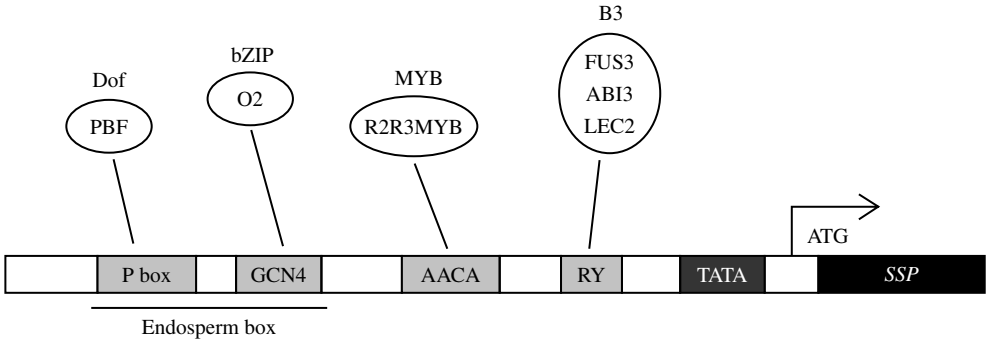

Figure 1. *Cis*-elements and *trans*-acting factors involved in SSP gene regulation in cereals and *Arabidopsis*. Interactions between *cis*-elements and TFs are indicated by solid lines.

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initiate embryo development, and is required for the maintenance of suspensor morphology, specification of cotyledon identity, progression through maturation and suppression of premature germination (Stone *et al.*, 2001). All four *abi3*, *lec1*, *lec2* and *fus3* mutants display severely affected seed maturation and share some common phenotypes, such as reduced expression of SSPs. However, they also show some specific phenotypes, such as the absence of chlorophyll degradation (*abi3*) and of anthocyanin accumulation (*fus3*, *lec1* and *lec2*), altered chemical composition (*lec2*), reduced sensitivity to ABA (*abi3* and *lec1*), intolerance to desiccation (*abi3*, *fus3* and *lec1*) and altered cotyledon identity (*fus3*, *lec1* and *lec2*) (Parcy *et al.*, 1997; Lotan *et al.*, 1998; Vicient *et al.*, 2000; Raz *et al.*, 2001; Kroj *et al.*, 2003; To *et al.*, 2006; Angeles-Nunez and Tiessen, 2011).

Numerous studies have been performed aiming to uncover the mechanisms through which these genes interact to control the various facets of seed maturation. LEC1 controls the expression of the SSP genes in a hierarchical manner, which involves ABI3 and FUS3 (Kagaya *et al.*, 2005). FUS3 and LEC2 seem to act in a partially redundant manner to control SSP gene expression by directly binding to the RY motif present in the promoter regions, and LEC2 locally regulates *FUS3* expression in regions of the cotyledons (Kroj *et al.*, 2003). However, FUS3 also regulates the synthesis of SSPs through TRANSPARENT TESTA GLABRA1 (TTG1) or some other intermediate protein (Tsuchiya *et al.*, 2004). Although ABI3 regulation of SSP genes is also dependent on the RY box, it is yet unclear whether it takes place through a direct interaction or by activation of downstream targets such as FUS3 or LEC2.

The complex interactions among the four major regulators are better understood by analysing *ABI3* and *FUS3* expression in various single, double and triple maturation mutants, which indicated that these regulators are interlocked in a complex hierarchical network of mutual interactions (To *et al.*, 2006). As shown in Fig. 2, LEC1 acts upstream of the main genetic network of seed maturation and positively regulates ABI3 and FUS3. One of the major roles of LEC2 is to upregulate *FUS3* and *ABI3* expression. Additionally, *ABI3* and *FUS3* positively regulate themselves and each other, thereby forming feedback loops essential for their sustained and uniform expression in the embryo. Notably, the gene regulatory controls in this network act locally and redundantly (To *et al.*, 2006). In the root tip, *FUS3* expression is redundantly controlled by *LEC2* and by *FUS3* itself; in the embryo axis *FUS3* expression is redundantly controlled by *LEC2* and *ABI3*; in the cotyledons, *FUS3* expression is under the control of all three regulators (To *et al.*, 2006). In addition to this set of regulatory controls, *LEC1* also locally regulates *FUS3* expression in cotyledons.

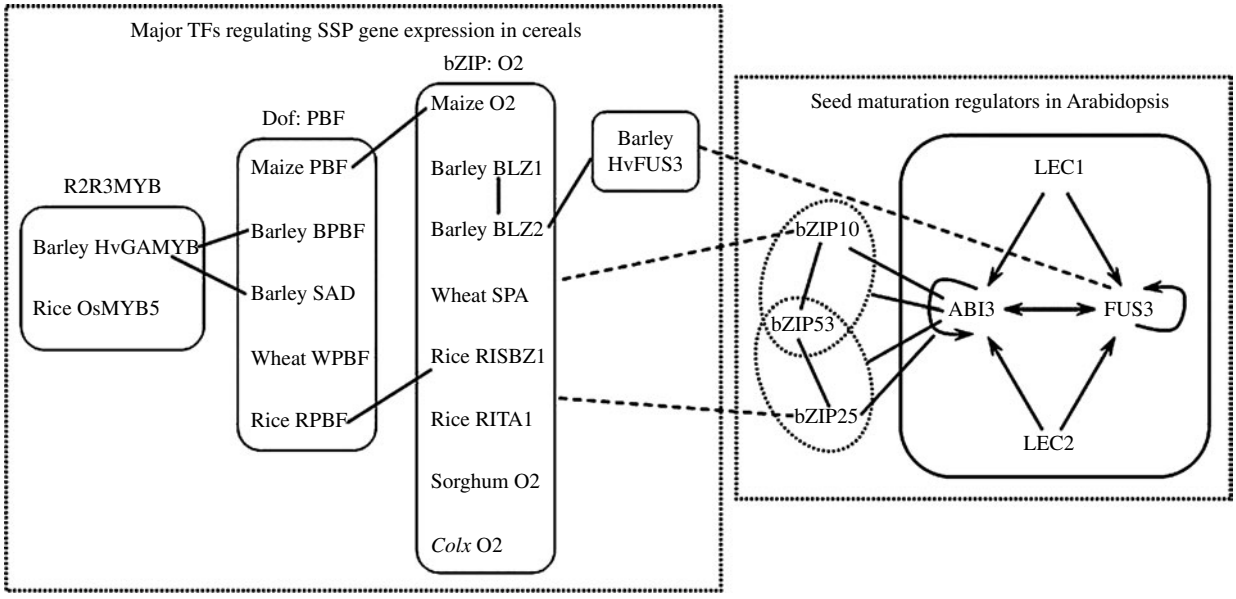

Figure 2. Major TFs involved in the regulation of SSP expression in cereals and *Arabidopsis*. Major TFs regulating SSP gene expression in cereals are located in the dashed box on the left. Major TFs involved in regulation of SSP expression in *Arabidopsis* are located in the dashed box on the right. Four master regulators (LEC1, LEC2, FUS3 and ABI3) of seed maturation in *Arabidopsis* are located in the solid box. An arrow indicates a promotional effect. Interactions between TFs are indicated by solid lines. The interactions between ABI3 and bZIP10/25–bZIP53 heterodimers are also indicated by solid lines. Conservation between bZIP10/25 and O2-like TFs, as well as between FUS3 and HvFUS3, is indicated by the dashed lines.

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Major TFs regulating SSP gene expression in cereals

The *cis* regulatory elements GCN4, P box and AACA, highly conserved in the promoters of genes encoding cereal SSPs, are bound by TFs of the basic leucine zipper (bZIP) (Vicente-Carbajosa *et al.*, 1998; Onate *et al.*, 1999), DNA binding with one finger (DOF) (Lijavetzky *et al.*, 2003; Yanagisawa, 2004) and R2R3MYB families (Diaz *et al.*, 2002), respectively (Fig. 1).

bZIP proteins contain a basic region and a leucine repeat involved in DNA binding and dimerization, respectively, other domains accounting for activation capabilities or for interaction with ancillary proteins of the transcription machinery (Onate *et al.*, 1999). One of the first-described endosperm-specific bZIP TFs in plants involved in regulation of SSP genes is O2 from maize, which can activate the transcription of maize 22 kDa α-zein gene and *b-32* ribosome-inactivating protein gene by recognizing the ACGT core motif and a distinct consensus sequence in their promoters, respectively (Lohmer *et al.*, 1991; Schmidt *et al.*, 1992). O2 also binds and activates promoters of SSP genes obtained from other species such as the wheat *LMWG-1D1* and rice *GluB-1* by binding to GCN4 elements (Holdsworth *et al.*, 1995; Wu *et al.*, 1998), thus indicating that the O2 has broad binding specificity. The central role of O2-like factors in seed-specific expression has been well documented in other cereal species, such as wheat storage protein activator (SPA) (Albani *et al.*, 1997), barley BLZ1 and BLZ2 (Vicente-Carbajosa *et al.*, 1998; Onate *et al.*, 1999), rice RISBZ1 and RITA1 (Izawa *et al.*, 1994; Onodera *et al.*, 2001), sorghum O2 (Pirovano *et al.*, 1994) and *Coix* O2 (Vettore *et al.*, 1998). Out of those, wheat SPA, barley BLZ1 and BLZ2, and rice RISBZ1 *trans*-activate expression of wheat *LMWG-1D1*, barley B-hordein gene *Hor2* and rice *GluB-1* by interacting with the GCN4 elements in their promoter regions, respectively (Albani *et al.*, 1997; Vicente-Carbajosa *et al.*, 1998; Onate *et al.*, 1999; Onodera *et al.*, 2001).

Dof proteins are a family of plant-specific TFs containing a highly conserved DNA-binding domain, the Dof domain (Yanagisawa, 2002), which includes one single Cys2–Cys2 zinc finger. Numerous Dof proteins are involved in diverse plant processes such as seed germination (Mena *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003), photosynthesis (Yanagisawa, 2000), secondary metabolism (Skirycz *et al.*, 2006), phytohormone expression) Baumann *et al.*, 1999) and SSP expression (Diaz *et al.*, 2005) in both monocots and dicots. The *Arabidopsis* and rice genomic projects indicate the presence of 37 and 30 Dof protein-encoding genes, respectively (Yanagisawa, 2002; Lijavetzky *et al.*, 2003). Prolamin-box binding factor (PBF), which belongs to the Dof protein family, is implicated in *trans*-activation of SSP genes from a wide spectrum of cereal grains. In maize, PBF is able to interact with the P box present in the promoters of 22 kDa α-zein and 27 kDa γ-zein genes (Vicente-Carbajosa *et al.*, 1997; Marzabal *et al.*, 2008). Similar types of PBFs have been isolated from barley, wheat and rice, which are designated BPBF (Mena *et al.*, 1998), WPBF (Mena *et al.*, 1998) and RPBF (Yamamoto *et al.*, 2006), respectively. In barley, BPBF, which activates transcription of a native *Hor2* promoter in the barley endosperm through P box-recognition (Mena *et al.*, 1998), is a repressor of the gibberellin-induced thiol-protease cathepsin-B-like gene in aleurone layer cells following germination (Mena *et al.*, 2002). In wheat, transient expression experiments in co-transfected BY-2 protoplast cells demonstrate that WPBF *trans*-activates transcription from native *α-gliadin* promoter through binding to the intact P box (Dong *et al.*, 2007). However, it seems that WPBF transcriptional activity is not specific to wheat storage protein genes since it is not only expressed in the grains but also in other tissues (Dong *et al.*, 2007). Rice RPBF activates the expression of several rice glutelin and prolamin genes by recognizing the P box in their promoters (Yamamoto *et al.*, 2006). In addition, scutellum and aleurone-layer-expressed DOF (SAD) from barley, *trans*-activates transcription of the cathepsin B-like cysteine protease *AI21* promoter in co-bombarded aleurone layers (Isabel-LaMoneda *et al.*, 2003). Also, transient expression experiments in co-bombarded developing barley endosperms demonstrate that SAD *trans*-activates transcription from B-hordein *Hor2* and trypsin-inhibitor BTI-CMe *Itr1* promoters through binding to the P box (Diaz *et al.*, 2005).

OsMYB5 is a MYB protein from rice that specifically binds to the AACA motif within the promoter region of the *GluB-1* gene (Suzuki *et al.*, 1998). In barley, GAMYB, a member of the R2R3MYB family, is not only a regulator of gibberellin-responsive genes during and after seed germination (Gubler *et al.*, 1995), but also activates gene expression during endosperm development through binding to the AACA motif in native *Hor2* and *Itr1* promoters (Diaz *et al.*, 2002). In contrast to endosperm-specific O2-like bZIP TFs and PBF-like TFs, HvGAMYB is also expressed in various non-seed tissues (Diaz *et al.*, 2002).

There have been many reports of cereal TFs involved in SSP gene regulation; however, because there are few mutants, most studies have been carried out *in vitro* (e.g. electrophoretic mobility shift assay for analysis of the interactions between *cis*-elements and TFs) or *in vivo* (e.g. transient assays using yeast or plant cells), with a few exceptions. Mutations of O2 lead to pleiotropic phenotypes such as opaque kernels, a substantial reduction in α-zein and increased total lysine and tryptophan contents (Kawakatsu and Takaiwa, 2010). In contrast, a knock-down (KD) mutant of RISBZI (KD-RISBZ1) or RPBF (KD-RPBF)

does not show a large reduction in the SSPs, whereas double KDs of these TFs results in a significant reduction of most SSP gene expression and accumulation (Kawakatsu *et al.*, 2009). In addition, the double KD line contains much less starch and a reduced lipid content compared with the wild type, indicating redundant functions of RISBZ1 and RPBF during rice grain filling (Kawakatsu *et al.*, 2009).

Interactions among TFs

Transcriptional regulation is normally exerted by the concerted action of multiple TFs responding to distinct signals. Consequently, the anticipated extent of gene expression arises from the effects of various TFs acting on a particular promoter and their combinatorial interactions. The importance of such interactions (e.g. ABI3–bZIP10/25, bZIP53–bZIP10/25, bZIP53–bZIP10/25–ABI3, PBF–O2, RPBF–RISBZ1, BPBF–HvGAMYB, SAD–HvGAMYB, BLZ1–BLZ2, HvFUS3–BLZ2) has already been reported in the regulation of seed-specific genes of both *Arabidopsis* and cereals (Fig. 2). In *Arabidopsis*, AtbZIP10 and AtbZIP25 are involved in the regulation of SSP gene expression in a concerted manner with ABI3 (Lara *et al.*, 2003). Recently, bZIP53 has been demonstrated to enhance seed maturation gene expression by specific heterodimerization with bZIP10 or bZIP25 (Alonso *et al.*, 2009). Furthermore, these bZIP heterodimers interact with ABI3, which further increases seed maturation gene activation (Alonso *et al.*, 2009). In maize, the interaction between PBF and O2 is involved in the zein protein expression during seed development (Vicente-Carbajosa *et al.*, 1997). In rice, RPBF activates rice glutelin and prolamin genes in cooperation with RISBZ1 (Yamamoto *et al.*, 2006). Moreover, the combinatorial interactions between them play an essential role during grain filling (Kawakatsu *et al.*, 2009). In barley, BPBF interacts with HvGAMYB through its C-terminal domain to activate barley-endosperm-specific genes during seed development (Diaz *et al.*, 2002). Another Dof protein, SAD, also interacts with HvGAMYB *in vivo* through its C-terminal domain. This interaction enhances the transcription activation of a gibberellin-induced protease promoter in aleurone layer cells (Isabel-LaMoneda *et al.*, 2003; Diaz *et al.*, 2005). Barley BLZ2 protein can interact with BLZ1 *in vivo* and activates transcription from the GCN4 motif of B-hordein promoters in the barley endosperm (Onate *et al.*, 1999). FUSCA3 from barley, HvFUS3, can interact with BLZ2 in a yeast two-hybrid system, and this interaction is essential for full *trans*-activation of the seed-specific genes *Hor2* and *Itr1* (Moreno-Risueno *et al.*, 2008). Therefore, cooperation of transcriptional regulators by protein–protein interactions provides an efficient mechanism to control gene expression in seeds and explains some of the molecular mechanisms underlying SSP gene expression.

Evolutionary conservation between dicot and monocot species

Most interestingly, the prolamins (the main group of SSPs in many cereal grains) and a major group of dicot seed albumins can be traced phylogenetically to a common ancestor (Kreis and Shewry, 1989). Their conservation relates not only to protein structure, but also to regulatory elements and *trans*-acting factors that are functionally exchangeable between the groups. The RY motif, which is widely distributed in the promoters of dicot species, is also conserved in the promoters of SSP genes of cereals, such as the barley *Hor2*, maize 22 kDa α-zein and 15 kDa β-zein, wheat α-gliadin and HMW-glutenin, rice *GluA-1* and *GluB-3*, and *Coix* α-coixin. Characterization of the TFs involved in seed gene expression provides further evidence for evolutionary conservation. In maize, Viviparous1 (VP1), which is the orthologue of *Arabidopsis* ABI3, is also involved in ABA signalling, establishment of dormancy and activation of maturation-specific genes during seed development (McCarty *et al.*, 1991; Suzuki *et al.*, 2003). Maize VP1, expressed in the embryo and aleurone layer, can complement *Arabidopsis abi3* mutants that are impaired in their expression of SSPs (Suzuki *et al.*, 2001). In addition, a detailed characterization of AtbZIP10 and AtbZIP25 of *Arabidopsis* showed that they are both structurally and functionally the most closely related to O2-like cereal TFs, and might represent their *Arabidopsis* counterparts involved in SSP gene regulation (Lara *et al.*, 2003). Both the barley and *Arabidopsis FUS3* genes maintain a conserved functionality for the regulation of SSP genes and anthocyanin biosynthesis in these two distantly related phylogenetic groups. Complementation of the loss-of-function mutant *fus3* in *Arabidopsis* by the barley *HvFus3* gene results in restored transcription of the *At2S3* gene promoter and normal accumulation of anthocyanins in the seed (Moreno-Risueno *et al.*, 2008).

Conclusions and outlook

The SSPs are of great importance in determining the quality and end-use properties of the seed. Transcriptional regulation of SSP genes is achieved by a variety of protein–DNA and protein–protein interactions. Understanding the expression pattern and regulation of these genes is important to underpin the attempts to change the content of seed reserves by genetic engineering. While studies on gene regulation governing seed maturation have been performed extensively

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in *Arabidopsis*, less progress has been made concerning the mechanisms regulating cereal SSP expression. One of the major reasons is a lack of loss-of-function mutants for cereal storage protein synthesis or the relevant regulatory genes. Thus, identification and characterization of mutants is necessary to better understand the molecular mechanism of cereal SSP gene regulation and to identify new regulators.

Transcriptional regulation of SSP genes does not constitute the full extent of the genetic network underlying seed maturation. Indeed, the spatial and temporal regulation of seed maturation requires the concerted action of several signalling pathways that integrate information from genetic programmes and from both hormonal and metabolic signals. Moreover, SSP levels are regulated by several steps at the transcriptional, translational and post-translational levels (modification, processing, trafficking and deposition). In recent years, our understanding of seed maturation regulation has greatly increased, thanks to the diversity of experimental approaches available. However, some other areas, such as DNA methylation, will have to be considered in future research. DNA methylation is a stable epigenetic modification associated with transposable element silencing and gene imprinting in plant and animals. Recently, two publications have provided evidence for the extensive occurrence of demethylation during *Arabidopsis* seed development, especially of the endosperm (Gehring *et al.*, 2009; Hsieh *et al.*, 2009). In one, it was demonstrated that large-scale methylation changes accompany endosperm development and endosperm-specific gene expression, and transposable element fragments are extensively demethylated in the endosperm (Gehring *et al.*, 2009). In the other, it is suggested that virtually the entire endosperm genome is demethylated, coupled with extensive local non-CG hypermethylation of small interfering RNA-targeted sequences (Hsieh *et al.*, 2009). These discoveries provide new insights into the mechanisms regulating SSP expression.

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