

Role of WRINKLED1 in the transcriptional regulation of glycolytic and fatty acid biosynthetic genes in Arabidopsis

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SUMMARY

The WRINKLED1 (WRI1) protein is an important regulator of oil accumulation in maturing Arabidopsis seeds. WRI1 is a member of a plant-specific family of transcription factors (AP2/EREBP) that share either one or two copies of a DNA-binding domain called the AP2 domain. Here, it is shown that WRI1 acts as a transcriptional enhancer of genes involved in carbon metabolism in transgenic seeds overexpressing this transcription factor. *PKp-β1* and *BCCP2*, two genes encoding enzymes of the glycolysis and fatty acid biosynthetic pathway, respectively, have been chosen to investigate the regulatory action exerted by WRI1 over these pathways. Using the reporter gene *uidA*, it was possible to demonstrate *in planta* that WRI1 regulates the activity of both *PKp-β1* and *BCCP2* promoters. Electrophoretic mobility-shift assays and yeast one-hybrid experiments showed that WRI1 was able to interact with the *BCCP2* promoter. To further elucidate the regulatory mechanism controlling the transcription of these genes, functional dissections of *PKp-β1* and *BCCP2* promoters were performed. Two enhancers, of 54 and 79 bp, respectively, have thus been isolated that are essential to direct the activity of these promoters in oil-accumulating tissues of the embryo. A consensus site is present in these enhancers as well as in other putative target promoters of WRI1. Loss of this consensus sequence in the *BCCP2* promoter decreases both the strength of the interaction between WRI1 and this promoter in yeast and the activity of the promoter *in planta*.

Keywords: Arabidopsis, WRINKLED1, transcriptional regulation, *BCCP2*, *PKp-β1*, seed.

INTRODUCTION

After the biochemical pathways producing storage lipids in oilseed species like *Brassica napus* or *Arabidopsis thaliana* were largely described (Voelker and Kinney, 2001), the elucidation of the regulation of oil synthesis has become a major challenge (Ohlrogge and Jaworski, 1997). Complementary studies carried out on developing seeds and/or embryos have established that the biosynthetic pathways for fatty acids and lipids are largely regulated at the level of transcription (Fawcett *et al.*, 1994; O'Hara *et al.*, 2002; Baud and Graham, 2006). In *A. thaliana*, microarray analyses have been used to characterize the 'contrapuntal' or differential timing of the expression of genes involved in fatty-acid biosynthesis and lipid metabolism during seed maturation (Ruuska *et al.*, 2002). Genes related to the biosynthesis and storage of triacylglycerols (TAGs) show several distinct temporal expression patterns. For instance, a number of genes encoding fatty acid synthesis enzymes display a bell-shaped pattern of expression at the onset of the maturation phase, whereas the expression of fatty-acid modifying enzymes and oleosins increases later (Baud and Lepiniec,

2009). The observation that the mRNAs of several genes encoding enzymes of the fatty acid biosynthetic pathway accumulate in a coordinated manner strongly suggests that those members of the pathway are co-regulated, and presumably share common *cis*- and *trans*-regulatory elements. This hypothesis has been strengthened by the isolation and characterization of the WRINKLED1 (WRI1) transcription factor (Focks and Benning, 1998; Cernac and Benning, 2004; Masaki *et al.*, 2005). WRI1 is a direct target of LEAFY COTYLEDON2 (LEC2) that specifies the regulatory action of this master regulator of seed maturation towards fatty acid metabolism (Baud *et al.*, 2007a). Putative targets of WRI1 encode enzymes of late glycolysis, the fatty acid synthesis pathway, and the biotin and lipoic acid biosynthetic pathways (Ruuska *et al.*, 2002; Baud *et al.*, 2007a).

WRI1 is a member of the APETALA2/ethylene-responsive element binding (AP2/EREBP) proteins, one of the largest transcription factor families in *A. thaliana* (Riechmann *et al.*, 2000). This family is best characterized by a common AP2 domain of about 60 amino acids that is important for DNA

binding (Jofuku *et al.*, 1994; Okamuro *et al.*, 1997; Riechmann and Meyerowitz, 1998; Sakuma *et al.*, 2002). While the AP2/EREBP transcription factor family is unique to plants, proteins containing homologues of the AP2 domain have been identified in cyanobacteria, ciliates and viruses, where they are predicted to function as mobile endonucleases (Magnani *et al.*, 2004; Wessler, 2005; Shigyo *et al.*, 2006). Based on the presence of the conserved AP2-like domains, 147 proteins were identified as belonging to the AP2/EREBP family in *A. thaliana* (Feng *et al.*, 2005). The AP2/EREBP family members are classified in groups based on the number of AP2 domains and the presence of other domains. The EREBP family (122 members), which has a single AP2 domain, is composed of two subgroups, namely the ERF (65 members) and DREB subfamilies (57 members). Proteins of the RAV family (six members) contain one AP2 domain and a second DNA binding domain (the B3-like domain). The AP2 family (19 members), which has two AP2 domains, is further divided into two monophyletic groups, the APETALA2-like and AINTEGUMENTA (ANT)-like groups. Depending on the publications, the WRI1-like group, composed of WRI1, At2g41710, At1g16060 and At1g79700, is either presented as a third monophyletic group of the AP2 family (Nole-Wilson *et al.*, 2005) or as a part of the ANT-like group (Shigyo *et al.*, 2006).

DNA binding specificity has been studied for proteins that contain a single AP2 repeat. Proteins of the ERF subfamily bind to ethylene response elements (ERE) or GCC boxes. These boxes consist of 11-bp sequences (TAAGAGCGGCC) exhibiting a GCCGCC core required for protein binding (Ohme-Takagi and Shinshi, 1995; Fujimoto *et al.*, 2000; Song *et al.*, 2005). The solution structure of the AP2 domain of AtERF1 bound to the GCC box has been determined (Allen *et al.*, 1998). Proteins of the DREB subfamily bind to dehydration response elements (DREs)/C-repeats containing the core sequence CCGAC (Baker *et al.*, 1994). The single AP2 repeat of the RAV1 transcription factor has been shown to bind to a CAACA motif, whereas the B3 domain binds to a CACCTG sequence (Kagaya *et al.*, 1999). Little is known about the interactions between members of the AP2 family of proteins and DNA. A consensus binding sequence of the AINTEGUMENTA (ANT) protein determined *in vitro* is 5'-gCAC(A/G)N(A/T)TcCC(a/g)ANG(c/t)-3' (Nole-Wilson and Krizek, 2000). This consensus is much longer than sites recognized by proteins containing a single AP2 repeat. Each AP2 repeat of ANT contacts juxtaposed subsites within the consensus sequence, demonstrating that ANT utilizes a mode of DNA recognition distinct from that used by proteins containing a single AP2 domain (Krizek, 2003). To date, given the lack of information about the binding properties of proteins of the AP2-like and WRI1-like groups, it has not been possible to determine whether all of the transcription factors of the AP2 family share a similar mode of DNA recognition.

In this paper, it is shown that WRI1 can be used in a seed-specific manner to enhance the transcription level of glycolytic and fatty acid biosynthetic genes in tissues where these genes are already expressed. Consistent with this, it is demonstrated that WRI1 is able to regulate *in planta* the activity of the *BCCP2* and *PKp- β 1* promoters. In addition, electrophoretic mobility-shift assays and yeast one-hybrid experiments show that WRI1 is able to interact with the *BCCP2* promoter. These results strongly suggest that WRI1, which is a limiting factor of lipogenic gene expression in seeds, directly induces the transcriptional activation of these genes at the onset of the maturation phase. Functional dissections of the *PKp- β 1* and *BCCP2* promoters allow isolating putative binding sites to be present in several target promoters of WRI1. Finally, the modification of the consensus sequence thus isolated modulates the strength of the interaction between WRI1 and the *BCCP2* promoter, both *in vitro* and in yeast, as well as the activity of the promoter *in planta*. Taken together, these results provide original insights into our understanding of the transcriptional activation of the glycolytic and fatty acid biosynthetic pathways in *A. thaliana*, and they pave the way for further analyses aimed at elucidating the regulatory network controlling oil accumulation in seeds.

RESULTS

Seed-specific overexpression of *WRI1* enhances the mRNA accumulation of glycolytic and fatty acid biosynthetic genes

Transcriptomic analyses (Ruuska *et al.*, 2002) and quantitative reverse transcriptase (RT)-PCR experiments (Baud *et al.*, 2007a) have previously been carried out on plant material exhibiting altered *WRI1* mRNA levels, e.g. *wri1* seeds or rosette leaves overexpressing *WRI1*. These approaches have led to the identification of putative target genes of WRI1 that encode enzymes of late glycolysis and the *de novo* fatty acid biosynthetic network in plastids. WRI1 has consequently been presented as a potential activator of these pathways in plants that could be exploited in biotechnological approaches designed to stimulate oil accumulation in seeds (Cernac and Benning, 2004). The ability of WRI1 to enhance the mRNA accumulation of glycolytic and fatty acid biosynthetic genes has been clearly established in seedlings (Cernac and Benning, 2004) and rosette leaves (Baud *et al.*, 2007a), where these genes are normally expressed at low/basal levels. It was then essential to check whether *WRI1* overexpression could increase the mRNA accumulation of these genes in maturing seeds, where they are already expressed at high levels. Unfortunately, *PRO35S_{dual}:WRI1* lines (Baud *et al.*, 2007a) could not be exploited for such a purpose. These transgenic lines exhibited a stunted, bushy phenotype, and were partially sterile. Fine physiological analyses of maturing seeds could not be carried with the very poor seed set present in developing siliques (Table 1).

Table 1 Characterization of the *Pro_{35S}uidA::WRI1* lines

	Wild type	<i>Pro_{35S}uidA::WRI1</i> lines			
		T31	T63	T66	T81
<i>WRI1</i> mRNA level in leaves (% <i>EF</i>)	ND	154 ± 12	674 ± 70	324 ± 24	543 ± 100
Number of seeds per plant (× 10 ⁻³)	24.7 ± 2.9	8.3 ± 1.1	2.9 ± 1.0	1.5 ± 0.3	11.0 ± 2.8
Seed dry weight (μg)	20.2 ± 0.4	20.2 ± 0.4	23.3 ± 1.0	21.3 ± 0.4	20.9 ± 0.6
Seed fatty acid content (μg mg ⁻¹ DW)	415.6 ± 5.7	366.7 ± 9.4	309.4 ± 5.5	323.0 ± 4.9	376.1 ± 17.9

Quantitative RT-PCR analyses of the relative *WRI1* mRNA levels were performed in rosette leaves. The results obtained are standardized to the constitutive *EF1αA4* gene expression level (% *EF*). Three independent cDNA preparations (biological replicates) were analysed; one technical replication was carried out with each cDNA population considered. Values are the means ± SE of three measurements. ND, not detected.

The seed dry weight (DW) determination was obtained by weighing batches of 20 seeds. The number of seeds produced per plant was estimated by weighing the entire seed production. Total lipid extraction was carried out on batches of 20 seeds. The total fatty acid content of seeds was determined by GC analysis of an aliquot. Values are the means ± SE of four replicates carried out with seeds from four distinct individuals. DW, dry weight.

Alternatively, *WRI1* cDNA was placed under the control of the strong and seed-specific oleosin *S2/OLEO4* (*At3g27670*) promoter (Schmid *et al.*, 2005). First, a *Pro_{S2}uidA* transgene was constructed that allowed the pattern of activity of the *S2* promoter to be verified: *Pro_{S2}* activity was only detected in the embryonic tissues of maturing seeds (M. Miquel and N. Reinhard Dichow, unpublished data). A *Pro_{S2}WRI1* transgene was then constructed and tested for its ability to complement the strong *wri1-1* mutation (Focks and Benning, 1998; Cernac and Benning, 2004). Five independent *wri1-1* plants carrying this construct were selected, and their progeny were subjected to fatty acid analyses (Figure 1). If fully complemented, segregating seeds of F₁ progeny are expected to exhibit at least a 75% reversion of the mutant phenotype (one insertion locus). A significant restoration of both fatty acid concentration (Figure 1) and fatty acid composition (data not shown) was measured in all five lines, demonstrating that a functional *WRI1* protein was synthesized. Transgenic plants carrying the *Pro_{S2}WRI1* construct in a Wassilewskija (Ws) wild-type background were then generated to avoid any indirect effect of the *wri1-1* mutation

on plant growth. Four primary transformants with a single insertion locus (see Experimental procedures) were characterized. Neither vegetative growth nor fertility was affected in these lines. Using a quantitative RT-PCR strategy, *WRI1* mRNA level was quantified in maturing siliques of these lines (Figure 2). Expression levels ranging from 370 to 1040% of the *EF1αA4* (*At5g60390*) mRNA level (constitutive expression; Nesi *et al.*, 2000) were detected 16 days after anthesis (DAA), indicating that *WRI1* was efficiently overexpressed in these lines (from 25- to 70-fold compared with the wild type). Using a similar procedure, the mRNA levels of five putative targets of *WRI1* were then analysed in these tissues. The genes considered encoded the BCCP2 subunit of heteromeric acetyl-CoA carboxylase (*At5g15530*), plastidial pyruvate kinases PKp-α (*At3g22960*) and PKp-β1 (*At5g52920*), phosphoglycerate mutase (PhoM, *At1g22170*) and enolase (ENOp, *At1g74030*). The five genes appeared to be significantly overexpressed in *Pro_{S2}WRI1* siliques (Figure 2). However, the increase in mRNA accumulation measured for these target genes of *WRI1* (1.5–3-fold increase on average, depending on the gene considered) was limited in comparison with the increase in *WRI1* mRNA level observed in the same lines (see above). To test the impact of these increased mRNA levels on seed physiology, corresponding enzymatic activities were measured in maturing seeds aged 16 DAA (Table 2). Pyruvate kinase, phosphoglycerate mutase and enolase activities were thus investigated: none of these enzymatic activities appeared to be significantly increased in *Pro_{S2}WRI1* seeds. Likewise, the TAG content of mature transgenic seeds was not improved compared with the wild-type content (Table 2).

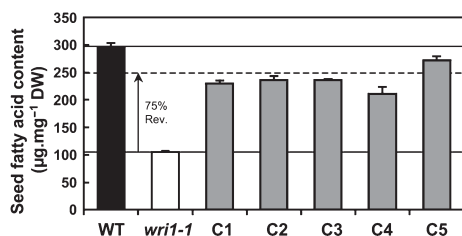


Figure 1. Rescue of oil phenotypes in mature *wri1-1* seeds by seed-specific overexpression of *WRI1* cDNA.

The fatty acid concentration of mature dry seeds was determined by GC analysis. Mutant *wri1-1* lines transformed with the *Pro_{S2}WRI1* construct are annotated C1–C5. Fully complemented, segregating seeds of F₁ progeny are expected to exhibit a 75% reversion (75% Rev.) of the mutant phenotype (one insertion locus). This threshold is presented on the graph as a dotted line. Values are the means and SE of three replicates carried out on batches of 20 seeds from three distinct individuals.

***WRI1* and its putative target genes exhibit resembling expression patterns**

In order to gain further insights into the regulation of *WRI1* targets, two of these genes, namely *PKp-β1* and *BCCP2*, were considered for an in-depth characterization of their

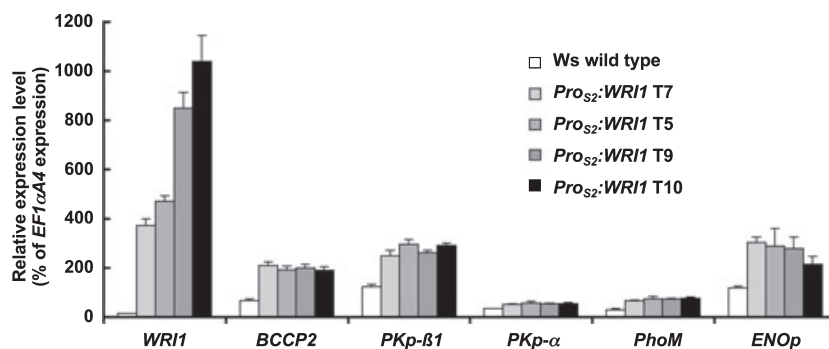


Figure 2. Overexpression of glycolytic and fatty acid biosynthetic genes in response to seed-specific overexpression of *WRI1* cDNA.

Quantitative RT-PCR analyses of the relative mRNA levels of *WRI1*, and of five lipogenic genes encoding *BCCP2*, *PKp-β1*, *PKp-α*, a phosphoglycerate mutase (*PhoM*) and an enolase (*ENOp*), were performed in siliques aged 16 days after anthesis. The results obtained are standardized to the constitutive *EF1α4* gene expression level. Three independent cDNA preparations (biological replicates) were analysed; one or two technical replications were carried out with each cDNA population considered. Values are the means \pm SEs of between three and six measurements.

transcriptional regulation. A quantitative RT-PCR approach was first carried out to finely characterize the relative mRNA accumulation profiles of these genes in a developmental series of wild-type siliques of the Columbia (Col-0) accession, ranging from 4 to 22 DAA (Figure 3a). The two genes studied exhibited identical patterns. Both *PKp-β1* and *BCCP2* relative transcript levels were relatively low during early silique development (up to 6 DAA). A sharp increase in expression was observed from 6 to 12 DAA, corresponding to the onset of seed maturation. Then, mRNA levels gradually decreased throughout the mid-maturation phase (12–18 DAA), and remained low during late maturation (18–22 DAA). The *WRI1* relative mRNA accumulation profile was determined on the same developmental series (Figure 3b). The profile obtained was very similar to the ones described above for *PKp-β1* and *BCCP2*. However, the successive rise and fall in *WRI1* relative mRNA levels were both initiated slightly earlier than the corresponding increase and decrease in *PKp-β1* and *BCCP2* transcript levels. The peak in *WRI1* expression was consequently observed at 10 DAA. These observations were fully consistent with the hypothe-

sized function of *WRI1* in triggering the transcription of *PKp-β1* and *BCCP2*. In addition, these results were confirmed using cDNAs obtained from a developmental series of isolated seeds ranging from 6 to 18 DAA (Figure S1).

Patterns of *PKp-β1* and *BCCP2* promoter activity in *wri1* and *Pro_{35S}duai:WRI1* backgrounds

To further evaluate the impact of changes in *WRI1* mRNA levels on the patterns of *PKp-β1* and *BCCP2* promoter activity, promoter fragments of 523 bp (*PKp-β1*) and 580 bp (*BCCP2*) were fused translationally to the *uidA* reporter gene. The chimerical constructs were assayed for their expression in transgenic *A. thaliana* lines, and we checked that the profiles of GUS activity thus obtained were consistent with the profiles of mRNA accumulation previously observed using qRT-PCR (see above). In a wild-type background, *Pro_{PKp-β1-423}* and *Pro_{BCCP2-580}* activities were detected in maturing embryos (Figure 4a). The staining observed was intense both in the hypocotyl and in the cotyledons of the embryo. On the contrary, neither *Pro_{PKp-β1-423}* nor *Pro_{BCCP2-580}* activity could be detected in leaf blades from

Table 2 Characterization of the *Pro_{S2}:WRI1* lines

	Wild type	<i>Pro_{S2}:WRI1</i> lines			
		T7	T5	T9	T10
PhoM activity (nmol NADH oxydized min ⁻¹)	16.5 \pm 1.0	18.4 \pm 0.8	20.7 \pm 0.8	19.8 \pm 0.7	20.3 \pm 1.0
ENO activity (nmol NADH oxydized min ⁻¹)	18.6 \pm 1.5	18.6 \pm 1.6	24.3 \pm 1.4	19.6 \pm 1.2	20.1 \pm 1.1
PKp activity (nmol NADH oxydized min ⁻¹)	14.2 \pm 0.8	13.9 \pm 1.2	16.6 \pm 0.9	14.2 \pm 1.0	15.8 \pm 1.2
Number of seeds per plant ($\times 10^{-3}$)	24.7 \pm 2.9	26.8 \pm 4.5	23.2 \pm 1.8	22.4 \pm 4.7	21.8 \pm 2.4
Seed DW (μ g)	20.2 \pm 0.4	19.5 \pm 0.5	19.1 \pm 0.3	19.6 \pm 0.7	19.4 \pm 0.3
Seed fatty acid content (μ g mg ⁻¹ DW)	415.6 \pm 5.7	396.3 \pm 7.7	393.1 \pm 5.9	404.2 \pm 9.4	402.3 \pm 5.3

Enzymatic activities (for 100 seeds) were carried out on batches of maturing seeds aged 16 days after anthesis. Values are the means \pm SE of between seven and 11 independent replicates. PhoM, phosphoglycerate mutase; ENO, enolase; PKp, plastidial pyruvate kinase.

The seed dry weight (DW) determination was obtained by weighing batches of 20 seeds. The number of seeds produced per plant was estimated by weighing the entire seed production. Total lipid extraction was carried out on batches of 20 seeds. Total fatty acid content of seeds was determined by GC analysis of an aliquot. Values are the means \pm SE of four replicates carried out with seeds from four distinct individuals. DW, dry weight.

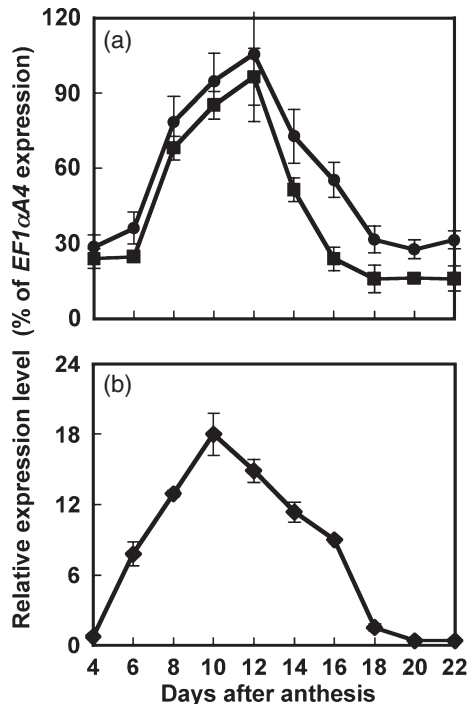


Figure 3. Comparison of *BCCP2*, *PKp-β1* and *WRI1* transcript levels in developing siliques.

Quantitative RT-PCR analyses of the relative mRNA levels of *BCCP2* (■) and *PKp-β1* (●) were performed in siliques of the Col-0 accession (a). The relative mRNA level of *WRI1* was investigated in parallel (b). The results obtained are standardized to the constitutive *EF1αA4* gene expression level. Two independent cDNA preparations (biological replicates) were analysed; between one and three technical replications were carried out with each cDNA population considered. Values are the means \pm SEs of between three and five measurements.

the rosette (Figure 4b). The constructs were then introduced into *wri1-1* and *wri1-3* mutant backgrounds to test whether these mutations could affect the activity of *PKp-β1* and *BCCP2* promoters. In contrast to the observations in wild-type plants, neither *ProPKp-β1-423* nor *ProBCCP2-580* activity could be detected in maturing *wri1* embryos (Figure 4a). The constructs were finally introduced in two independent *Pro35S_{duar}:WRI1* overexpressing lines (T63 and T81). As *PKp-β1* and *BCCP2* are already expressed at high levels in maturing seeds of the wild type, testing an increased induction of *PKp-β1* and *BCCP2* transcription by *WRI1* overexpression in this tissue was not convenient. On the contrary, it was possible to observe a strong induction of both *ProPKp-β1-423* and *ProBCCP2-580* activities in rosette leaves (Figure 4b). These results established that *WRI1* is necessary and sufficient to enhance the activity of both *PKp-β1* and *BCCP2* promoters *in planta*.

Isolation of key regulatory elements in the promoters of *BCCP2* and *PKp-β1*

To identify the domains within the 580-bp *BCCP2* promoter that might be important for the transcriptional regulation of

tissue specificity, 5' deletions were generated and translational fusions to the *uidA* reporter gene were constructed. For each construct, several stable transformants were obtained and analysed for GUS activity (Figure 4c). Deletions to positions –402, –202 and –180 did not affect the *uidA* expression pattern and apparent intensity. Deletions to positions –168 and –160 did not affect the *uidA* expression pattern in mid-maturing embryos, but apparent intensity was markedly decreased, so that the GUS activity could not be detected in young torpedo-shaped embryos. The GUS activity was further decreased, with deletion to position –147: the staining observed in maturing embryos was extremely pale. A further deletion to position –126 resulted in a complete loss of GUS activity. These observations led us to conclude that the region between –180 and –126 contained key elements necessary for the induction of *BCCP2* in maturing embryos of *A. thaliana*. A similar approach was then carried out to identify domains within the 523-bp *PKp-β1* promoter that might be important for its transcriptional regulation (Figure 4d). Deletions to positions –422, –317, –274, –266 and –246 did not affect the *uidA* expression pattern and apparent intensity. Deletion to positions –233 did not affect the *uidA* expression pattern, but the apparent intensity was decreased. A further deletion to position –177 resulted in an almost complete loss of GUS activity: a faint staining could be detected in some cotyledon and hypocotyl cells of mid-maturing embryos. These data strongly suggest that the region between –256 and –177 contained some of the elements necessary for induction of *PKp-β1* in maturing embryos of *A. thaliana*. A comparison of the two promoter regions thus identified was then undertaken to isolate common *cis*-regulatory elements putatively involved in the simultaneous induction of both *BCCP2* and *PKp-β1* (Figure 5a). A 15-bp sequence (5'-CAAAAGGAGCGGTTT-3') was detected in the promoter of *BCCP2* between –179 and –165 that was very similar to the 15-bp sequence (5'-CAAAAGTACGAGTTT-3') present in the *PKp-β1* promoter between –253 and –239. In each of the promoter regions considered, a second version, slightly less conserved, of this 15-bp motif was observed, either on the plus strand (*BCCP2*) or on the minus strand (*PKp-β1*) of the DNA sequence examined. Finally, database comparison using PLACE showed the presence of soybean embryo factor 3 (SEF3) recognition sequences (Allen *et al.*, 1989) in each of the two promoter regions studied. Interestingly, these SEF3 motifs were entirely (*BCCP2*) or partially (*PKp-β1*) included in one of the 15-bp motifs described above.

Characterization of the key regulatory elements identified in the *BCCP2* promoter

Deletion series of the *BCCP2* promoter have shown that nucleotides from –180 to –126 contain a seed-specific enhancer. This promoter region encompasses two 15-bp motifs, the complete removal of which dramatically

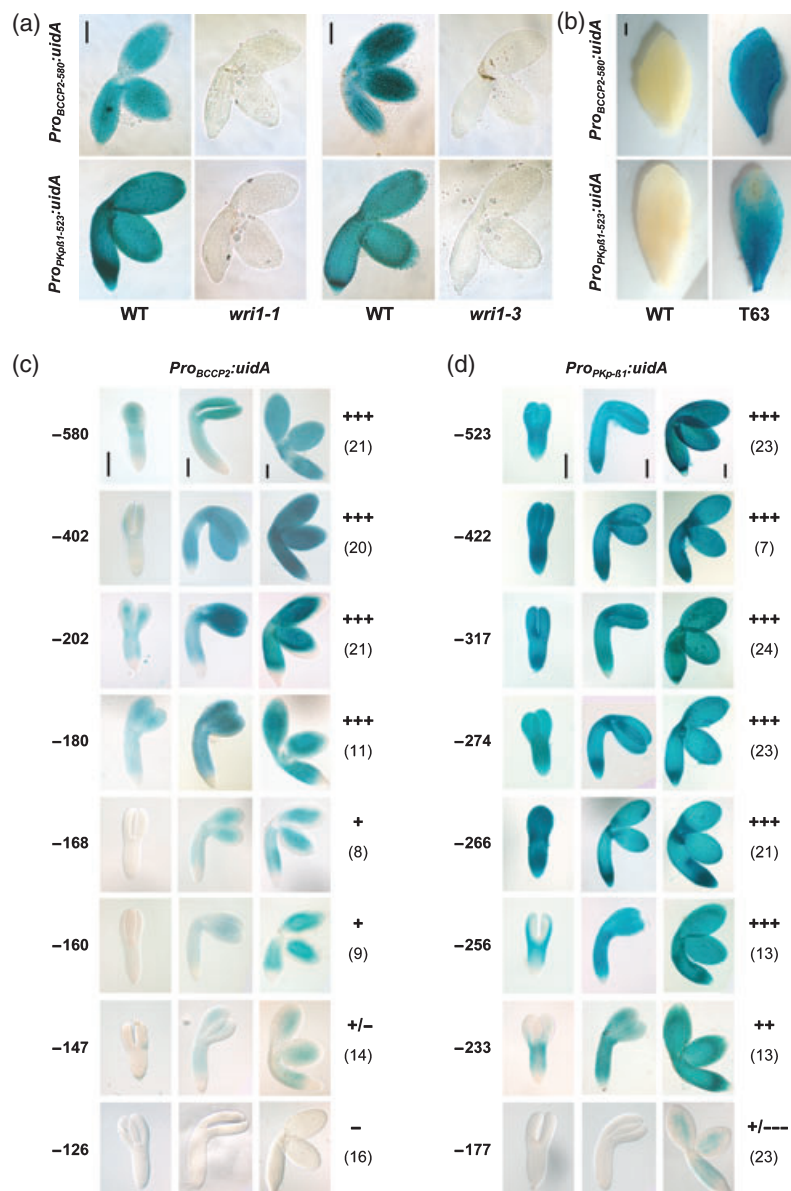


Figure 4. Transcriptional regulation of *BCCP2* and *PKp-β1*.

(a) The pattern of activity of *Pro_{BCCP2-580}:uidA* and *Pro_{PKpβ1-523}:uidA* cassettes in wild-type (WT), *wri1-1* and *wri1-3* mutant embryos was observed during the seed maturation phase. Scale bars: 100 μ m.

(b) The pattern of activity of *Pro_{BCCP2-580}:uidA* and *Pro_{PKpβ1-523}:uidA* cassettes was observed in wild-type (WT) and *Pro_{35S_{Dual}::WRI1}* (T63) rosette leaves. Scale bar: 5 mm.

(c) Dissection of the *BCCP2* promoter: a series of 5' deletions was generated, and translational fusions to the *uidA* gene were prepared. The corresponding transgenic embryos were assayed for GUS activity at torpedo, bent-cotyledon and maturing stages. The length of the promoter tested is indicated on the left-hand side of the panels; position +1 refers to the translational start of the *BCCP2* gene. The number of independent transgenic lines analysed is indicated, between brackets, on the right-hand side of the panels. Representative pictures are presented. +++, the embryos displayed a strong staining pattern, similar to the one observed with full-length *BCCP2* promoter; +, the embryos displayed a weak staining pattern; +/-, the embryos displayed a very weak staining pattern; -, the embryos were not stained. Scale bars: 100 μ m.

(d) Dissection of the *PKp-β1* promoter: a series of 5' deletions was generated and translational fusions to the *uidA* gene were prepared. The corresponding transgenic embryos were assayed for GUS activity at the torpedo, bent-cotyledon and maturing stages. The length of the promoter tested is indicated on the left-hand side of the panels; position +1 refers to the translational start of the *PKp-β1* gene. The number of independent transgenic lines analysed is indicated, between brackets, on the right-hand side of the panels. Representative pictures are presented. +++, the embryos displayed a strong staining pattern, similar to the one observed with full-length *PKp-β1* promoter; ++, the embryos displayed a decreased although unaltered staining pattern; +/-, the embryos displayed an extremely weak staining pattern. Scale bars: 100 μ m.

For histochemical detection of GUS activity, tissues were incubated for 4 h (embryos) or 16 h (leaves) in a buffer containing 2 mM (embryos) or 0.2 mM (leaves) of potassium ferrocyanide and potassium ferricyanide. For maturing embryos, the result of the GUS activity was observed with Nomarski optics on whole mounts of excised embryos.

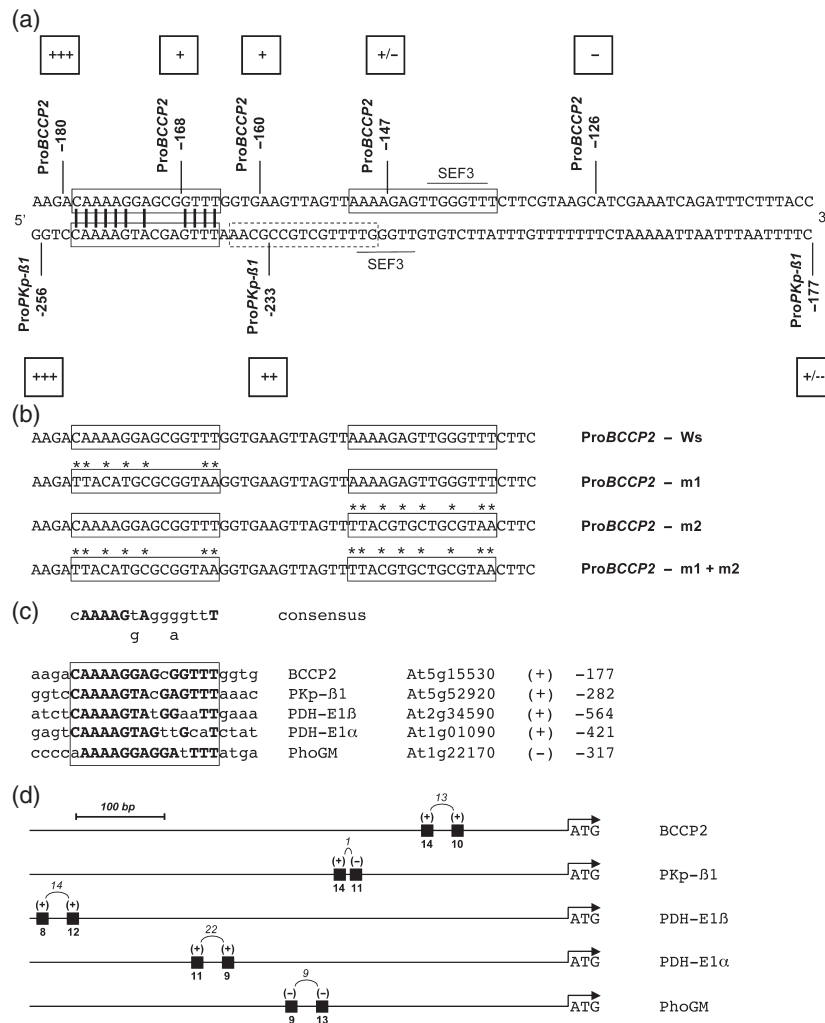


Figure 5. Putative regulatory elements in the *BCCP2* and *PKp-β1* promoters.

(a) Comparison of the key regulatory elements identified in promoters of the *BCCP2* and *PKp-β1* genes. The relative intensities of GUS staining observed while dissecting these promoters have been reported in the open boxes. The putative soybean embryo factor 3 (SEF3) binding sites, detected by the analysis of the promoter sequences with the SignalScan server (PLACE database), are underlined. The 15-bp motifs observed in the two regulatory regions of *BCCP2* and *PKp-β1* and promoters are framed with either solid (presence of the motif on a positive DNA strand) or broken lines (presence of the motif on a negative DNA strand).

(b) Mutagenized versions of the *BCCP2* promoter designed to investigate the role of the key regulatory elements identified in this promoter.

(c) Sequence of the consensus sequence identified in target promoters of WRI1.

(d) Occurrence of the 15-bp consensus sequence identified in this study within five promoter sequences of putative target genes of WRI1. Each consensus sequence is represented by a closed box. Figures placed below each box indicate the number of nucleotides (x out of 15) matching the consensus sequence previously described. +, presence of the motif on a positive DNA strand; -, presence of the motif on a negative DNA strand. Figures placed above the boxes indicate the number of nucleotides separating two consecutive consensus motifs.

decreases the activity of the promoter *in planta* (Figure 4c). To further investigate the role of these motifs, the effects of mutations in these key regulatory elements were tested in the context of a 202-bp promoter cloned in front of the *uidA* reporter gene. The motifs of interest were altered by *in vitro* mutagenesis, as summarized in Figure 5(b): the primers used were designed so as to obtain a specific alteration of conserved nucleotides without compromising the hybridization of these primers. The effects of the mutations on promoter function were tested in stable transformants (Figure 6). A gene construct that included seven nucleotide

changes in the 15-bp motif at position -179 (mutation m1) did not result in significant changes of GUS activity in maturing embryos. Likewise, a gene construct including eight nucleotide changes in the 15-bp motif at position -151 (mutation m2) did not modify the GUS pattern in maturing embryos. Interestingly, when these two set of nucleotide changes were combined (mutation m1 + m2), the *uidA* expression pattern remained unaffected, but the apparent intensity was markedly decreased throughout the maturation phase, so that GUS activity was not detected in young torpedo-shaped embryos. These observations confirmed

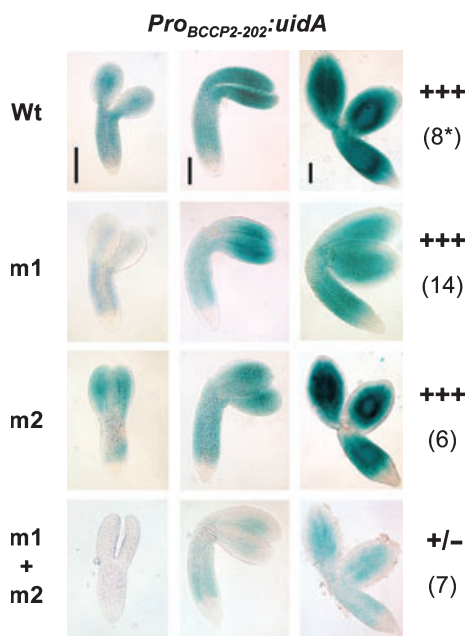


Figure 6. Effects of mutations affecting the 15-bp regulatory motif in the *BCCP2* promoter.

The basic gene construct used in this study (first row of pictures, Wt) corresponds to the *Pro_{BCCP2-202}:uidA* construct presented in Figure 4(a). *Note that the *Pro_{BCCP2-202}:uidA* lines observed in these experiments were obtained independently from the lines previously characterized in the frame of the promoter dissection experiments. Mutations described in Figure 5(b) were made in the promoter sequence, and corresponding transgenic lines were obtained. Embryos were assayed for GUS activity at the torpedo, bent-cotyledon and maturing stages. For the histochemical detection of GUS activity, excised embryos were incubated for 4 h in a buffer containing 2 mM of potassium ferrocyanide and potassium ferricyanide. The result of GUS activity was observed with Nomarski optics on whole mounts of embryos. The number of independent transgenic lines analysed is indicated, between brackets, on the right-hand side of the panels. Representative pictures are presented. +++, the embryos displayed a strong staining pattern, similar to the one observed with the full-length *BCCP2* promoter; +/-, the embryos displayed a decreased staining pattern. Scale bars: 100 μ m.

the importance of the 15-bp regulatory elements previously identified. They also suggested that these rather long consensus sites tolerate variations of their nucleotide sequences, and retain some activity even when partially modified by mutagenesis.

Interaction of WRI1 with the *BCCP2* promoter

Analysis of the interaction between WRI1 and the *BCCP2* promoter was firstly performed using a yeast one-hybrid approach. Yeast strains that presented the *HIS3* reporter gene under the control of a *BANYULS* (*BAN*; *At1g61720*), *HYDROXYSTEROID DEHYDROGENASE1* (*HSD1*, *At5g50600*) (both used as negative controls) or *BCCP2* promoter were constructed and transformed with different plasmids, allowing the expression of WRI1 or its closest homologue *At2g41710* (Figure 7a). A transformation of the strains presenting the *HIS3* reporter gene under the control of *BAN* or *HSD1* promoters with WRI1 fused to the GAL4 activation

domain (AD) gave no positive interaction results, showing that WRI1 was not able to interact with these promoters used as negative controls. On the contrary, the expression of WRI1 fused to AD in the strain presenting the *HIS3* reporter gene under the control of the *BCCP2* minimal promoter (180 bp) resulted in the specific growth of the strain on medium lacking histidine, thus demonstrating the interaction between WRI1 and this promoter sequence. This interaction seemed to be specific, as the closest homologue of WRI1, namely *At2g41710* (Feng *et al.*, 2005), was not able to interact with the *BCCP2* minimal promoter. A second set of experiments was then designed in order to determine whether the drastic reduction of promoter activity observed when short versions (<180 bp) of the *BCCP2* promoter were fused to the *uidA* reporter gene (Figure 4c) could be correlated with a reduced interaction with WRI1. Yeast strains that presented the *HIS3* reporter gene under the control of various versions of the *BCCP2* promoter were thus transformed with WRI1 fused to AD (Figure 7b). When grown on a selective medium lacking histidine and containing 25 mM 3-aminotriazole (AT), strains presenting a 160-bp version of the *BCCP2* promoter exhibited very limited growth compared with the strains containing a 180-bp minimal promoter. Strains presenting a 126-bp version of the *BCCP2* promoter were not able to grow on this medium. Taken together, these results demonstrated that the strength of the interaction between WRI1 and the *BCCP2* promoter could be modulated by nucleotide elements located in the *BCCP2* promoter, between -180 and -126.

To independently verify the one-hybrid results that suggested there was an interaction between WRI1 and the *BCCP2* promoter, electrophoretic mobility-shift assays (EMSAs) were performed. A recombinant WRI1 DNA-binding domain (DBD) was produced, and EMSAs were performed using the *BCCP2* promoter (DNA fragment corresponding to the region from -193 to -127 bp, relative to the *BCCP2* translational start codon) as the probe (Figure 8). Retardation could be observed (lane 3), which demonstrated the ability of WRI1 to bind the *BCCP2* promoter. Competition experiments showed that unlabelled *Pro_{BCCP2}* probes of the wild type (Ws) competed for WRI1 binding (Figure 8, lanes 4–6). On the contrary, the use of a mutagenized version (m1 + m2 mutations; see Figure 5b) of *Pro_{BCCP2}* probes resulted in a limited competition (Figure 8, lanes 7–9).

DISCUSSION

WRI1 directly enhances the expression of genes involved in glycolysis and fatty acid biosynthesis

Factors controlling the overall level of oil stored in seeds of *A. thaliana*, and the integration of this biochemical process in the complex framework of seed development, are only partially characterized (Santos Mendoza *et al.*, 2008). Complementary expression analyses and transcriptomic

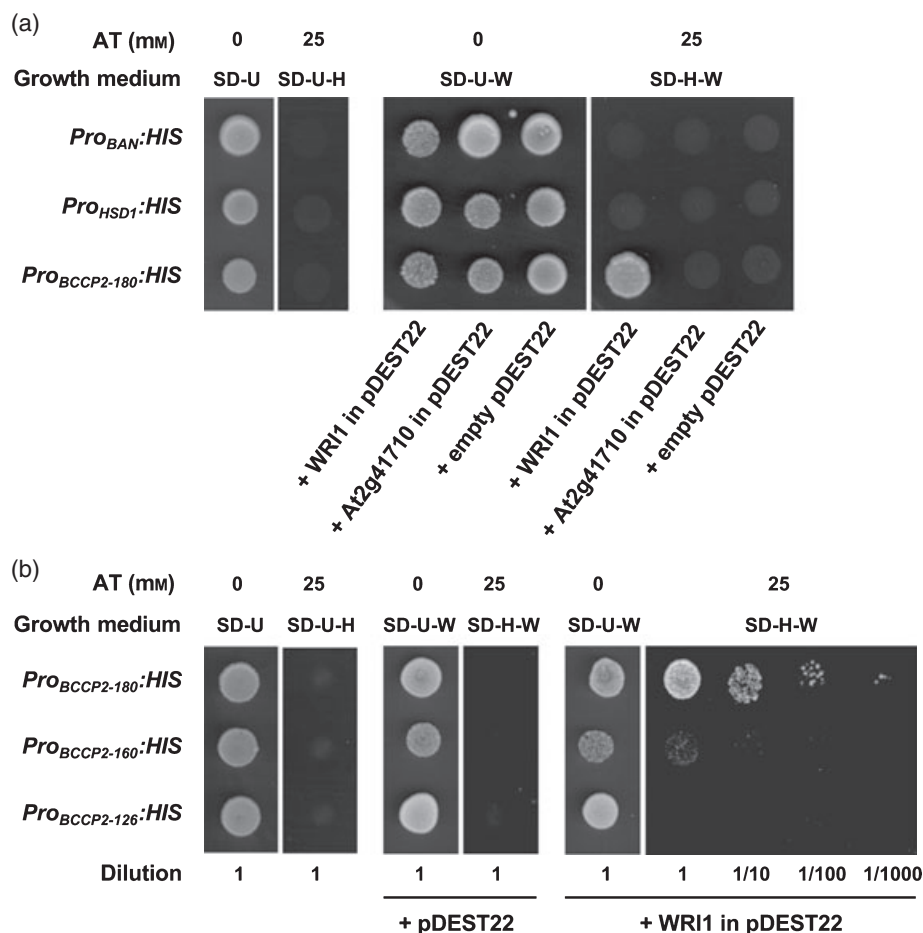


Figure 7. Analysis of WRI1 interaction with the *BCCP2* promoter in yeast one-hybrid experiments.

Yeast clones were grown on appropriate media to maintain the expression of the vectors, and to test for the activation of the *HIS3* reporter gene. Data presented are representative from the results obtained for five independent colonies.

(a) Specific binding of WRI1 to the *BCCP2* promoter. Yeast strains presenting the *HIS3* reporter gene under the control of either a functional *BAN*, *HSD1* or *BCCP2* promoter were first plated on a medium lacking histidine to verify the lack of self-activation. These yeast strains were then transformed with either the empty pDEST22 expression vector, or with versions of this vector allowing the expression of WRI1 or At2g41710, before being plated on a medium lacking histidine.

(b) Identification of a region within the *BCCP2* promoter important for the interaction of WRI1 with this promoter. The same procedure as described above for (a) was applied to test the activation of the *HIS3* reporter gene when different lengths of the *BCCP2* promoter were used to drive the expression of the reporter gene.

AT, 3-aminotriazole; H, histidine; SD, synthetic drop-out medium; U, uracil; W, tryptophan.

approaches have demonstrated that the biosynthetic pathways for fatty acids and lipids are largely regulated at the transcriptional level in maturing seeds (Ruuska *et al.*, 2002). The 'contrapuntal' or differential timing of the expression of genes involved in oil metabolism during seed development most probably reflects distinct regulatory pathways controlling the transcription of different sets of genes involved in fatty acid synthesis and TAG assembly, respectively. As groups of enzymes within a pathway sometimes exhibit similar regulation, it has been postulated that those members of a given pathway might share common *cis*- and *trans*-regulatory elements. For instance, a number of genes encoding enzymes of the glycolysis and the fatty acid biosynthetic pathway display a bell-shaped pattern of expression between 5 and 13 DAA (Ruuska *et al.*, 2002). This is

corroborated in our study by qRT-PCR experiments aimed at characterizing the steady-state accumulation of *BCCP2* and *PKp-β1* mRNAs among a developmental series of maturing siliques (Figure 3a) or seeds (Figure S1). The WRI1 transcription factor, previously shown to represent a node in the regulatory network controlling oilseed metabolism (Cernac and Benning, 2004), was proposed to trigger the transcription of the set of genes involved in the conversion of sucrose into fatty acids. This hypothesis was based on the analysis of the expression profiles of putative target genes of WRI1 characterized in various *wri1* mutant backgrounds, and in tissues overexpressing *WRI1* ectopically (Ruuska *et al.*, 2002; Baud *et al.*, 2007a). In this study, the use of the *uidA* reporter gene, the expression of which is driven by full-length promoter sequences of either *BCCP2* or *PKp-β1*,

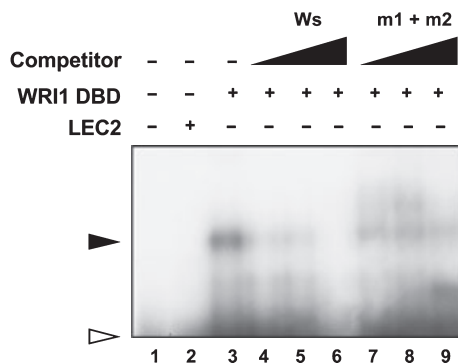


Figure 8. *In vitro* binding of WRI1 to the *BCCP2* promoter.

The recombinant WRI1 DNA-binding domain (DBD) forms specific gel retardation complexes (black arrowhead) with the promoter of *BCCP2* (lane 3), whereas LEAFY COTYLEDON2 does not (negative control; lane 2). WRI1-DBD was incubated with a radiolabelled fragment of the *BCCP2* promoter (from -193 to -127 relative to the translational start codon) containing the 15-bp motifs identified in this study (lanes 3–9). Non-labelled competitor DNA corresponding to either wild type (Ws; lanes 4–6) or mutagenized (m1 + m2; lanes 7–9) versions of the *BCCP2* promoter was added in 10-fold (lanes 4 and 7), 20-fold (lanes 5 and 8) or 100-fold (lanes 6 and 9) molar excess. The gel retardation complexes presented are representative of three independent experiments carried out with distinct recombinant protein preparations. It is to be noted that an additional type of gel retardation complexes has been observed in one out of three experiments carried out.

further establishes that WRI1 is able to strongly modulate the activity of these two promoters *in planta* (Figure 4a,b). Finally, EMSA (Figure 8) and yeast one-hybrid experiments (Figure 7a) demonstrate that WRI1 is able to interact with the *BCCP2* promoter, strongly suggesting that this gene is a direct target of WRI1 *in planta*. It is noteworthy that the fine characterization of the changes in mRNA accumulation for *BCCP2*, *PKp-β1* and *WRI1* throughout silique development shows that the induction of the regulatory factor precedes the induction of its targets in the Col-0 accession (Figure 3). This slight delay in the detection of mRNAs corresponding to target genes of WRI1 may presumably illustrate biochemical processes occurring in embryo cells, such as translation of the WRI1 peptide, and post-translational regulations (e.g. translocation of WRI1 towards the nucleus or putative recruitment of its interacting partners).

Whereas Cernac and Benning (2004) have reported that expression of *WRI1* cDNA under the control of the cauliflower mosaic virus 35S-promoter leads to slightly increased seed oil content, neither the *Pro*_{35S}*WRI1* nor the *Pro*_{S2}*WRI1* transgene used in the present study efficiently stimulates oil accumulation in the corresponding transgenic seeds (Tables 1 and 2). Several factors like growth conditions or strength and specificity of the different promoters used (classical *Pro*_{35S} versus *Pro*_{35S}*WRI1* or *Pro*_{S2}) may explain these apparent discrepancies. However, it is also interesting to note that the transgenes considered have been introduced in different *A. thaliana* accessions (Col-0 versus Ws backgrounds). In the Ws background (this study), the endogenous expression level of glycolytic and fatty acid

biosynthetic genes is significantly higher than in the Col-0 accession (Figure S1). Likewise, fatty acid concentration is more elevated in mature dry seeds of the Ws accession (see Figure 1c in Baud *et al.*, 2007a). Therefore, it is tempting to speculate that expression of lipogenic genes may be more limiting in a Col-0 background than in a Ws background.

The transcriptional machinery controlling the lipogenic pathway may be composed of multiple and distinct activating factors

When considering the mRNA levels of target genes of WRI1 in a *wri1* null mutant background (e.g. *wri1-5*), it is interesting to note that the relative accumulation of these transcripts is dramatically altered, although not completely abolished (see Figure 6 in Baud *et al.*, 2007a), suggesting that basal transcriptional activity of these lipogenic genes still occurs. This is certainly the reason why *wri1* seeds are still able to synthesize and store appreciable quantities of fatty acids (30–45% of the wild-type content, depending on the allele considered; Baud *et al.*, 2007a). Two main hypotheses can be put forward to explain this remnant transcriptional activity. First, transcription factors of the AP2/EREBP family, closely related to WRI1, may be able to substitute for WRI1, binding its target promoters and triggering the transcription of the corresponding lipogenic genes. In this case, the low abundance of mRNAs for target genes of WRI1 observed in *wri1* seeds may be explained by a low affinity of the substitute transcription factor(s) for the *cis*-elements usually bound by WRI1 and/or by a low abundance of this (these) substitute element(s). To test this hypothesis, we recently investigated the function of the closest homologue of WRI1 among the AP2 family, which is encoded by the gene *At2g41710* (Feng *et al.*, 2005). Transgenic lines overexpressing this gene ectopically were prepared, homozygous mutants were isolated, and these mutant lines were then crossed with *wri1* to generate double mutants. This material was subjected to fine physiological and metabolic analyses: it appeared that *At2g41710* does not participate in the induction of target genes of WRI1 (SB, unpublished data). The inability of the *At2g41710* protein to interact with the promoter of *BCCP2* (Figure 8a) is in agreement with these data. Although the model involving substitute factors homologous with WRI1 to explain a basal and WRI1-independent transcriptional activity of lipogenic genes cannot be completely ruled out, the observations previously mentioned make it most unlikely. An alternative hypothesis may involve one or several constitutively expressed activator(s) of the glycolytic and fatty acid biosynthetic pathways that would be unrelated to WRI1, and would ensure a basal activation of these pathways in every plant cell. This operating system would be sufficient to provide the fatty acid pool required to ensure housekeeping functions. In this model, WRI1 would behave as a transcriptional enhancer, boosting fatty acid synthesis in specific tissues where the

demand for fatty acids is high, as in the TAG-accumulating tissues of the maturing embryo. According to this model, the activator(s) and the enhancer would bind distinct *cis*-regulatory elements within the promoter of lipogenic genes. A progressive removal of these *cis*-regulatory elements may explain the gradual loss of activity observed when shortening the *BCCP2* and *PKp-β1* promoters (Figure 4c,d). Finally, the poor over-accumulation (1.5–3-fold increase, depending on the gene considered) of the transcripts encoding lipogenic enzymes observed in transgenic seeds overexpressing *WRI1* to high levels (25–70-fold increase; Figure 2) might be the consequence of a limiting abundance of these transcriptional activators, which also constitute key elements of the transcriptional machinery. Obviously, other mechanisms relying on negative feedback modulating the stability of *WRI1* mRNAs, translation, and/or half-life of *WRI1* proteins may also explain this phenomenon. These aspects should be further investigated with care. For this purpose, the development of tools allowing the detection and quantification of *WRI1* proteins will be valuable.

Identification of a *cis*-regulatory element required for the induction of the glycolytic and fatty acid biosynthetic pathways in seeds

Functional dissections of the *BCCP2* and *PKp-β1* promoters have led to the identification of enhancing regions of 54 and 79 bp, respectively, that are essential to direct the activity of these promoters in oil-accumulating tissues of the embryo. Comparison of these enhancer elements using PLACE reveals the presence of shared nucleotide sequences that may constitute putative recognition sequences bound by the transcriptional machinery triggering fatty acid biosynthesis. The hexanucleotide AACCCA that can be observed on the minus strand of each enhancer element has previously been described as the core of the SEF3-recognition sequence (Allen *et al.*, 1989). This DNA binding factor was identified in nuclear extracts from developing soybean seeds, and was initially presented as a positive regulator of transcription of the β -conglycinin, α -subunit gene. SEF3-like activities were reported in seeds of other oleaginous species, such as tobacco and sunflower (Lessard *et al.*, 1991). In soybean, SEF3 was shown to bind specifically to a site composed of two AACCCA elements separated by 27 bp (Allen *et al.*, 1989). Nevertheless, complementary studies then established that mutations abolishing the protein binding of this SEF3 factor *in vitro* did not affect the *in vivo* promoter activity in a significant manner (Fujiwara and Beachy, 1994). Similarly, in this study, we show that mutations in the AACCCA element of the *BCCP2* promoter (m2; Figure 5b) do not impact on promoter activity (Figure 6). These results strongly suggest that isolated core SEF3 recognition sequences do not constitute essential *cis*-regulatory elements of the lipogenic pathway.

By carefully analysing the results of the functional dissections carried out on the enhancer regions of the

BCCP2 and *PKp-β1* promoters, it is possible to identify a DNA sequence highly conserved between the two promoters that is important for their activity (Figure 5). This 15-bp motif is also found in the promoters of other putative target genes of *WRI1* encoding the PDH-E1 β (At2g34590) and PDH-E1 α (At1g01090) subunits of pyruvate dehydrogenase, or the plastidial phosphoglycerate mutase At1g22170, which were previously shown to respond to variations of the *WRI1* transcript level (Baud *et al.*, 2007a). The consensus site thus defined is 5'-cAAAAG(t/g)Agg(a/g)gtT-3'. In each promoter considered, a second version of this motif, slightly less conserved, can be observed in the immediate vicinity of the first one (Figure 5d). The results of site-directed mutagenesis carried out on the *BCCP2* promoter suggest that these elements might be partially redundant (Figure 6). Consistent with this finding, one-hybrid analyses indicate that the successive removal of these two motifs in the *BCCP2* promoter gradually diminishes the strength of the interaction between *WRI1* and this promoter (Figure 7b). Last, the low competition observed in EMSA experiments when using mutagenized *ProBCCP2* probes (Figure 8) confirms the importance of these motifs for the binding of *WRI1* to its target promoter. Interestingly, both the length and structure of the motif identified are reminiscent of the consensus binding site of the ANT protein (Nole-Wilson and Krizek, 2000). Nevertheless, further analyses are now required to extend the predictive value of the consensus sequence identified. A close examination of the promoters of some genes encoding enzymes of the glycolysis (e.g. plastidial enolase, At1g74030) or the fatty acid biosynthetic network (e.g. KASII, At1g74960) has enabled the detection of a similar 15-bp motif (data not shown). In the case of the enolase, quantitative RT-PCR experiments showed that this gene was also upregulated in *Pro35S_{duai}:WRI1* transgenic lines, whereas it was downregulated in *wri1* mutant seeds (data not shown). These results suggest that the 15-bp motif could be utilized in a predictive manner to identify new putative targets of *WRI1*. However, without a fine structure–function study that would unravel key nucleotides within this rather long and complex motif, it will be difficult to perform *in silico* analyses of the *A. thaliana* genome to identify all the promoters exhibiting such a *cis*-regulatory element. At that stage, it is consequently too early to determine whether or not the transcriptional machinery involving *WRI1* co-ordinately induces all of the genes involved in fatty acid biosynthesis. Alternatively, master regulators of the maturation program like LEC1 might directly control some of the genes involved in lipogenesis (Mu *et al.*, 2008).

CONCLUSION

As the economic viability of using plant oils as renewable resources is critically dependent on yield, numerous biotechnological approaches have concentrated on the manipulation of the total oil content in seeds of *A. thaliana* and

B. napus. One of the most significant improvements obtained so far results from the seed-specific overexpression of a yeast gene coding for cytosolic glycerol-3-phosphate dehydrogenase (Vigeolas *et al.*, 2007). Approaches relying on the overexpression of the genes coding acylating enzymes of the Kennedy also resulted in significant although limited improvements of the seed oil content (Zou *et al.*, 1997; Jain *et al.*, 2000; Jako *et al.*, 2001; Taylor *et al.*, 2002). However, all of these results are based on growth-chamber or glasshouse cultures, and have not yet been demonstrated in field experiments. To date, attempts to stimulate fatty acid production by overexpression of individual genes involved in *de novo* fatty acid biosynthesis have been unsuccessful, even under controlled growth conditions (Dehesh *et al.*, 2001; Thelen and Ohlrogge, 2002). These setbacks suggest that a transcriptional activation of the entire fatty acid biosynthetic network may be required to enhance the rate of acyl-chain production (Baud and Lepiniec, 2009). The WRI1 transcription factor, regarded as a central regulator of the lipogenic pathway, may thus constitute an effective tool towards manipulating the quantity of fatty acids produced in seeds (Ohlrogge and Jaworski, 1997; Cernac and Benning, 2004). To fully exploit this tool, it is now urgent to further characterize the transcriptional machinery regulating the lipogenic pathway in plants (Baud and Lepiniec, 2008). Identification of protein partners of WRI1, if any, and of the motifs bound by these regulatory elements will undoubtedly constitute useful pieces of information for the design of new transgenic plants. Ultimately, the combination of constructs driving the seed-specific overexpression of transcription factors regulating the lipogenic pathway, and of key enzymes involved in TAG assembly, may appear as an efficient approach to obtain oleaginous seeds with improved agronomic traits.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana seeds of the Ws and Col-0 accessions were obtained from the Station de Génétique et d'Amélioration des Plantes (INRA, <http://www.international.inra.fr>). Plants were cultured as described previously (Baud *et al.*, 2007a). Material used for RNA extraction was frozen in liquid nitrogen immediately after harvest, and then stored at -80°C prior to extraction.

Constructs and plant transformation

Construction of the *Pro_{BCCP2}:uidA* transgene: the DNA fragment used corresponds to the region from -580 to -1 bp, relative to the *BCCP2* translational start codon. DNA was amplified with the proofreading *Pfu* Ultra DNA polymerase (Stratagene, <http://www.stratagene.com>) from Ws genomic DNA using ProBCCP580 (5'-attB1-GATATGTTTTTACCAGAAAGCC-3') and ProBCCP100 (5'-attB2TGTGAGACAGTGGACGATG-3'), where attB1 and attB2 refer to the corresponding Gateway recombination sequences. The PCR product was introduced by a BP recombination into the pDONR207 entry vector (Invitrogen, <http://www.invitrogen.com>), which was then transferred into the binary vector pBI101-R1R2-GUS (J.-C.

Palauqui and B. Dubreucq, IJPB, unpublished data) by an LR recombination reaction, so as to obtain a transcriptional fusion between the promoter and *uidA*.

Construction of the *Pro_{BCCP2}:uidA* transgene: a similar procedure was adopted. Primer sequences were as follows: ProBCCP402, 5'-attB1CAAAAAGACTGTTGGATTGTG-3'; ProBCCP200, 5'-attB1GG-CACGATTGAAGTATAGAAG-3'; ProBCCP180, 5'-attB1ACAAAAG-GAGCGGTTTG-3'; ProBCCP168, 5'-attB1GTTTGGTGAAGTTAGTTAAAAGAGTTG-3'; ProBCCP160, 5'-attB1AAGTTAGTTAAAAGAGTTGGGTTTC-3'; ProBCCP147, 5'-attB1GAGTTGGGTTTCTTCGT AAGC-3'; ProBCCP126, 5'-attB1ATCGAAATCAGATTTCTTACC-3'.

Construction of the *Pro_{PKP-β1}:uidA* transgene: the DNA fragment used corresponds to the region from -523 to -1 bp, relative to the *PKP-β1* translational start codon. DNA was amplified using ProPKPD523 (5'-attB1CATTCTTCCGCGGAATAC-3') and ProPKPD100 (5'-attB2TGTGATTTGAAGAGAAGGAATTC-3') before introduction into pDONR207 and subsequent transfer into the binary vector pBI101-R1R2-GUS.

Construction of the *Pro_{PKP-β1}:uidA* transgene: a similar procedure was adopted. The primer sequences were as follows: ProPKPD422, 5'-attB1GAAAAGGCTCAGCTTCTCTG-3'; ProPKPD317, 5'-attB1TTACAAGTCAAACTAAACACCG-3'; ProPKPD274, 5'-attB1AAATGGGCTTATAAATTGGTC-3'; ProPKPD266, 5'-attB1TTATAAATGGTCCAAAAGTACG-3'; ProPKPD256, 5'-attB1GTCCAAAAGTACGAGTTTAAACG-3'; ProPKPD233, 5'-attB1CCGTCGTTTGGGTTGTGTC-3'; ProPKPD177, 5'-attB1CAACTACTTAATGATTGT CATGC-3'.

The resulting binary vectors were electroporated into the *Agrobacterium tumefaciens* C58C¹ (pMP90) strain, and then used for agroinfiltration of *A. thaliana* inflorescences (Bechtold *et al.*, 1993). Primary transformants were selected on MS medium containing kanamycin (50 mg l^{-1}) and were then transferred to soil for further characterization. For each construct, several independent transgenic lines were considered. Although some variations in the intensity of reporter gene expression were observed among lines carrying a given construct, the patterns of expression were mostly similar.

Construction of the *Pro_{S2}:WRI1* transgene was previously described in Baud *et al.* (2007a). The corresponding binary vector was electroporated into the *Agrobacterium tumefaciens* C58C¹ (pMP90) strain, and was then used for the agroinfiltration of *A. thaliana* inflorescences (Bechtold *et al.*, 1993). Primary transformants were selected on MS medium containing hygromycin (50 mg l^{-1}) and then transferred to soil. The progeny of these primary transformants (T_2 seeds) were subjected to segregation analyses, and lines segregating 3:1 for hygromycin resistance were selected (heterozygous lines, one insertion locus). T_2 lines were then grown in a glasshouse, and their progeny (T_3 seeds) were subjected to segregation analyses. Lines producing 100% resistant plantlets were selected (homozygous lines, single insertion locus) and used for further analyses.

Introduction of mutations in the *BCCP2* promoter

The *in vitro* mutagenesis reactions were carried out using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. Mutations introduced into the *BCCP2* promoter are summarized in Figure 5(b). The DNA fragment corresponding to the region from -202 to -1 bp, relative to the *BCCP2* translational start codon, cloned into the pDONR207 vector (see above), was used as a template. Sequences of the mutagenic oligomers (complementary strand) are shown below (in the 5' → 3' direction); modified sequences are set in bold. Primers used to introduce the m1 mutation are as follows: mbccp2P181f, CGATTGAAGTATAGAAGATTACATGCGCGGTAAAGTGAAGTTAG;

mbccp2P181r, CTAATTCACCTTACCGCGCATGTAATCTTCTATCA GTTCAATCG. Primers used to introduce the m2 mutation are as follows: mbccp2P151f, CGGTTTGGTGAAGTTAGTTTACGTGCTG CGTAACTTCGTAAGC; mbccp2P151r, GCTTACGAAGTTACGCAG CACGTAAACTAACTTACCAAAACCG. The correct introduction of mutations was confirmed by sequencing.

Microscopy and image analysis

Microscopic observations of seeds and embryos were carried out as described previously (Baud *et al.*, 2007a). For the histochemical detection of GUS activity, tissues were incubated in 0.1 M phosphate buffer, pH 7.2, containing 2 mM 5-bromo-3-indolyl- β -D-glucuronide (X-Gluc; Duchefa, <http://www.duchefa.com>), 0.1% (v/v) Triton X-100: water, 0.2 (leaves) or 2 mM (embryos) each of potassium ferrocyanide and potassium ferricyanide, and 10 mM Na₂EDTA. A vacuum was applied for 1 h before incubating for 3 h at 37°C in the dark.

Real-time quantitative RT-PCR

RNA extractions, conversion into first-strand cDNA and real-time quantitative RT-PCR reactions were performed as described by Baud *et al.* (2004). The results obtained were standardized to the constitutive *EF1 α* gene (*At5g60390*) expression level, amplified with the EF1F and EF1R primers (Baud *et al.*, 2003). Primer sequences were as follows: PhoM10, 5'-GCAAGTTCACGAATGG CGTAGG-3'; PhoM11, 5'-CCGCAGCGAGTTCCCG-3'; qENOF1, 5'-TTTAGCAAGCGGACAG-3'; qENOR1, 5'-TTCTCATGGTGAT- CGG-3'. Primers used to amplify *WRI1*, *BCCP2* and *PK β* were as described by Baud *et al.* (2007a). Primers used to amplify *PK α* (22960up6 and 22960low6) were as described by Baud *et al.* (2007b).

Yeast one-hybrid experiments

The reporter plasmid was constructed by inserting fragments of the *BCCP2* promoter into the pHISi vector (Alexandre *et al.*, 1993). These fragments were amplified by PCR using the proofreading Pfu Ultra DNA polymerase (Stratagene), digested by *EcoRI* and *SacI*, and inserted into pHISi between the *EcoRI* and *SacI* sites. These plasmids were then digested with *NcoI* and integrated into the yeast strain YM4271 (Liu *et al.*, 1993), at the *URA3* locus. The resulting yeast strains, selected on a medium lacking uracil, contained the *HIS3* reporter gene under the control of the promoter sequences studied. The 180-bp fragment of the *BCCP2* promoter (from -180 to -1 bp, relative to the *BCCP2* ATG codon) was amplified with *EcoRI*bccp180 (5'-GGAATTCCACAAAAGGAGCGGTTTGG-3') and *SacI*bccpLOW (5'-TCCCCGCGGGGATGTTGAGACAGTGGACGAT- G-3'), the 160-bp fragment was amplified with *EcoRI*bccp160 (5'-GGAATTCCAAGTTAGTTAAAAGAGTTGGGTTTC-3') and *SacI*bccpLOW, and the 126-bp fragment was amplified with *EcoRI*bccp180 (5'-GGAATTCCATCGAAATCAGATTTCTTTACC-3') and *SacI*bccpLOW. The *BAN* promoter used corresponded to the region between -193 and +43 bp, relative to the *BAN* transcription start site (Baudry *et al.*, 2004). The *HSD1* promoter used corresponded to the region between -212 and -155 bp, relative to the *HSD1* ATG codon (Baud *et al.*, 2009).

WRI1 and At2g41710 cDNAs were then cloned in the pDEST22 vector (Invitrogen), to be expressed in yeast as fusions with the GAL4 AD. WRI1 cDNA was amplified with the proofreading Pfu Ultra DNA polymerase (Stratagene) from a mixture of seed cDNAs (from the Ws accession), as previously described (Baud *et al.*, 2007a). The At2g41710 cDNA was amplified using 5'-attB1TA-ATGGCGTCGGTGTC-3' and 5'-attB2TCATTCTCTTGTGGGAGG-3'. PCR products were introduced by a BP recombination into the pDONR207 entry vector, and then transferred into the pDEST22

vector by an LR recombination reaction. Yeasts already presenting the *HIS3* reporter gene under the control of a functional *BAN*, *HSD1* or *BCCP2* promoter were transformed with pDEST22 using the AcLi/SSDNA/PEG method (Gietz and Woods, 2002). Transformants were selected on appropriate media. AT was added in media lacking histidine: several concentrations were tested, from 5 to 50 mM.

Fatty acid analyses

Pools of 20 seeds were used. Extraction and analyses of fatty acid methyl esters by gas chromatography were performed as described previously (Li *et al.*, 2006).

Electrophoretic mobility-shift assays

The expression plasmid was constructed by inserting a truncated version of WRI1 cDNA encoding the WRI DBD (amino acids 52–237) in the pET_{trx1a} vector (for details, see <http://www.embl-hamburg.de/~geer/lof/webPP/vectordb/Gunter-vectors/>). To this end, WRI1 cDNA was amplified with the proofreading Pfu Ultra DNA polymerase (Stratagene) from a mixture of seed cDNAs (from the Ws accession) using *NcoI*WRIaa52-F (5'-CATGCCATGGCATGCCATAACCCGACAAGCC-3') and *XhoI*WRIaa237-R (5'-CCGCTCG-ACGGTCAACCTTTCTTCTTAACCGG-3'). The product obtained was digested by *NcoI* and *XhoI*, and then cloned in pET_{trx1a} digested by *NcoI* and *XhoI*. Protein expression and purification were adapted from Hamès *et al.*, 2008. The WRI1 DBD was expressed using *Escherichia coli* strain RosettaBlue(DE3)pLysS (Novagen, <http://www.merckbiosciences.co.uk/g.asp?f=NVG/home.html>). After induction by 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in Luria Bertani buffer (LB) NaCl 0.5 M, cells were grown overnight at 17°C. For cell lysis, the pellet of a 1-l culture was sonicated in 8 ml lysis buffer A (250 mM NaCl, 20 mM Tris-HCl, pH 8, 5 mM imidazole, 5% glycerol) and centrifuged for 40 min at 21 000 g. The clear supernatant was incubated for about 4 h with 1.5 ml Ni-NTA resin (Qiagen, <http://www.qiagen.com>). The resin was transferred into a column, washed with 15 ml of buffer A plus 60 mM imidazole and eluted with 5 ml of buffer A plus 300 mM imidazole. The fractions containing the protein were pooled and dialysed in 1 l of buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM MgCl₂, 0.25 mM EDTA, 0.02% Igepal, 20% glycerol). Recombinant LEC2 proteins used as negative controls were obtained as described in Baud *et al.* (2009).

Probes were prepared as follows: overlapping oligonucleotides were annealed in water from 100°C to room temperature (20°C), filled by DNA polymerase I with [α -³²P]dCTP, and purified on a column of Sephadex G-50 (GE Healthcare, <http://www.gehealthcare.com>). DNA-binding reactions were performed following the procedure described by Braybrook *et al.* (2006). Briefly, 125 ng of WRI1 DBD recombinant protein were incubated with 0.55 fmol of [³²P]-labeled α ProBCCP2 probe (corresponding to the region located between -193 and -127 bp, relative to the *BCCP2* ATG codon) in binding buffer (20 mM Tris-HCl, pH 8, 250 mM NaCl, 2 mM MgCl₂, 1% glycerol, 1 mg ml⁻¹ BSA, 1 mM DTT). For competition assays, the unlabeled competitor was incubated briefly with protein before the addition of the labelled probe. After the addition of the labelled probe, reactions were incubated for 30 min at room temperature. Binding reactions were fractionated at 4°C by 5% PAGE.

Enzymatic assays

Enzymatic measurements were carried out on maturing seeds isolated from four siliques aged 16 DAA. The material was homogenized in 300 μ l of extraction buffer (Focks and Benning, 1998): phosphoglycerate mutase and enolase assays were conducted

successively on the same extract using a protocol derived from Burrell *et al.* (1994), as described in Focks and Benning (1998). Pyruvate kinase activities were assayed as described in Baud *et al.* (2007b).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of *BCCP2*, *PKp-β1* and *WRI1* transcript levels in developing seeds.

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