Identification of cis-Elements That Regulate Gene Expression during Initiation of Axillary Bud Outgrowth in Arabidopsis[w]

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Growth regulation associated with dormancy is an essential element in plant life cycles. To reveal regulatory mechanisms of bud outgrowth, we analyzed transcriptomes of axillary shoots before and after main stem decapitation in Arabidopsis (*Arabidopsis thaliana*). We searched for any enriched motifs among the upstream regions of up-regulated and down-regulated genes after decapitation. The promoters of down-regulated genes were enriched for TTATCC motifs that resemble the sugar-repressive element, whereas the promoters of up-regulated genes were enriched for GGCCCAWW and AAACCCTA, designated Up1 and Up2, respectively. Transgenic plants harboring a reporter gene driven by a tandem repeat of the elements were produced to analyze their function in vivo. Sugar-repressive element-mediated gene expression was down-regulated by the application of sugars but was unaffected after decapitation. In contrast, expression driven by the repeat containing both Up1 and Up2 was up-regulated after decapitation, although the Up1 or Up2 repeat alone failed to induce reporter gene expression in axillary shoots. In addition, disruption of both Up1 and Up2 elements in a ribosomal protein gene abolished the decapitation-induced expression. Ontological analysis demonstrated that up-regulated genes with Up elements were disproportionately predicted to function in protein synthesis and cell cycle. Up1 is similar to an element known to be a potential target for TCP (TEOSINTE BRANCHED1, CYCLOIDEA, PCFs family) transcription factor(s), which regulate expression of cell cycle-related and ribosomal protein genes. Our data indicate that Up1-mediated transcription of protein synthesis and cell cycle genes is an important regulatory step during the initiation of axillary shoot outgrowth induced by decapitation.

Regulation of axillary shoot growth is a fundamental aspect of the plant body plan (Horvath et al., 2003; Leyser, 2003). The outgrowth of the axillary bud is inhibited by the apical bud since decapitation of the shoot apex releases dormant axillary buds causing initiation of their outgrowth. Such growth habit is referred to as apical dominance or paradormancy (Cline, 1997).

A number of mutants have been identified that result in enhanced outgrowth of axillary buds in a variety of plant species (Shimizu-Sato and Mori, 2001). However, few such mutants are characterized physiologically. Molecular genetic approaches have successfully identified genes that are involved specifically in bud dormancy. In maize (*Zea mays*) and rice (*Oryza sativa*), a transcription factor TEOSINTE BRANCHED1 (TB1) that belongs to TB1, CYCLOIDEA, PCF (TCP)-domain protein family is necessary to repress the outgrowth of axillary buds (Doebley et al., 1997; Hubbard et al., 2002; Takeda et al., 2003). In Arabidopsis (*Arabidopsis thaliana*), *MORE AXILLARY GROWTH* (*MAX*) genes and auxin signaling genes are involved in the regulation of axillary bud outgrowth (Lincoln et al., 1990; Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004). Interestingly, pea (*Pisum sativum*) *RAMOSUS1* gene is shown to be the *MAX4* ortholog, demonstrating that this regulation is common in Arabidopsis and pea. Nevertheless, it is still unknown how these factors regulate the axillary bud outgrowth.

Physiological and developmental phases are characterized by the expression of phase-specific genes. Accordingly, genes whose expression is associated with dormancy and/or subsequent growth could provide insight into growth regulation in axillary buds. Several dormancy-associated genes have been isolated and characterized from pea axillary buds (Stafstrom et al., 1998; Madoka and Mori, 2000). In addition, genes with predicted roles in DNA synthesis, protein synthesis, and cell cycle are used as molecular markers for axillary bud activity (Stafstrom and Sussex, 1992; Devitt and Stafstrom, 1995; Shimizu and Mori, 1998). A large number of genes associated with the end of dormancy have been identified in grape (*Vitis vinifera*) by using microarray techniques (Pacey-Miller et al., 2003). Although the expression of dormancy- or outgrowth-associated genes has been characterized, the regulatory mechanisms by which gene expression is linked to developmental phases are still unknown.

Most dormant bud cells are blocked in G_1 phase, and the cell cycle machinery is reactivated in concert with the initiation of the outgrowth (Devitt and Stafstrom, 1995; Cline, 1997). Indeed, many of the known marker genes for bud outgrowth encode cell cycle- or protein

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synthesis-related proteins. Therefore, activation of such gene expression might reflect the change in the developmental phase to bud outgrowth. Several transcriptional regulators of cell cycle- and protein synthesis-related gene expression have been identified (Ito et al., 2001; Veylder et al., 2002; Mathieu et al., 2003; Trémousaygue et al., 2003); however, it remains unknown whether these regulators activate the expression of bud outgrowth-associated genes. To understand the regulation of bud outgrowth, it is crucial to identify the regulatory checkpoints that are in turn the downstream targets of internal and external signals.

To characterize transcriptional regulation during bud growth initiation, we analyzed gene expression profiles in Arabidopsis axillary buds. We identified several cis-elements that are enriched in the promoters of down-regulated and up-regulated genes after main stem decapitation. Both in silico and in vivo analyses demonstrated Up1, which is thought to be the potential target element of TCP transcription factors, functions in defining gene expression profiles in axillary buds. Our results demonstrate that Up1 is one of the important cis-elements for the bud outgrowth-associated gene expression.

RESULTS

Microarray Analysis in Axillary Shoots

Prior to assessing the gene expression profile associated with bud outgrowth in Arabidopsis, we analyzed the effects of main stem decapitation on the growth of axillary buds. After decapitation, axillary rosette leaves developed quickly (Fig. 1A). Enhancement of axillary rosette leaf growth was evident by 2 d after decapitation. The axillary bud in the uppermost rosette node (first node) initiated elongation 2.5 d earlier in decapitated plants than intact plants, whereas initiation of elongation of the axillary bud in the fourth and fifth nodes (below the first node) occurred more than 17 d earlier in decapitated plants.

Next, we explored dormancy-associated gene expression following decapitation. This was achieved by analyzing the expression patterns of At*DRM1* (At1g28330) and At*DRM1* homolog (At2g33830), which are Arabidopsis orthologs of dormancy-associated genes isolated from pea (Stafstrom et al., 1998). Plants subjected to decapitation contained at least five visible axillary shoots (both axillary buds and expanding axillary rosette leaves). The axillary shoots from each successive rosette leaf along the primary shoot axis were harvested independently and used for expression analysis. Regardless of bud position along the primary axis, expression levels of At*DRM1s* were reduced within 24 h after decapitation and then the expression was up-regulated thereafter (Fig. 1B). Although the kinetics of the down-regulation varied among the samples, the lowest expression levels were observed by 12 to 24 h after decapitation at the third and fourth positions. At the first and second buds, the expression levels reached the lowest level by 6 to 12 h and the low expression levels were maintained until 24 h after decapitation.

Based on these results, we performed oligonucleotide-directed Affymetrix microarray analysis using total

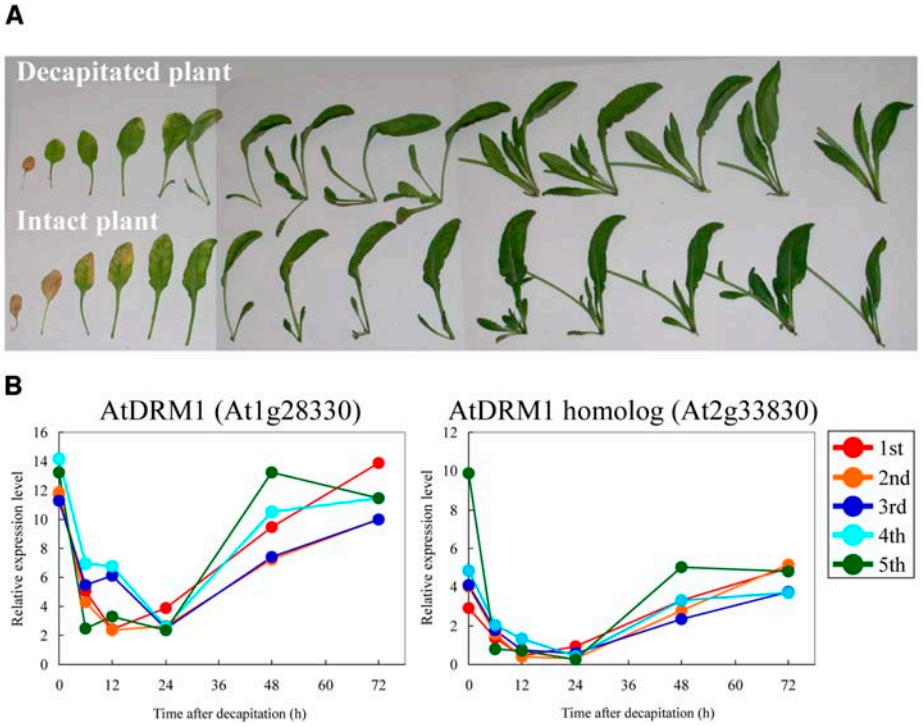

Figure 1. The effect of main stem decapitation on axillary shoot outgrowth. A, Rosette leaves with axillary shoots of decapitated (top) and intact (bottom) 50-d-old plants are shown. The rosette leaves were displayed in order of emergence, oldest leaf to the left. B, QRT-PCR analysis of At*DRM1* (left) and At*DRM1* homolog (right) in axillary shoots after decapitation of the main stem. Expression analysis was carried out in a series of five axillary shoots numbered basipetally with first (top) to fifth (basal) rosette leaves. The first is represented as red, second as orange, third as blue, fourth as pale-blue, and fifth as green. A sample of time 0 (h) indicates intact 5-d-after bolting plants. Experiments were repeated twice and similar results were obtained (see Supplemental Fig. 2C). A representative result is shown.

RNA prepared from axillary shoots. Due to the difficulty of dissected bud sampling, axillary buds from all positions were equally mixed before microarray analysis. We chose the time point of 24 h after decapitation for the following 2 reasons. One was that the down-regulation of At*DRM1s* expression was evident at this time point regardless of bud position. The second reason was that no visible bud outgrowth was yet observed at this time point. The first visible effect of decapitation on the growth (enlargement of the axillary leaves) was observed 2 d after decapitation.

Of the 22,591 genes represented on the microarray, the expression of 13,728 genes was judged as ''Presence'' by the analytical software MicroArray Suite in duplicate experiments in axillary shoots both before and after decapitation (see Supplemental Table IA). Similar results were obtained from duplicate microarray experiments using independent plant materials (see Supplemental Fig. 1). The accuracy of the microarray data was confirmed by comparing these data with those obtained from quantitative reverse transcription (QRT)-PCR for 7 down-regulated and 12 up-regulated genes (see Supplemental Fig. 2, A and B). We expected that the down-regulated genes would be enriched for those with expression associated with bud dormancy, whereas the up-regulated genes would represent those associated with induction of bud outgrowth. Indeed, At*DRM1s* were found among the down-regulated genes, and the up-regulated genes included a large number of ribosomal protein and cell cycle-related genes.

Regulatory Elements Controlling Gene Expression Associated with Decapitation

To identify potential transcriptional regulatory elements involved in a change in expression profile after decapitation, we searched for 6- to 8-base sequences that are overrepresented in the various lengths of upstream regions of down- or up-regulated genes (see ''Materials and Methods''). First, genes exhibiting at least a 2-fold difference in the expression level after decapitation were selected from the microarray data (see Supplemental Table I, B and C). We found 27 candidate elements that were enriched in the 500-base 5′ of their predicted translation start sites of the down-regulated gene group compared to those of the other expressed genes. Among them, 13 candidates contained a part of the 6-base sequence, TTATCC (Table I). This sequence was similar to an element involved in sugar-mediated repression of gene expression in rice (Lu et al., 1998). Therefore, we designated this sequence sugar-repressive element (SRE; see below). SRE was found in 272 of the 1,592 down-regulated genes (*P* value, 1.11e-6; see Supplemental Table IIA). In the up-regulated gene set, 85 candidate elements were significantly enriched in the 500-base promoters. Seventeen and 27 candidates had similar sequences, GGCCCAWW and AAACCCTA, respectively (Table II). We designated the 2 sequences as Up1 and Up2, respectively. Up1 and Up2 were similar to the elements site II motif and *telo*-box, which are related to ribosomal protein gene expression (Trémousaygue et al., 2003). Up1 and Up2 were observed in 162 (*P* value, 3.64e-11) and 193 (*P* value, 9.96e-20) of the 1,184 up-regulated genes, respectively (see Supplemental Table II, B and C). Overrepresentation of Up1 and Up2 was also observed similarly in the data from the second experiment (data not shown). These 3 elements were still significantly enriched in the 1,000-base promoters of genes in which expression was changed 2-fold, although the false positive probability values were higher than those for the 500-base region.

We further carried out in silico expression analysis to compare the expression of each gene set containing the selected element to that of the remaining expressed gene set (Fig. 2; see Supplemental Table III). The expression profiles of each gene set with the selected element were compared by linear regression analysis (see supplemental data). The regression lines of the selected gene sets suggested that SRE negatively regulates gene expression after decapitation, whereas both Up1 and Up2 regulate expression positively (see supplemental data).

Expression Analysis of the Synthetic Promoter:*GUS* Fusion Genes

To verify whether these elements function in vivo, we produced transgenic plants carrying the *β-glucuronidase* (*GUS*) reporter gene driven by tandem repeats of the element(s) (Fig. 3A). The *pSRE* seedlings showed GUS staining in older leaf blades, and the staining reduced significantly when transferred to medium with sugars (see Supplemental Fig. 3A). This result reveals that SRE is involved in sugar-dependent

Table I. *Thirteen SRE-like sequences found in 1,592 down-regulated genes*

Sequence	Expected	Observed	*P* Value	Sequence	Expected	Observed	*P* Value
CTTATC	208	314	8.52E-10	AATATC	335	427	0.03%
AAATATC	160	248	2.13E-07	TATCCA	201	275	0.06%
TTATCC	186	272	1.70E-06	CTTATCT	92	148	0.08%
CTTATCC	45	90	0.01%	TCTTATC	95	152	0.10%
AAAATATC	72	129	0.01%	CTTATCCA	16	42	1.66%
TTATCCA	81	137	0.02%	CTTATCTT	37	72	2.92%
TATCTT	413	512	0.02%				

Table II. *Up1- or Up2-like sequences found in 1,184 up-regulated genes*

Up1-Like	Expected	Observed	*P* Value	Up2-Like	Expected	Observed	*P* Value
AGGCCCA	63	133	8.41E-11	AAACCCT	136	269	2.11E-23
AAGCCCA	70	139	1.21E-09	TAAACCCT	35	111	1.14E-20
GGCCCA	129	207	8.07E-08	AAACCCTA	88	193	2.64E-19
GCCCAA	129	203	7.35E-07	AAAACCCT	62	150	8.19E-18
GCCCAAT	47	97	2.29E-06	TAAACCC	72	159	4.96E-16
GGCCCAT	54	106	4.25E-06	AAACCC	263	398	1.89E-15
CCCAAT	140	213	5.00E-06	AAAACCC	120	224	2.67E-15
GCCCATT	54	104	2.00E-03	AACCCT	199	319	1.80E-14
ATGGGCC	46	91	0.01%	AACCCTA	121	218	4.66E-13
GGCCCAA	55	102	0.01%	ACCCTA	152	253	2.29E-12
GCCCAT	129	192	0.02%	TAAAACCC	33	91	5.38E-12
GGCCCAAT	25	59	0.03%	AAAACC	357	484	2.29E-11
GGCCCATT	27	63	0.05%	TTAAACCC	23	72	2.89E-11
AAGGCCC	34	70	0.10%	TAAACC	244	346	1.15E-08
CCCATT	149	211	0.21%	AACCCTAA	74	142	3.90E-08
AGGCCCAA	28	62	0.25%	ACCCTAA	90	160	1.06E-07
CCCATTT	65	110	0.63%	CCCTAA	153	231	1.12E-06
				TTAAACC	85	149	1.41E-06
				CTTAAACC	21	59	1.48E-06
				CCCTAAA	64	117	3.00E-03
				TAAAACC	107	172	3.00E-03
				AAAAACC	139	210	4.00E-03
				AAAAACCC	39	78	0.26%
				ATAAACCC	25	57	0.46%
				ACCCTAG	35	68	1.48%
				ACCCTAAA	36	70	3.95%
				AACCCTAG	31	63	4.30%

repression of gene expression as was shown in rice (Lu et al., 1998). On the other hand, the GUS staining of *pU1*, *pU2*, and *pU1U2* seedlings was similar to those of transgenic plants carrying a reporter gene driven by site II and/or *telo*-box (Manevski et al., 2000; Trémousaygue et al., 2003; see Supplemental Fig. 3B).

We then analyzed GUS staining patterns of the lateral buds that attached to the main stem. In the *pSRE* lines, GUS staining was observed in the lateral buds, cauline leaves, and the main stem, but not in the elongating lateral stem. On the other hand, weak GUS staining was observed in the elongating lateral stem of *pU1* lines. The *pU2* plants showed no visible staining. The *pU1U2* lines exhibited a similar but enhanced GUS staining pattern to the *pU1* lines (Fig. 3B). Interestingly, the *pU1U2* lines exhibited the opposite GUS staining pattern to the *pSRE* lines (Fig. 3B).

Next, we analyzed the effect of decapitation on GUS staining in axillary shoots using the same lines. In intact *pSRE* plants, GUS staining was observed at the base of rosette leaves and this staining pattern was unaffected by decapitation (data not shown). We failed

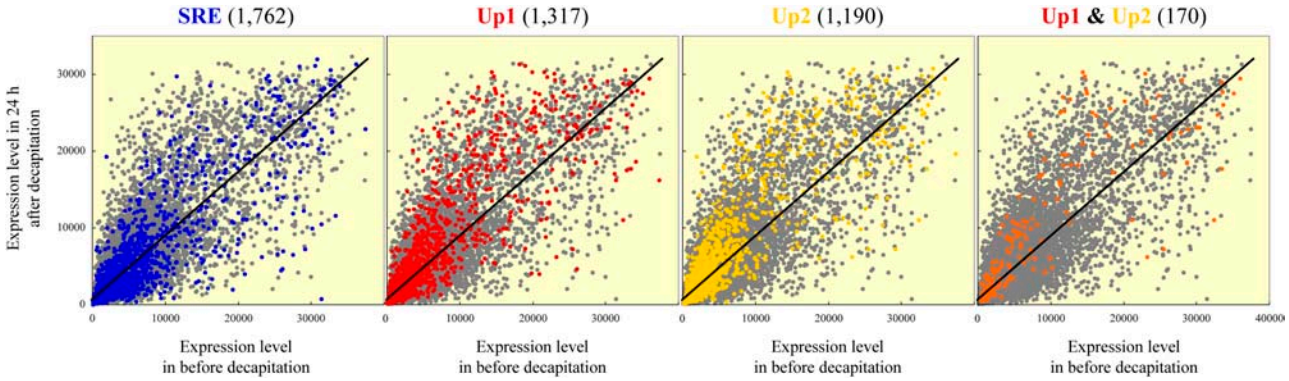

Figure 2. Gene expression profiles of SRE-, Up1 alone-, Up2 alone-, and Up1/Up2-containing genes in axillary shoots before and 24 h after decapitation. Numbers of genes included in each group are indicated in parentheses at the top of the plots. *x* and *y* axes indicate the relative expression levels before and 24 h after decapitation, respectively. Each dot represents the expression level of individual genes. Colored dots represent the genes containing the indicated element in 500-base upstream region. Gray dots represent the remaining expressed genes. Black line indicates that the regression line of the expression profiles of the entire genes.

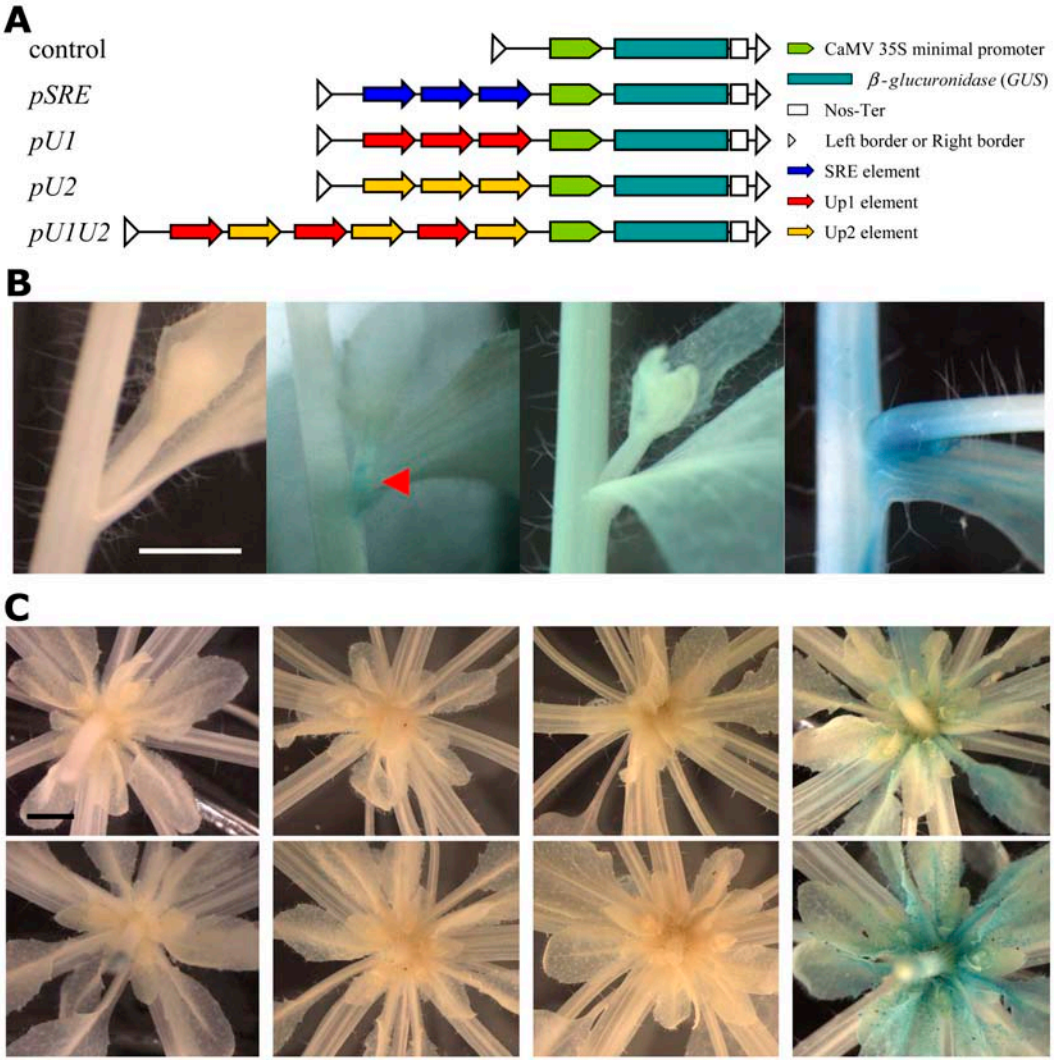

Figure 3. Gene expression analysis of the synthetic promoter:*GUS* fusion genes. A, Construction of the synthetic promoter:*GUS* fusion genes. Each construct was designated as indicated on the left. B, GUS staining of lateral shoots at the main stem of 5-d-bolted plants. From left to right, the plants harbor the transgene as follows: control, *pU1*, *pU2*, and *pU1U2*. A red arrowhead indicates GUS staining. The scale bar in the left photograph equals 1 mm and photographs were taken at the same magnification. C, GUS staining of axillary shoots of plants before (top) and 24 h after (bottom) decapitation. From left to right, the plants are shown same as B.

to observe any GUS staining in axillary shoots of *pU1* and *pU2* lines even after decapitation (Fig. 3C). However, in the axillary shoot, all *pU1U2* plants exhibited GUS staining in the intact plant, and this was significantly enhanced after decapitation (Fig. 3C). These results suggest that Up1 and Up2 synergistically act as positive cis-elements in response to decapitation. On the other hand, SRE alone might not be sufficient to confer the down-regulation after decapitation.

Loss of Function of Up Elements Abolishes Decapitation-Induced Gene Expression

To ascertain Up elements are necessary for growth-associated gene expression, we produced and analyzed transgenic lines harboring the *GUS* gene driven by native 1-kb promoter regions with Up elements or their deletion derivatives (Fig. 4A). Since double Up1s and one Up2 are located in the promoter of At4g17390 (60S ribosomal protein L15), we examined 3 kinds of internal deletion promoter:*GUS* fusions as described in Figure 4A.

We analyzed the *GUS* expression pattern histochemically in axillary buds of intact and decapitated plants (Fig. 4B). Ups wt lines showed weak GUS staining in the petioles of rosette leaves, and this staining was strongly induced by decapitation (Fig. 4B). In contrast, both intact and decapitated plants displayed no visible GUS staining in the internal deletion Ups promoter lines, Δ Up1, Δ Up2, and Δ Ups (Fig. 4B). These loss-of-function experiments indicate that Up1 and Up2 are

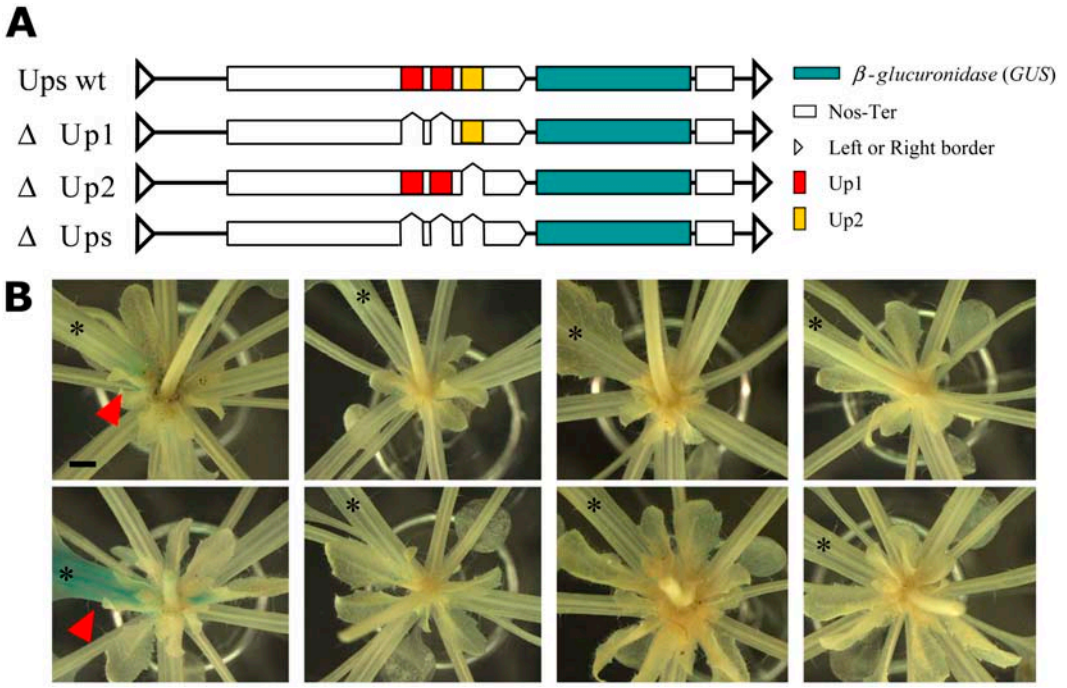

Figure 4. Loss-of-function experiments of Up1 and Up2 in native promoters. A, A diagram of the constructs of the internal deletion promoter:*GUS* fusions used in this article. Each construct was described as indicated on the left. B, Histochemical analysis of axillary buds. From left to right, the plants harbor the transgene as follows: Ups wt, Δ Up1, Δ Up2, and Δ Ups. The top and bottom pictures show plants before and 24 h after decapitation, respectively. Asterisks indicate the rosette leaf emerging apical end. Red arrowheads indicate GUS staining. The scale bar in the top left photograph equals 1 mm and photographs were taken at the same magnification.

necessary for the expression of the 60S ribosomal protein gene after decapitation.

Gene Ontology of Genes with Up Elements in the Promoters

We classified all genes into 13 functional groups according to the gene ontology of the MIPS and KEGG databases (Nakabayashi et al., 2005; Fig. 5). Then, we analyzed the 162 Up1- or 193 Up2-containing genes that were up-regulated after decapitation. Both gene groups included a higher proportion of genes involved in cell cycle, DNA processing, and protein synthesis than did the whole gene set (Fig. 5; see Supplemental Table IV).

In particular, components of mRNA splicing and translation initiation were highly overrepresented in the up-regulated genes containing Up1 or Up2 (see Supplemental Table IV). These included basic components of spliceosomes and translation initiation factors and a number of ribosomal proteins. A subset of genes for DEAD box RNA helicases, snRNPs, and tRNA synthetases were also included in these groups. It is also worth mentioning that a certain number of up-regulated genes with Up2 are predicted to function as mitochondrial carrier proteins, suggesting that mitochondrial functions are also controlled by this regulation.

QRT-PCR Expression Analysis of Genes Containing Up Elements in Axillary Buds

To examine the kinetics of genes related to protein synthesis and cell cycle with Up elements in their

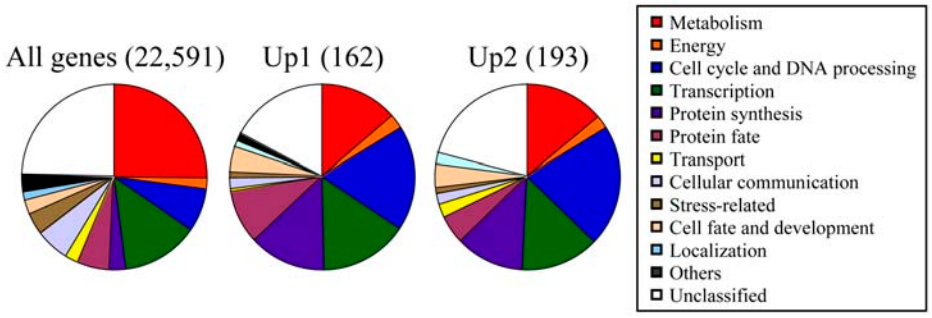

Figure 5. Gene classification based on ontology. Genes were classified to 13 groups as described at the right. The number of genes in each group is indicated in parentheses.

promoters, we performed QRT-PCR expression analysis in axillary shoots following decapitation (Fig. 6; see Supplemental Table IV). A transient accumulation of the transcripts was observed for all protein synthesis and cell cycle-related genes within 24 h after decapitation. However, the kinetics of up-regulation was different among the bud positions. In six protein synthesis-related genes, a transient accumulation occurred by 6 h after decapitation at the first and second buds, and by 12 to 24 h at the third to fourth positions (Fig. 6A). Similar kinetics was observed in four cell cycle-related genes (Fig. 6B, middle and right). In contrast, induction of 2 Up1-containing genes, DRP5A (At1g53140) and kinesin-related protein (At2g22610), was different from those of others (Fig. 6B, left). A transient up-regulation was found at the third to fifth buds, and at the fourth and fifth ones the induction occurred by 6 h after decapitation. Interestingly, for 10 of the genes, expression was reinduced after 48 h in the upper 4 buds. QRT-PCR analysis revealed that the Up element-mediated induction of gene expression divided into two phases.

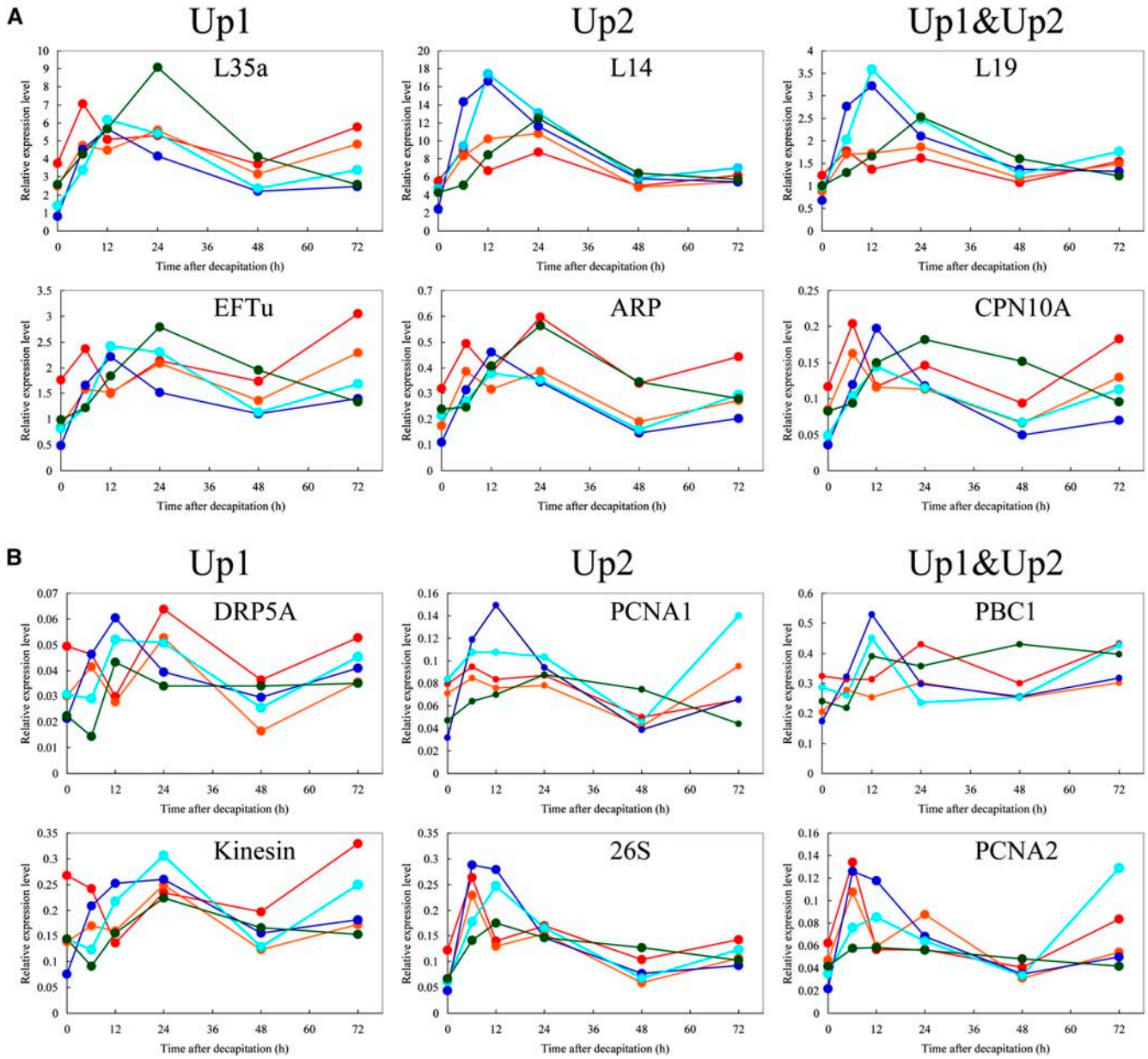

Figure 6. Gene expression analysis of Up1- or Up2-containing genes in axillary shoots after decapitation as determined by QRT-PCR. A, Protein synthesis-related genes with Up elements. B, Cell cycle-related genes with Up elements. Five-day-after bolting plant was indicated as 0 h. For more detail, see the legend of Figure 1B. Each gene abbreviates as described in Supplemental Table V. Experiments were repeated twice and similar results were obtained (see Supplemental Fig. 2, D and E). A representative result is shown.

DISCUSSION

Transcriptome Analysis of Axillary Bud Outgrowth

The genetic approach has successfully elucidated the molecular mechanism of a variety of biological processes in many organisms. In Arabidopsis, axillary bud dormancy is well studied genetically to identify genes involved in the process (Shimizu-Sato and Mori, 2001). In this study, we took a different approach to identify cis-elements involved in axillary bud outgrowth by gene expression profiling. This approach is independent of highly redundant genetic processes, so it is possible to obtain a different spectrum of regulatory factors compared to the genetic approach. For example, we found that many TCP genes are abundantly expressed in Arabidopsis axillary shoots (see Supplemental Table IA). This might preclude the identification of such genes in genetic screens. Integration of these cis-elements into the genetic model will facilitate further understanding of this process. It is also worth mentioning that our analysis demonstrates that Up1 and Up2 affect the expression of a large number of genes. This indicates that their interacting transcription factors might be involved in global regulation of axillary bud outgrowth. As such, they are the possible targets of outgrowth regulation in response to intrinsic and external signals.

In silico analysis performed in this study underestimates the numbers of genes regulated by these elements, because we analyzed only exact matches to the documented sequences of SRE, Up1, and Up2. Analysis using related (similar) sequences to these elements gave similar results, indicating that these related elements might play the same role in regulating gene expression.

Down-Regulation of Gene Expression after Decapitation

Transcriptome analyses suggest that SRE is involved in the down-regulation of gene expression in axillary shoots after decapitation. The promoters of At*DRM1* and its homolog contain an SRE and an SRE-like sequence, respectively (see Supplemental Table IIA), and their expression levels reduced after decapitation (Fig. 1B). The synthetic promoter harboring a tandem SRE repeat led to the sugar-dependent repression of the reporter gene expression, indicating that this element is a functional cis-element in vivo (see Supplemental Fig. 3A). However, we did not find any effect of decapitation on expression driven by the SRE repeat. This suggests that SRE alone is not sufficient to down-regulate gene expression in response to decapitation. Considering these matters, SRE might require other regulatory elements associated with down-regulation. Our gene expression profiling suggests that SRE is one of the potential regulatory elements involved in the down-regulation of gene expression after decapitation. The nutrient hypothesis (Cline, 1991) suggests that continued growth of the apical bud causes nutrient deprivation and prohibits outgrowth of axillary buds and that decapitation causes an increase in nutrient levels in axillary buds, which in turn permits subsequent outgrowth. Since the expression of genes with SRE in their promoters was reduced by sugar, sugar content in growing buds might be higher than in dormant buds, consistent with the idea that sugar is one of the regulatory substances, which are required for bud growth. Accordingly, SRE may contribute to the nutritional regulation of gene expression in Arabidopsis axillary buds.

Gene Expression Associated with Growth Initiation in Axillary Buds

We identify 2 regulatory elements, Up1 and Up2, associated with the induction of gene expression after the main stem decapitation. Up1 and Up2 are almost identical to the cis-elements, site II motif and *telo*-box, respectively (Trémousaygue et al., 2003). The site II motif is the potential target sequence of the TCP protein family (Kosugi and Ohashi, 1997, 2002; Cubas et al., 1999; Trémousaygue et al., 2003), which is composed of two subfamilies, PCF subfamily (class I) and CYC/TB1 subfamily (class II). Both subfamilies of TCP proteins are able to bind to similar sequences, although they have preferences for certain DNA sequences (Cubas et al., 1999; Kosugi and Ohashi, 2002). Interestingly, the class II subfamily includes TB1 in maize and rice, which have been shown to repress outgrowth of axillary shoots (Doebley et al., 1997; Takeda et al., 2003). Our analyses indicate that the involvement of TCP proteins in the regulation of bud outgrowth is also conserved in Arabidopsis.

The *telo*-box is the DNA binding motif of the transcriptional enhancer AtPurα, which regulates gene expression in combination with TCP proteins (Tremousaygue et al., 1999, 2003; Manevski et al., 2000). The site II motif and *telo*-box are colocated in the promoters of about 70% of 216 ribosomal protein genes in Arabidopsis (Trémousaygu et al., 2003). These two elements are also found in the promoter region of several cell cycle-related genes, such as *PCNA* genes (Kosugi et al., 1995; Manevski et al., 2000). Ontological analysis of the up-regulated genes having Up1 and/or Up2 suggests a role for these elements in cell cycle, DNA processing, and protein synthesis during axillary bud growth. Consistent with this idea, the transcripts of these genes accumulate after decapitation. In silico and in vivo analyses of expression profiles demonstrate that Up element-mediated transcriptional regulation of ribosomal protein and cell division genes is a regulatory checkpoint in bud outgrowth induced by decapitation and that TCP proteins might function in this checkpoint.

Most cells in dormant buds are arrested in G_1 phase (Devitt and Stafstrom, 1995). TCP proteins are implicated in regulating the transition from G_1 to S phase (Kosugi and Ohashi, 1997). Indeed, DNA synthesis-related genes were highly enriched in Up1-containing genes up-regulated during growth initiation in buds

(see Supplemental Table IVA). However, we also found that some genes related to M phase have Up1 in their promoters. DRP5A, which encodes a dynamin-like protein, may be involved in plastid division during mitosis (Hong et al., 2003). In plants, kinesin-related proteins localize to the phragmoplast, which is found in telophase of mitosis (Liu and Lee, 2001). QRT-PCR analysis revealed that the up-regulation of these M phase-gene expressions is delayed compared with those of other cell cycle-related genes (Fig. 6B) Taken together, it is likely that TCP protein(s) act at multiple steps in the cell cycle.

Release from dormancy and subsequent growth are not equivalent processes, and they are categorized into distinct physiological phases (Cline, 1997). The expression analysis by QRT-PCR shows that induction of Up1/Up2-containing genes occurs transiently within 24 h following decapitation and the expression of these genes is reinduced after 48 h (Fig. 6). The outgrowth of the axillary shoot does not become apparent until at least 48 h after decapitation. Transcriptome analysis in this study might reflect the early induction of gene expression. Therefore, these data indicate that the early induction of Up1/Up2-mediated transcription is associated with release from bud dormancy and the latter induction reflects the subsequent outgrowth. The predicted role of TB1 is the suppression of axillary bud outgrowth (Doebley et al., 1997; Takeda et al., 2003). Accordingly, it is possible that the Up1-mediated induction of gene expression is the consequence of release from the repression of Arabidopsis TB1 ortholog. Alternatively, another TCP protein such as PCFs, which is a transcriptional activator, is possibly involved in the outgrowth regulation in Arabidopsis axillary buds.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used in this study. Buds located at the axils of cauline leaves are referred to as lateral buds, and buds located at the axils of rosette leaves are referred to as axillary buds. We also refer to small rosette leaves emerging from the axil of axillary buds as axillary rosette leaves. We assigned the order of axillary buds acropetally. Plants were grown as described in Kushiro et al. (2004). In our growth conditions, plants bolted about 30 d after seeds were sown onto soil. Decapitation of the main stem was carried out 5 d after bolting, and the point of the decapitation was below the lowest lateral buds.

Microarray Analysis

Duplicated microarray analyses using the ATH1 GenomeArray (Affymetrix, Santa Clara, CA) were carried out using independent plant materials and total RNA samples were extracted from 8 to 10 axillary shoots (both axillary buds and axillary rosette leaves) of intact (before) or 24 h after decapitated plants using RNeasy Plant mini kit (QIAGEN, Hilden, Germany). The procedures for labeling, hybridization, washing, and signal detection are described elsewhere (Kushiro et al., 2004).

Data Analysis

Signal values of individual genes were obtained by statistical algorithms in Microarray Suite software version 4.0 (MAS, Affymetrix). The reliability of hybridization signals was judged by the detection call on MAS. First, the genes detected as "Present" in two stages of duplicated experiments were selected (designated as the whole expressed genes). Genes were regarded as down-regulated and up-regulated if their expression level after decapitation were altered by at least 2-fold compared with that before decapitation.

The candidate cis-elements were searched using "Find potential regulatory sequences tool" in analyzing software GeneSpring version 4.2 (Silicon Genetics, Redwood, CA). Searches were carried out using "Find new regulatory sequence function" without allowing the single point discrepancies and the base gaps. The statistical test was carried out toward the upstream of other genomic elements with the probability cutoff 0.05. For the particular candidate sequences (SRE, Up1, and Up2; see results), the number of genes whose promoter contained the sequence and its probability value were obtained using "Enter a specific regulatory sequence function" in this analysis tool with same parameters as mentioned above.

The comparison of expression profiles between the selected gene group and remaining expressed genes was carried out by linear regression analysis using Microsoft Excel (Santa Rosa, CA). Detailed description of the in silico analyses is shown in supplemental data.

Throughout the data sets, genes were identified by the AGI gene code from the Munich Information Center for Protein Sequence (MIPS; http://mips.gsf.de/). Gene annotation was confirmed using the database of the Salk Institute Genomic Analysis Laboratory (SIGnAL; http://signal.salk.edu/smission.html). The procedure for gene ontological analysis is described elsewhere (Nakabayashi et al., 2005).

Construction of Transgenic Lines

Several promoter:*GUS* fusion genes used in this article were constructed using the Gateway Cloning Technology (Invitrogen, Carlsbad, CA). Both gain-of-function and loss-of-function constructs were created using the upstream sequences of At1g08570 (which encodes putative thioredoxin) for SRE and At4g17390 (which encodes 60S ribosomal protein L15) for Up1/Up2.

Synthetic promoter:*GUS* fusions contained 3 copies of each cis-element located upstream of the CaMV 35S minimal promoter (−90 to −1). The 35S minimal promoter amplified by PCR was cloned into the pENTR 1A vector (Invitrogen), and the created vector was named as pENTR90. The regions containing each cis-element were amplified from the upstream region of each open reading frame as follows: SRE 5′-TCTGCGAGAAGTCATGAACC-TTATCCATTTATTAGTTTTCTTCT-3′, Up1 5′-TAAAGCTAACGGAAAAA-GGCCCAATAAGGGCTTTCTTTGTCTGATGG-3′, Up2 5′-GGGCTTTCT-TTGTCTGATGGAAACCCTAATTCCCATGGAGCCA-3′, Up1 and Up2 5′-TAAAGCTAACGGAAAAAGGCCCAATAAGGGCTTTCTTTGTCTGATG-GAAACCCTAATTCCCATGGAGCCA-3′; underlining indicates the cis-elements. The 3 copies of each region were cloned into pENTR90. The destination vector, pGate101, was created from pBI101 (CLONTECH, Palo Alto, CA) and Gateway Vector Conversion System (Invitrogen). The synthetic promoter:*GUS* fusions were created from pENTR90 having a fusion promoter and pGate101 via the LR reaction described in the manufacturer's manual.

The internal deletion promoter constructs were made using PCR-based mutagenesis techniques as described in Picard et al. (1994). Wild-type promoters were amplified using each primer set as follows: the Up elements forward primer, 5′-CACCATTGCACAATATCTTCTAGG-3′, and reverse primer, 5′-TGCGATTTTCCGGCGTCGC-3′. For construction of the internal deletion promoter, mutagenic primers were used as follows: the Up1-deletion 5′-TTGTAGCGAAACATAAAGC-3′ and 5′-AACGGAAAAAAAGGGCT-TTC-3′, and the Up2-deletion 5′-TGTCTGATGGATTCCCATGG-3′. The promoters were cloned into the pENTR/D-TOPO vector (Invitrogen), and converted into pGate101 by the procedure described above.

The binary vectors constructed above were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, which was then used to transform wild-type Columbia plants by floral dipping (Desfeux et al., 2000). GUS staining was performed by a standard protocol (Jefferson, 1987). Transgenic plants were incubated with the staining buffer at 37°C for 24 h. Analyses were performed using at least 5 and 10 independent transgenic lines for the analysis. All lines showed similar GUS staining patterns in axillary buds.

QRT-PCR

The procedures of first-strand cDNA synthesis and QRT-PCR were described elsewhere (Kushiro et al., 2004). Duplicate experiments were performed using independent plant materials. AGI gene codes, abbreviations,

gene-specific primers, Taq-Man probes, and annotations used in this article are listed in Supplemental Table V.

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