DNA binding and dimerization specificity and potential targets for the TCP protein family

Shunichi Kosugi1 and Yuko Ohashi2,*
1Molecular Genetics Department, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
2Core Research for Evolutional Science and Technology (CREST), Ochanomizu, Chiyoda-ku, Tokyo, 101-0062 Japan

Summary
The TCP domain is a plant-specific DNA binding domain found in proteins from a diverse array of species, including the cycloidea (cyc) and teosinte branched1 (tb1) gene products and the PCF1 and PCF2 proteins. To understand the role in transcriptional regulation of proteins with this domain, we have analysed the DNA binding and dimerization specificity of the TCP protein family using rice PCF proteins, and further evaluated potential targets for the TCP protein. The seven PCF members including five newly isolated proteins, were able to be grouped into two classes, I and II, based on sequence similarity in the TCP domain. Random binding site selection experiments and electrophoretic mobility shift assays (EMSAs) revealed the consensus DNA binding sequences of these two classes to be distinct but overlapping; GGNCCAC for class I and GTGGNCCC for class II. The TB1 protein from maize, which belongs to class II, had the same specificity as the rice class II proteins, suggesting the conservation of binding specificity between TCP domains from different species. The yeast 2-hybrid assay and EMSA revealed that these proteins tend to form a homodimer or a heterodimer between members of the same class. We searched predicted 5’ flanking sequences of Arabidopsis genes for the consensus binding sequences and found that the consensus sites are distributed in the genome at a considerably lower frequency. We further analysed eight promoters containing the class I consensus TCP sites. The transcriptional activities of six promoters were decreased by a mutation of the TCP binding site, which is consistent with the observation that the class I TCP site can confer transactivation function on a heterologous promoter. These results suggest that the two classes of TCP protein are distinct in DNA binding specificity and transcriptional regulation.

Keywords: TCP, PCF, CYC, TB1, rice, Arabidopsis, EMSA, GUS Accession numbers for databases: PCF3, AB071804; PCF5, AB071805; PCF6, AB071806; PCF7, AB071807; PCF8, AB071808

Introduction
Plant growth and development are regulated by a number of transcription factor families, including NAC, AP2, GRAS, YABBY, ARF and TCP, whose conserved DNA binding domains have not been found in other kingdoms (Riechmann et al., 2000). These plant-specific factors could play a significant role in the developmental regulation unique to plants. Hence, the biochemical characterization of these families is important to an understanding of their biological functions as well as regulatory networks involving targets and regulators.

The TCP domain is unique to plants and is found in proteins involved in growth and development, such as the products of cycloidea (cyc) and the closely related dichotoma (dich) from Antirrhinum, and teosinte branched1 (tb1) from maize, as well as PCF proteins from rice (Cubas et al., 1999; Doebley et al., 1997; Kosugi and Ohashi, 1997; Luo et al., 1996; 1999). Antirrhinum has asymmetrical (zygomorphic) flowers with marked differences in petal shape along the dorsoventral axis, while cyc and dich double mutants produce radially symmetrical (actinomorphic) flowers (Coen, 1996; Luo et al., 1996). Mutants for the cyc gene alone produce semiradial flowers with ventralized petals in lateral positions and weakly lateralized petals in dorsal positions, whereas mutations in dich alone affect only the shape of the dorsal petals. These genes therefore functionally overlap but contribute differ-
ently to the flower development due to their differential expression specific to certain subdomains of the flower organ, and probably due to a difference in protein function. (Luo et al., 1999).

The tb1 gene affects the growth and development of axillary buds in maize, and has greatly contributed to domestication of an ancient strain, teosinte (Doebely et al., 1995; Wang et al., 1999; White and Doebley, 1998). tb1 mutants exhibit a phenotype of multiple long primary lateral shoots bearing terminal tassel-like inflorescences, which contrast with wild type lateral branches with arrested buds or developing female inflorescences (ears). In teosinte, the level of expression of the tb1 gene is reduced by about half (Doebely et al., 1997). Thus, it has been implied that tb1 functions as a repressor of the growth of lateral shoots and development of female inflorescences. This hypothesis is supported by another phenotype of the cyc mutant: an arrested stamen at the dorsal position. The arrest of the stamens is probably caused by an arrest of the cell cycle because the cell cycle regulators cyclin D3b, cyclins B1 and B2, cdc2b and cdc2c are not detectable in the arrested stamens (Gaudin et al., 2000).

The biochemical function of the TCP domain has been elucidated from the study of rice PCF1 and PCF2 proteins, whose TCP domains can be classified into a class distinct from those of CYC and TB1. PCF1 and PCF2 have been isolated as DNA-binding proteins that specifically interact with cis elements, site Ila and site IIb, necessary for the meristematic tissue-specific expression of the rice promoter of proliferating cell nuclear antigen (PCNA), a component of DNA replication and DNA repair synthesis machinery (Kosugi and Ohashi, 1997; Kosugi et al., 1995). The PCF proteins seem to play an important role as transcription factors in the cell cycle-dependent expression of the rice PCNA promoter, as well as the E2F transcription factor family evolutionarily conserved in animals (Kosugi and Ohashi, 2002). The TCP domain in the PCF1 and PCF2 proteins constitutes a predicted basic helix-loop-helix (bHLH) structure and is required for both the DNA binding and dimerization (Kosugi and Ohashi, 1997). This bHLH is structurally and functionally distinct from the canonical bHLH domain contained in MyoD and c-Myc from mammals and B and R gene products from maize, which bind to the E-box consensus sequence (CANNTG) (Cubas et al., 1999; Kosugi and Ohashi, 1997). These observations suggest that CYC and TB1 are also transcription factors with DNA binding and homo and hetero dimerization activities.

To investigate the biochemical properties of TCP proteins in detail, we determined the DNA binding and dimerization specificity of the TCP protein family using the PCF proteins including five newly isolated members, consisting of two classes (class I and class II). The consensus DNA binding sequences of the class I and class II TCP proteins shared a core sequence (GGNCCC) but differed in the flanking dinucleotide, and completely matched the sequences of sites Ila and IIb. A computer search for these consensus sequences revealed that the Arabidopsis genome contains few consensus site-containing promoters. Using eight representative promoters, we evaluated the biological function of the consensus TCP binding sites using transgenic tobacco cells and intact plants, and revealed that the binding sites of the class I TCP protein function as positive cis elements in six promoters.

**Results**

**Cloning of the rice TCP proteins PCF**

PCF1 and PCF2 have been shown to bind the site Ila and site IIb elements in the rice PCNA promoter through the TCP domain present in their N-terminal region (Kosugi and Ohashi, 1997). To isolate other TCP proteins from rice, we screened a cDNA library derived from rice meristematic leaves in a GAL4 activation domain-tagged vector by the yeast 1-hybrid system using the site Ila, site IIb, CYC1-HIS3 reporter gene. A total of five distinct cDNA clones that encoded the TCP domain were isolated and their deduced proteins were designated PCF3, PCF5, PCF6, PCF7 and PCF8. As observed for PCF1 and PCF2, these proteins shared homology limited to the TCP domain in the N-terminal region. A comparison of the amino acid sequence of the TCP domain revealed that these proteins can be categorized into two classes with distinct sequence similarity, class I and class II (Figure 1), consistent with the observation for the TCP domains in Arabidopsis (Cubas et al., 1999). PCF1, PCF2 and PCF3 are members of the class I TCP family, while the others are class II proteins. The CYC protein from Antirrhinum and TB1 from maize can be classified as class II type, although the isolated PCF proteins (PCF5-8) do not contain an R domain found in CYC and TB1.

**DNA binding specificity of class I and class II TCP proteins**

To determine the sequence specificities for the binding of the class I and II TCP proteins, we conducted a random binding site selection analysis using recombinant PCF2 and PCF5 proteins, as representative of rice class I and II TCP proteins, respectively. After the fifth round of selection, 76 and 83 oligonucleotides chosen for PCF2 and PCF5 binding were sequenced, respectively, and their consensus sequences were aligned (Figure 2). The consensus DNA binding sequences of PCF2 and PCF5 were GGNCCCA and G(t)/cGGNCCC, respectively, and shared
the core sequence 5′-GGNC-3′ flanked by Ac at the 3′ end and its complement GT at the 5′ end, respectively, on the different side. The consensus PCF2 binding sequence exhibited a perfect match with site IIa (GGGCCCAC) and site IIb (GGTCCCAC) in the rice PCNA promoter, which is consistent with the DNA binding specificity of PCF1 and PCF2 (Kosugi and Ohashi, 1997). The site IIa sequence also matches the PCF5 consensus binding sequence due to the palindromic GGGCCC of site IIa.

We further confirmed the integrity of the consensus sequences by electrophoretic mobility shift analyses (EMSA) using mutated oligonucleotide probes (Figure 3a). Recombinant thioredoxin proteins fused with PCF1, PCF2 and PCF3 for the class I proteins, and with PCF5, PCF6 and TB1 for the class II proteins were used for EMSAs. A double stranded oligonucleotide probe, pcs1, which possessed both the consensus sequences, GTGGTCCCAC, bound all six proteins with a high affinity (Figure 3b). The probes pcsm1 and pcsm2, in which a single nucleotide at position −2 (G) and +1 (C) of pcs1 was changed to T and A, respectively, had no activity to bind the proteins, indicating the importance of the core sequence for the binding of both classes of proteins.

Single nucleotide changes at positions +4 (pcsm3 and pcsm4) and +5 (pcsm5) resulted in a decrease in binding to class I proteins (PCF1, PCF2 and PCF3). Although PCF3 appeared to effectively bind these mutant probes (pcsm3, pcsm4 and pcsm5), the amount of free probe was much higher than with the wild-type pcs1, indicating that these three class I proteins have a similar DNA binding specificity. In contrast, the mutant probes did not cause any change in the binding activities of the class II proteins (PCF5, PCF6 and TB1). The negative effect of the mutation at position +4 or +5 on the binding of the class I proteins was greatly enhanced by simultaneous mutation at positions −5/−4 (pcsm6, pcsm8 and pcsm9). Although positions −4 and −5 were not biased toward GT in the oligonucleotide pool selected by PCF2 (Figure 2), a single mutation at positions −5/−4 (pcsm6) led to a decrease in the binding of the class I proteins to an extent similar to that of the pcsm3, pcsm4 and pcsm5 probes (Figure 3b). In contrast, the pcsm6 probe did not bind the class II proteins. These results indicate that the consensus binding sequences determined for PCF2 and PCF5 represent the optimal DNA binding sequence of each class of TCP protein.

Homodimer and heterodimer

PCF1 and PCF2 form a homodimer and heterodimer through the TCP domain to bind DNA (Kosugi and Ohashi, 1997). Using the six rice PCF proteins, we analysed the protein–protein interaction by the yeast 2-hybrid assay with reciprocal interaction between bait and prey vectors (Figure 4). All the proteins exhibited substantial activity for homodimerization, except for PCF3 whose bait construct hardly expressed any protein in yeast. In contrast, the interaction to form a heterodimer was limited to PCF1/PCF2, PCF1/PCF3, PCF5/PCF6 and PCF6/PCF7. Heteromeric interaction appeared to occur preferentially among the same class but not between the two classes, although there was some inconsistency dependent on the bait or prey vector. Preferential interaction of the class II PCF members with TB1 was also observed when TB1 was used as prey (data not shown). Additionally, when TB1 alone was expressed as bait, strong activation of the reporter, giving 25 units of β-galactosidase activity, was observed, suggesting that TB1 contains a strong transactivation domain. Also, PCF6 could contain a weak transactivation domain, as shown in Figure 4.

To confirm the formation of a heterodimer complex in vitro, EMSA was performed with in vitro translated products of PCF1, PCF2 and PCF7. When the translation product of PCF1 or PCF2 was incubated with the pcs1 probe, a high and a low mobility complex was detected (Figure 5). When a co-translation product of PCF1 and PCF2 was used, a major shifted band was detected at the intermediate position, indicating that the heterodimer of PCF1 and PCF2 is predominant. In contrast, EMSA with a co-translation product of PCF1 and PCF7 resulted in no formation other than the bands derived from the PCF1 and PCF7 homodimer. The results of the in vitro analysis showed a good correlation with the results of the yeast 2-hybrid assay.

The consensus binding sequence of the class I protein mediates transcriptional activation

The above results suggest a specific function for each class of the consensus binding sequences mediated by the binding of the corresponding class of proteins. We then examined in vivo the function of the consensus binding sequences of the two classes of TCP proteins by transgenic analyses with cultured tobacco cells. Reporter genes were constructed by inserting two, four or six copies of the pcsm5, pcsm6 or pcsm7 oligonucleotide upstream of the
CaMV35S-54 core promoter fused to the β-glucuronidase (GUS) gene. Pcsm5 and pcsm6 were used as class II- and class I-specific binding sequences, respectively, and pcsm7 was used as a mutant control that can bind proteins of neither class. Suspension cells established from a bulk of transgenic calli carrying the pcsm6-GUS construct showed an increase in GUS activity especially when the copy number of pcsm6 was increased to six, although the level of the activity was low. In contrast, pcsm5-GUS or pcsm7-GUS exhibited no increase in GUS activity even when the copy number of pcs5 or pcs7 was increased (Figure 6). These results suggest that the class I consensus binding sequence can confer transcriptional activation.

Potential target genes for TCP proteins

The Arabidopsis genome encodes 24 predicted TCP proteins whose TCP domains can be categorized into two classes, as observed for the rice PCF proteins. To determine potential targets for the TCP proteins, we searched the Arabidopsis genome database for the consensus binding sequences of the TCP protein using the Pattern Matching program of the Arabidopsis Information Resource (http://www.Arabidopsis.org/). Although we found >100 predicted 5’ upstream regions that contained the consensus sequences within 600 bp upstream of their translation start, the frequency of occurrence in the Arabidopsis genome was quite low. When we searched 1269 predicted 5’ upstream regions from randomly selected Arabidopsis BAC clone sequences derived from different chromosomes, only 14 and 7 genes (3 genes are common) were found to contain the class I and class II consensus sites within the proximal 600 bp regions, respectively. These frequencies were 7 and 14 times lower than the expected value (about one copy per 13 600 bp promoters) of the class I or class II consensus.
sequence when the GC content was 50%. However, because 5’ flanking sequences of *Arabidopsis* genes are generally AT-rich, these unexpected low frequencies of the GC-rich TCP sites are assumed to be attributed to a low GC content in the 5’ flanking sequences.

We then selected eight genes whose functions or properties were likely to be associated with cell growth or development and isolated the 5’ upstream regions by PCR, although TCP site-containing genes in *Arabidopsis* have not been biased toward functionally or structurally related genes (Figure 7a). *Atgh3* is highly homologous to

---

**Figure 6.** Transcriptional activity mediated by the consensus PCF binding sequences. Cultured tobacco cells were transformed with the 35S-54-GUS reporter construct under the control of 2-, 4- or 6-tandem repeats of the pcs5, pcs6 or pcs7 sequence. The bulk of the cells consisting of 150–200 independent transgenic calli were cultured in a liquid medium containing kanamycin sulfate (50 mg l⁻¹) with shaking. The suspension-cultured cells at mid-log phase were harvested and assayed for GUS activity. The experiment was repeated twice with similar results.

**Figure 7.** Transcriptional activity of *Arabidopsis* promoters containing the consensus PCF/TCP binding sites. (a) Alignment of the consensus TCP binding sites found in 5’ upstream regions of *Arabidopsis* genes. The consensus PCF/TCP binding sequences are boxed and the relative positions of nucleotides are numbered at the top. Bold capital letters in the boxed sequences indicate nucleotides that match the consensus sequence. Numbers marked at the right represent positions of the last nucleotides relative to the translation start sites of the genes. (b) Promoter activity mediated by consensus TCP sites. Cultured tobacco cells were transformed with the GUS reporter construct under the control of the *Arabidopsis* promoters shown in (a) and mutants for the consensus TCP sites. The GUS assay was performed as described in the legend to Figure 6. Solid and white bars represent the activities of the wild-type and mutant promoters, respectively. The experiment was repeated twice with similar results.

the auxin-responsive soybean GH3 gene (Liu et al., 1994). Atpts2 and Atxet encode homologues of the 26S proteasome regulatory subunit S2 and xyloglucan endotransglycosylase/hydrolase, respectively. The HY5 gene encodes a bZIP transcription factor involved in photomorphogenesis in Arabidopsis (Oyama et al., 1997). The TUB3, TUB7, NR1 and NR2 genes are beta-type tubulin and nitrate reductase genes of Arabidopsis (Lin et al., 1994; Snustad et al., 1992). The 5' upstream region of these genes contained the complete sequence of at least the class I consensus site, except for TUB3, TUB7 and NR2, in which 1 bp at position +4 or +5 was mismatched (Figure 7a).

These 5' upstream regions were fused with the GUS coding region and introduced into cultured tobacco cells. The GUS assay with suspension cells established from a bulk of transgenic calli revealed that all of the 5' upstream regions had the transcriptional activity in the tobacco cells (Figure 7b). These promoters were mutated at the consensus TCP sites by the conversion of nucleotides GG at positions ±3 and ±2 to TC and assayed with the cultured tobacco cells. Six of the mutant promoters had reduced activity relative to the corresponding wild-type promoters. The activities of the Atgh3 and Atpts2 promoters were especially decreased by the mutation. The activity of the rice PCNA promoter (OsPC) was also decreased by a simultaneous mutation of the two consensus sites (site Ila and site Iib), as previously observed with transgenic plants (Kosugi et al., 1995). In contrast, the activity of the TUB7 promoter was not affected and that of the NR1 promoter was slightly increased by the mutation (Figure 7b). These results indicate that the class I binding site contributes to the transcriptional activation of many but not all of the class I site-containing promoters, which is consistent with the result shown in Figure 6.

The Atgh3 gene, a homologue of the auxin-inducible GH3 gene, contained potential auxin-response elements, TGTCTC (~248) and CGTCTC (~198), 5 bp and 55 bp, respectively, downstream of the consensus TCP site. It was thus expected that the Atgh3 promoter responds to auxin and the consensus TCP site affects the response. Transgenic tobacco plants carrying the Atgh3-GUS and mAtgh3-GUS constructs were produced, and leaves excised from these plants were assayed for GUS activity induced by auxin, an α-naphthalene acetic acid. The Atgh3 promoter exhibited a high degree of auxin-inducibility in a dose-dependent manner (Figure 8a). Although the mAtgh3 promoter also retained auxin-inducibility, the level of expression was lowered to on average about one-third that of the wild-type promoter, as observed for the transgenic cultured cells. On the other hand, an assay with transgenic plants carrying the TUB7-GUS construct showed that the TUB7 promoter has strong activity in meristematic tissues such as immature leaves and weak activity in terminally differentiated mature leaves (Figure 8b). The expression in both mature and immature leaves was less affected by the mutation of the consensus TCP site (Figure 8b), which is consistent with the observation for the transgenic cultured cells.

Taken together, these results indicate that the TCP binding site in the Atgh3 promoters, and probably also in the other TCP-site regulated promoters, is functional in both cultured cells and intact plants.
Discussion

The rice TCP proteins, PCFs

We have isolated five new members of the PCF family, the TCP protein family from rice. These proteins in addition to the previously isolated PCF1 and PCF2 are highly homologous only in the TCP domain present in the N-terminal region. Based on the TCP domain, the PCF family can be categorized into two groups, class I and II. The Arabidopsis genome encodes 24 predicted TCP proteins, 13 of which can be grouped as class I and 11 as class II based on similarity in the amino acid sequence of the TCP domain. The high degree of conservation and clear classification of the TCP domain among diverged plant species suggest that the two classes play a fundamental role in the cellular processes of plant growth and development, and have different biological roles due to different biochemical properties.

DNA binding and dimerization specificities of the two classes of TCP proteins

The random binding site selection analysis with PCF2 and PCF5 and EMSAs with six different TCP members revealed the consensus DNA binding sequences of the two classes of TCP proteins (GGNCCCAC for class I and GTGGGCAC for class II). The two sequences exhibit no evident similarity to any binding sites of other DNA binding proteins and any known functional promoter elements other than sites IIA and IIb, cis-elements of the rice PCNA promoter. The core (GGNCCC) shared by these sequences has a strict role in the binding of both classes, and the flanking dinucleotide (GT) of the class II consensus sequence at positions −5 and −4 is essential for the binding of the class II TCP proteins. In contrast, the dinucleotide (AC) of the class I consensus sequence at positions +4 and +5 has a more moderate effect on the binding of the class I proteins, depending on the presence of GT at positions −4 and −5. The class I proteins, thus, can bind also to the class II consensus sequence at least when the class I consensus site at either position +4 or +5 is conserved. A palindromic sequence such as pcs1, which has both the class I and class II consensus sequences, seems to have maximal activity for the DNA binding of the class I TCP.

The results of the yeast 2-hybrid assay and the EMSA with in vitro co-translation products of PCF1 and PCF2 suggest that PCF proteins can readily form heterodimers between specific members among the same class, but not between the two classes. If this is true of all other members of the TCP family, the heterodimers formed by TCP proteins in vivo would bind to either of the consensus binding sequences.

Recently it was reported that an Arabidopsis class II TCP protein, PTF1, was isolated as a DNA binding protein of the chloroplast psbD light-responsive promoter (Baba et al., 2001). The authors have shown that an ACC repeat located around −40 of the promoter is essential for the binding of PTF1 using a yeast 1-hybrid assay with several multimerized promoter fragments. This DNA binding specificity seems to be inconsistent with our findings on the consensus binding sequence. In the DNA fragments that exhibit the interaction with PTF1, however, there are sequences similar to the class II TCP consensus site: GTGGACCT in the wild type and mt-1 and TAGGTCCC in mt-3. Although these sites would be incomplete for efficient binding to TCP proteins, their multimer could have an increased affinity for the binding. It may be necessary to conduct more detailed analyses for evaluation of the binding specificity of PTF1.

Transcriptional regulatory role of TCP proteins

Analysis of the promoters containing the class I consensus site has revealed that most class I sites can function as positive cis elements with some degree of contribution to the transcription. Overall, the activity of the Arabidopsis promoters seems to correlate with the binding potential of the class I TCP proteins. The Atpts2 promoter, which exhibited the greatest contribution by the consensus site to the transcription, contains a perfectly palindromic sequence, which could strongly bind to the class I TCP proteins. In contrast, the activities of TUB3 and TUB7 promoters, which contain imperfect TCP consensus sites, did not depend on the TCP sites. It has been shown that, among potential binding sites for the yeast alpha2/Mcm1 repressor complex found in the yeast genome, all of the functional sites are located in proximal promoter regions and exhibit a perfect or nearly perfect match with the consensus binding sequence (Zhong et al., 1999). This finding supports our observation that suggests an effect depending on relative position of the TCP site as well as the importance of the binding potential. For the NR1 promoter, the class I binding site appears to be involved in negative regulation, although it perfectly matches the consensus sequence. A similar finding has been made by linker scanning mutation analyses of the NR promoters, in which the NR1 and NR2 promoters containing TCP site-mutations have slightly increased (LS1-6) and considerable decreased (LS2-12 and LS2-13) activities relative to the wild-type promoters, respectively (Hwang et al., 1997). These observations suggest that the transcriptional regulatory effect of some TCP binding sites could depend on the relative position, which could affect the interactions with other promoter elements.

Although the class I binding sites function mainly as positive cis-elements, class I TCP proteins (probably also
class II proteins) seem to have no ability to activate transcription by themselves. We have observed that none of the PCF proteins examined could transactivate a reporter gene driven by the consensus TCP sites in cotransfection assays with cultured tobacco cells and mesophyll protoplasts (S. Kosugi and Y. Ohashi, unpublished data). This raises a possibility that the observed mutational effects of TCP sites on the transcription are due to an impaired interaction with unrelated transcriptional activators that bind to a sequence overlapping the TCP site. However, the observation that TCP site-dependency appears to correlate with the binding potential of the TCP site in the promoters analysed suggests that the decreased activity of mutated promoters is an actual effect of disrupting the interaction with TCP proteins. Thus, the transcriptional activation mediated by the class I site would require other factors that could interact with class I TCP proteins. Alternatively, only a few class I PCF proteins containing a transactivation domain might be responsible for the transactivation.

It is not clear how the class II site affects promoter activity because we have not assessed this using promoters containing only class II sites. The observation that a reporter construct under the control of a multimerized class II site exhibited no significant transcriptional activity suggests that the class II consensus site cannot confer the ability to activate transcription. However, PCF6 and TB1 could confer a moderate and strong transactivation function in yeast, respectively, when expressed as bait, as shown in Figure 4 and in the text. The transactivation in yeast may be mediated by a domain exposed only in the monomer form because neither PCF7 nor TB1 has the ability to transactivate a TCP site-reporter gene in plants. These class II TCP proteins might acquire their transactivation function via a factor that changes their conformation to expose the transactivation domain.

**Potential targets for TCP proteins**

We have found that the consensus TCP binding sites are distributed in predicted proximal promoter regions in the *Arabidopsis* genome at a considerably lower frequency. These TCP site-containing genes have not been biased toward functionally or structurally related genes. However, the *Atgh3*, *Atpts2* and rice PCNA promoters, whose activities exhibited a great dependence on the TCP sites, appear to share a common feature in their regulation. The *Atgh3* gene is a homologue of the soybean GH3 gene, an auxin-inducible gene of unknown function. It has been shown that an *Arabidopsis* mutant overexpressing the *DFL1* gene, a closely related homologue of *Atgh3*, exhibits inhibited growth of shoots, hypocotyls and lateral roots in light and resistance to exogenous auxin treatment (Nakazawa et al., 2001). Another *Arabidopsis* GH3 homologue FIN219, more distantly related to *Atgh3* and *DFL1*, has been shown to act to inactivate COP1, a photomorphogenic repressor, and mediate a far-red light-specific inhibition of hypocotyl elongation (Hsieh et al., 2000). These findings suggest that GH3 homologues are involved in organ growth in response to auxin and light. Like GH3 and FIN219, Atgh3 shows an auxin-responsive expression, which is likely to be mediated by elements similar to the auxin response elements (AuxRE, consensus: TGTTCCT, Ulmasov et al., 1999), located immediately downstream of the TCP site. The TCP site in the *Atgh3* promoter contributes to the increase in the level of expression. Like *Atgh3*, *Atpts2*, a gene encoding a homologue of the 26S proteasome subunit S2, may also be auxin-inducible because there are several AuxRE-like elements around the TCP site. The ubiquitin-proteasome pathway is known to play a pivotal role in cell cycle progression, auxin response, and photomorphogenesis through degradation of the HY5 regulator in plants (Genschik et al., 1998; Gray and Estelle, 2000; Osterlund et al., 2000; Zhao et al., 2001). Consistently, the *Atpts2* product contains a potential bipartite nuclear localization signal in the N-terminus, which is generally found in nuclear proteasome components involved in degradation of cell cycle regulators. Also, it has been shown that a tobacco 26S proteasome subunit *NtPS1* gene is expressed preferentially in meristematic tissues (Bahrami and Gray, 1999). The rice PCNA gene is a cell cycle-regulated gene induced at the G1(G0)–S phase boundary and is also induced by stimulation of cell growth by auxin and/or cytokinin treatment (Kosugi et al., 1991). These observations imply that the class I TCP proteins are likely to be involved in up-regulation of several growth-associated genes including auxin-induced genes.

As for the class II TCP proteins, the *tb1* and *cyc* genes have been presumed to work to inhibit the growth of axillary buds of maize and stamens at dorsal positions of *Antirrhinum* flower buds, respectively. The class II TCP proteins, thus, seem to be involved in repressing several growth-associated genes, in contrast with the potential activation function of class I proteins. It is also likely that the growth inhibition is rather indirect and the class II TCP proteins directly activate repressor genes for organ growth such as *DFL1* and *FIN219*. In another case, the geminivirus AL2 gene product was found to confer the ability to transactivate through a sequence motif termed the conserved late element (CLE, consensus: GTGGTCCC), which is present in the coat and movement protein promoters of many geminiviruses, even though AL2 binds single-stranded DNA in a sequence-nonspecific manner (Arguèllo-Astorga et al., 1994; Hartitz et al., 1999; Ruiz-Medrano et al., 1999). Because CLE completely matches the class II consensus TCP site, the transactivational effect of the AL2 gene product may have been exerted by directly
competing with the repressive effect of some class II TCP proteins or by being recruited to CLE through interaction with a class II TCP protein.

The overlap in the binding specificity of the class I and class II TCP proteins also suggests that the two classes coordinate or competitively regulate transcription through the binding of consensus TCP sites. The individual roles and actual targets of the TCP proteins will be revealed in future studies incorporating these observations.

Experimental procedures

Plant materials

Suspension-cultured tobacco cells were established from calli of Nicotiana tabacum cv. Sumsun NN. The calli were developed from leaf strips grown on MS basal medium (Murashige and Skoog, 1962) supplemented with 200 mg I^-1 of NaH_2PO_4 and 1.0 mg I^-1 of 2,4-dichlorophenoxyacetic acid (2,4-D), cultured with liquid medium containing the same constituents with shaking at 100 r.p.m. at 28°C, and maintained by weekly subculturing in a fresh medium.

Cloning of PCF members from rice

An oligo-dT primed cDNA library derived from rice meristematic leaves (Oryza sativa cv. Nipponbare) was constructed in the λHibrizAP vector (Stratagene, La Jolla, CA, USA) using the HybriZAP 2.1 cDNA synthesis kit (Stratagene), and in vivo excised plasmids based on the pAD-GAL4-2.1 vector were prepared, according to the instructions provided by the manufacturer (Stratagene). Yeast 1-hybrid screening was conducted as described previously (Kosugi and Ohashi, 1997). A total of 50 μg of the cDNA library was introduced into yeast containing the (site lla)12-CYC1-HIS3 reporter gene, to produce approximately 5 × 10^6 Leu^- transformants. We obtained eight independent clones that exhibited the Leu^+ phenotype, of which five were identified as for PCF1 and PCF2 cDNAs (Kosugi and Ohashi, 1997) cloned in the pBluescript KS+ vector (Stratagene) were inserted into the EcoRI and Xhol sites of the pAD-GAL4-2.1 vector to generate pAD-PCF1 and -PCF2. The bait plasmids (pGB-PCF5s) were generated by inserting the EcoRI-Xhol or EcoRI-Sal fragments from the above pAD clones into the EcoRI and Sal sites of the pGBK7 vector (Clontech, Palo Alto, CA, USA).

To generate reporter constructs (pcsm5)-54-GUS (pcsm6)-54-GUS and (pcsm7)-54-GUS, double stranded oligonucleotides pcsm5, pcsm6 and pcsm7 (Figure 3) were self-ligated after phosphorylation of the 5'-end and 3'- and 5'-copies of these oligonucleotides aligned head to tail were inserted into the Xhol site of the 35S-54-GUS vector (Kosugi et al., 1995). To clone into a binary vector, HindIII-EcoRI fragments from these constructs were replaced with the corresponding fragment of the pHIB101 vector (Clontech).

The 5' upstream regions of the Arabidopsis genes were isolated by PCR, based on sequence information in GenBank and the AtDB database.

Construction of plasmid

The pET-32a vector (Novagen, Madison, WI, USA) was used for production of bacterially expressed proteins, as for PCF1 and PCF2 (Kosugi and Ohashi, 1997). The EcoRI-Xhol fragments of the PCF3 and PCF8 cDNAs and BamHI-Xhol fragments of the PCF5 cDNAs from the originally isolated pAD-GAL4-2.1 clones were inserted into the corresponding sites of the pET-32a vector. An EcoRI-Xhol fragment of the T71 cDNA was produced from a PCR product amplified with a primer set (5'-GGTGACTAGTTTTAATACGTAGTACGATCTC3') and a T71 cDNA clone (kindly provided by Dr Doebely) as a template, and cloned into the pET-32a vector.

The originally isolated PCF3, PCF5, PCF6, PCF7 and PCF8 clones (pAD-PCF3, 5, 6, 7, 8) were used as the prey of the yeast 2-hybrid assay. The EcoRI-Sal fragment of the full-length PCF1 and PCF2 cDNA (Kosugi and Ohashi, 1997) cloned in the pBluescript SK+ vector (Stratagene) were inserted into the EcoRI and Xhol sites of the pAD-GAL4-2.1 vector to generate pAD-PCF1 and -PCF2. The bait plasmids (pGB-PCF5s) were generated by inserting the EcoRI-Xhol or EcoRI-Sal fragments from the above pAD clones into the EcoRI and Sal sites of the pGBK7 vector (Clontech, Palo Alto, CA, USA).

To generate reporter constructs (pcsm5)-54-GUS (pcsm6)-54-GUS and (pcsm7)-54-GUS, double stranded oligonucleotides pcsm5, pcsm6 and pcsm7 (Figure 3) were self-ligated after phosphorylation of the 5'-end and 3'- and 5'-copies of these oligonucleotides aligned head to tail were inserted into the Xhol site of the 35S-54-GUS vector (Kosugi et al., 1995). To clone into a binary vector, HindIII-EcoRI fragments from these constructs were replaced with the corresponding fragment of the pBI101 vector (Clontech).

To generate reporter constructs (pcsm5)-54-GUS (pcsm6)-54-GUS and (pcsm7)-54-GUS, double stranded oligonucleotides pcsm5, pcsm6 and pcsm7 (Figure 3) were self-ligated after phosphorylation of the 5'-end and 3'- and 5'-copies of these oligonucleotides aligned head to tail were inserted into the Xhol site of the 35S-54-GUS vector (Kosugi et al., 1995). To clone into a binary vector, HindIII-EcoRI fragments from these constructs were replaced with the corresponding fragment of the pBI101 vector (Clontech).
CTTTTCTTTATAATTCACCTCCTCTTC-3' for m1TUB7 and
5'-CTCTTTTTCTCTTATAAAGACAGTCTTCTTCTCTC-3' for
m2TUB7, in which the mutated nucleotides are underlined. The
mutated fragments were cloned into the pBI101 vector to gener-
ate mATH3, mAptx2, mAAXt, mHy5, mNR1, mNR2, mTUB3, m1TUB7 and m2TUB7. For the rice PCNA promoter
construct containing the double-mutation of site Ila and site Iib
(mOsPC), the previously generated construct M2ab (Kosugi et al.,
1995) was used.

**Production of recombinant proteins and electrophoretic
mobility shift assays (EMSAs)**

The production and purification of recombinant thioredoxin (Trx)
fusion proteins with PCF1, PCF2 and PCF3 were performed as
described previously (Kosugi and Ohashi, 1997). Fusion proteins
with PCF5, PCF8 and TB1 were purified from the inclusion bodies
produced in bacteria under denaturing conditions, as described
previously (Kosugi and Ohashi, 2000).

The conditions for the DNA binding reaction and electrophor-
esis were as described (Kosugi and Ohashi, 1997), except that
100 ng of salmon sperm DNA per 10 μl of reaction solution was
used in place of poly(dI-dC) as a non-specific DNA competitor.
DNA probes were generated by annealing oligonucleotides
containing the sequences represented in Figure 3 with cohesive
SalI or XhoI sites at the 5’ ends, and by filling with α-32P-dCTP
using the Klenow enzyme.

**Random binding site selection analyses**

Random binding site selection for the PCF2 and PCF5 proteins
was performed using the oligonucleotide BS18N, as described
previously (2000). The purified Trx-PCF2 or Trx-PCF5
protein (100 ng) was incubated with 1.5 μg of BS18N in 50 μl of
1 × EMSA buffer containing 0.05% BSA, 0.5 μg of poly(dI-dC) and
0.5 μg of salmon sperm DNA. The DNA-protein complex was
produced in bacteria under denaturing conditions, as described
previously (Kosugi and Ohashi, 1997). Fusion proteins
containing the sequences represented in Figure 3 with cohesive
SalI or XhoI sites at the 5’ ends, and by filling with α-32P-dCTP
using the Klenow enzyme.

**Yeast manipulations and yeast 2-hybrid assay**

Yeast manipulations including culture, transformation, and the
yeast 2-hybrid assay with strain SFY526 were performed as described (Kosugi and Ohashi, 1997).

In vitro coupled transcription-translation reactions

Proteins were synthesized by coupled transcription-translation
with a TNT rabbit reticulocyte lysate kit (Promega) using T7 RNA
polymerase and the pG8-PCF1, -PCF2 and -PCF7 plasmids. Reactions were performed with 1 μg of total plasmid DNA in
25 μl of solution.

**Agrobacterium-mediated plant transformation and assay
of β-glucuronidase activity**

Agrobacterium-mediated transformation of tobacco leaf discs and
assays for GUS activity were performed as described previously
(Kosugi et al., 1995). Transformation of suspension-cultured
tobacco cells was conducted by coculture of 5 ml of the tobacco
suspension cells at a log phase with 100 μl (OD600 1.0) of
Agrobacterium carrying GUS reporter gene constructs cloned in
the binary vector at 28°C for 2–3 days, followed by selection on
MS agar plates containing 75 mg l−1 kanamycin and 500 mg l−1
carbenicillin.

**Acknowledgements**

We thank Dr Doebley (University of Minnesota, St Paul) for kindly
providing the TB1 cDNA plasmid.

**References**

Argüello-Astorga, G.R., Guevara-González, R.G., Herrera-Estrella,
origins have a group-specific organization of iterative elements:

Involvement of a nuclear-encoded basic helix-loop-helix
protein in transcription of the light-responsive promoter of

alpha-type subunit gene during tobacco development and


domain: a motif found in proteins regulating plant growth and

the origin of maize: evidence for epistasis and the evolution of

Doebley, J., Stec, A. and Hubbard, L. (1997) The evolution of

Gaudin, V., Lunness, P.A., Fobert, P.R., Towers, M., Riou-
The expression of D-cyclin genes defines distinct
developmental zones in snapdragon apical meristems and is
locally regulated by the *Cycloidea* gene. *Plant Physiol.* 122,
1137–1148.

Genschik, P., Criqui, M.C., Parmentier, Y., Derevier, A. and Fleck,
Identification Destruction Box Pathway Metaphase Arrest
Produced Proteasome Inhibitor Mg132. *Plant Cell* 10, 2063–
2076.

Gray, W.M. and Estelle, I. (2000) Function of the ubiquitin-
25, 133–138.

golden mosaic virus transactivator (TrAP) is a single-stranded
DNA and zinc-binding phosphoprotein with an acidic activation

Hsieh, H.L., Okamoto, H., Wang, M., Ang, L.H., Matsui, M.,
gene, defines a link between phytochrome A and the
downstream regulator COP1 in light control of *Arabidopsis*

Sequences necessary for nitrate-dependent transcription of
862.

Kosugi, S. and Ohashi, Y. (1997) PCF1 and PCF2 specifically bind


