A T9G Mutation in the Prototype TATA-Box
TCACTATATATAG Determines Nucleosome Formation and Synergy with Upstream Activator Sequences in Plant Promoters1[W]

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We had earlier reported that mutations to G and C at the seventh and eighth positions in the prototype TATA-box TCACTATATATAG inhibited light-dependent activation of transcription from the promoter. In this study, we characterized mutations at the ninth position of the prototype TATA-box. Substitution of T at the ninth position with G or C enhanced transcription from the promoter in transgenic tobacco (Nicotiana tabacum) plants. The effect of T9G/C mutations was not light dependent, although the 9G/C TATA-box showed synergy with the light-responsive element (lre). However, the 9G/C mutants in the presence of lre failed to respond to phytochromes, sugar, and calcium signaling, in contrast to the prototype TATA-box with lre. The 9G/C mutation shifted the point of initiation of transcription, and transcription activation was dependent upon the type of activating element present upstream. The synergy in activation was noticed with lre and legumin activators but not with rbcS, Pcc, and PR-1a activators. The 9G mutation resulted in a micrococcal nuclease-sensitive region over the TATA-box, suggesting a nucleosome-free region, in contrast to the prototype promoter, which had a distinct nucleosome on the TATA-box. Thus, the transcriptional augmentation with mutation at the ninth position might be because of the loss of a repressive nucleosomal structure on the TATA-box. In agreement with our findings, the promoters containing TATAGATA as identified by genome-wide analysis of Arabidopsis (Arabidopsis thaliana) are not tightly repressed.

The core promoter is essential for efficient and accurate initiation of transcription in eukaryotic genes. The core promoter sequence is generally located between −35 and +35 bp with respect to the transcription start site (Smale, 2001) and mainly comprises two important elements: the TATA-box and the initiator element. Various functional DNA motifs, known as core promoter elements, assist in the recruitment, assembly, and initiation of the RNA polymerase II transcription machinery. Transcription by RNA polymerase II involves the recruitment of general transcription factors such as TFIIA, -B, -D, -E, -F, and -H, coactivators (such as the mediator complex), and polymerase II to the promoter to form a preinitiation complex (PIC; Hahn, 2004). Several of the general transcription factors have been proposed as targets of transcriptional activators, including TFIID, TFIIB, and TFIIF. TFIID is a multisubunit protein complex consisting of TBP and approximately 14 associated factors that plays a critical role not only in promoter binding but also as a transducer in conveying the upstream regulating signals to the downstream general transcription machinery. The core promoter and its elements are highly variable, so they can be a point of regulation for gene-specific transcription (Zhu et al., 1995; Nakamura et al., 2002; Kiran et al., 2006; Bjornsdottir and Myers, 2008). Efficient transcription generally requires the synergistic action of multiple activators bound at distinct sites upstream (or downstream) of the promoter (Struhl, 1999). Activators contain a DNA-binding domain that specifically recognizes enhancer elements and a physically separate activation domain that stimulates transcription of the target gene (Brent and Ptashne, 1985; Hope and Struhl, 1986; Hope et al., 1988). Activation domains have been proposed to enhance transcription by a variety of mechanisms. These include simple recruitment of the polymerase II machinery to promoters (Klein and Struhl, 1994; Struhl, 1996; Ptashne and Gann, 1997), altering the conformation of components of the polymerase II machinery (Roberts and Green, 1994; Chi and Carey, 1996), modifying chromatin structure (Workman et al., 1991; Croston et al., 1992; Kingston et al., 1996), and affecting one or more steps after the transcriptional activation.
transcriptional initiation event (Rougvie and Lis, 1990; Yankulov et al., 1994; Krumm et al., 1995). These possible mechanisms are not mutually exclusive, but their relative importance in vivo has yet to be established.

Our analysis (Sawant et al., 1999) identified TCAC-TATATATAG as the prototype TATA-box sequence present most commonly in highly expressed plant genes. The TATA-box sequence determines the site of transcription initiation and the efficiency of transcriptional complex formation. In recent years, the significance of this element in regulating gene expression in development-specific (Duan et al., 2002), tissue-specific (Hochheimer and Tjian, 2003; Grace et al., 2004), or organ-specific (Kloeckener-Gruissem et al., 1992) manner has also been reported. Light induces the expression of many genes in plants, such as ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) and chlorophyll a/b binding (cab) proteins. Response to light is mediated by at least four distinct families of photoreceptors, which include phytochrome, cryptochromes, phototropins, and unidentdified UV B photoreceptors (Jiao et al., 2007). These photoreceptors absorb the light signals and, in conjunction with molecular systems of the plant, regulate the expression at the transcriptional and posttranscriptional levels. Studies of light-mediated genome-wide expression during seedling development in Arabidopsis (Arabidopsis thaliana; Ma et al., 2001; Jiao et al., 2005) and rice (Oryza sativa; Jiao et al., 2005) revealed that light controls many growth and developmental pathways. These pathways are believed to target different transcription factors and/or light-responsive elements (lres) within the promoter of a gene. These lres are conserved DNA modular arrays (CMAs) that are associated with light-responsive promoter regions and contain an I- and G-box cis-element. A 52-bp LRE, called CMA5, has been reported to confer light-specific activation of transcription to even heterologous promoters (Martinez-Hernandez et al., 2002). The core promoter region of several light-regulated genes has a distinct TATA-box and/or initiator element. Nakamura et al. (2002) showed that the majority of the photosynthesis nuclear genes lacked TATA-boxes. Instead, pyrimidine-rich initiator elements that overlap the transcription initiation sites were essential for light-responsive transcription. In spite of many studies on light-mediated regulation, the role of the TATA-box sequence in light-regulated core promoters is poorly understood.

We previously examined (Kiran et al., 2006) the effect of point mutations in a 13-bp prototype TATA-box sequence (Sawant et al., 1999) in light-mediated transcription. The nucleotides at the seventh and eighth positions in the prototype TATA-box were reported as critical to light-dependent transcription. When T at the seventh or A at the eighth position was mutated to G/C, the promoters continued to express in the dark to a level comparable to that of the prototype TATA-box sequence in both the presence and absence of the lre. However, the mutations led to failure of the promoters to express in the light. These findings established that the seventh and eighth positions in the TATATATA-dependent core promoter were critical to light-mediated transcription and were not important for gene expression in the dark. In this study, the ninth position in the prototype TATA-box was mutated to G/C, and the effect on transcription was examined in the presence of different upstream activator sequences after transformation using gusA as the reporter gene. This study shows that the ninth position plays an important role in the formation of nucleosome(s), assembly of the transcriptional complex, and interactions with the upstream activator sequences.

RESULTS
Substitution of G/C at the Ninth Position in the Consensus TATA-Box Leads to Enhancement of Transcription

In an earlier work (Kiran et al., 2006), we had demonstrated the critical role that nucleotides at the seventh and eighth positions play in the consensus TATA-box TCAC-TATATATAG for light-mediated transcription, both in the presence and absence of the 52-bp lre (Martinez-Hernandez et al., 2002). This study investigates the role of the ninth nucleotide in expression from the same prototype minimal promoter (Pmec) containing TCAC-TATATATAG as a consensus TATA-box (Supplemental Fig. S1). The Pmec used in this study is a 139-nucleotide-long typical TATA-dependent minimal promoter (Kiran et al., 2006), as shown by us by mutating TATATATA to GAGAGAGA. The lre was fused upstream of Pmec, Pmec 9G, and Pmec 9C and cloned in binary vector pBI101 containing the gusA reporter gene. The resultant constructs, lre-Pmec, lre-Pmec 9G, and lre-Pmec 9C, were then transformed into tobacco (Nicotiana tabacum) to obtain several transgenic lines. More than 12 independent transgenic tobacco lines for each construct (lre-Pmec, lre-Pmec 9G, and lre-Pmec 9C) were analyzed for the expression of the gusA reporter gene. Seedlings were grown either continuously in the dark or with a 16-h-light/8-h-dark cycle for a period of 11 d before fluorimetric GUS analysis.

In contrast to mutations at the seventh and eighth positions (Kiran et al., 2006), mutation to G and C at the ninth position exhibited enhancement of transcription in light. An increase by 8- to 9-fold was observed in lre-Pmec 9G and by 3- to 4-fold in lre-Pmec 9C in comparison with lre-Pmec (Fig. 1A). In the dark, the expression of lre-Pmec 9G and lre-Pmec 9C increased by about 5- and 3-fold, respectively (Fig. 1A). To study this further, we generated several independent transgenic lines of the prototype promoter and 9G mutation without any activator region. The transgenic lines of Pmec 9G mutation in the TATA-box showed more than
22-fold higher expression as compared with the transgenic lines of Pmec with the prototype TATA-box (Fig. 1B). Thus, the results indicate that the mutation in the TATA-box at the ninth position results in a more favorable sequence architecture at the TATA-box, thus enhancing the level of transcription. Semiquantitative reverse transcription-PCR for the gusA transcript showed that the GUS activity correlated well with the level of gusA mRNA in all cases (Fig. 1C).

The T9G/C Mutant Promoter Shows an Altered Transcription Initiation Site

In order to study the mechanistic differences at the minimal promoters of Ire-Pmec 9G and Ire-Pmec 9C in comparison with Ire-Pmec, we mapped the transcription start site of the three promoters. The result (Fig. 2) showed that Ire-Pmec 9G and Ire-Pmec 9C had multiple sites of transcription initiation. Ire-Pmec 9G shared one common site with the Ire-Pmec prototype construct, which showed only one transcription initiation site as reported earlier (Kiran et al., 2006). The mapping of transcription sites is indicated on the sequence as arrows in Supplemental Figure S2. The multiple transcription start sites in 9G mutants suggest that the difference in the TATA-box sequence may lead to differences in the PIC formed on the core promoter.

Phytochrome-Mediated Transcription Enhancement Is Lower in the T9G/C Mutant Promoter

The Ire used in this study exhibits a 2- to 3-fold activation of transcription in light in comparison with dark in the three constructs. We further examined the contribution of light of different wavelengths in activating transcription from these three constructs. Two transgenic lines of each construct were grown in continuous dark for a period of 9 d followed by exposure to continuous dark or to red, far-red, blue, or white light (16 h of light/8 h of dark) for 2 d before the seedlings were harvested to perform GUS assay. The prototype construct Ire-Pmec showed 8.5-, 3.1-, and 5.4-fold activation of transcription in red, far-red, and blue light, respectively, in comparison with dark, indicating the involvement of phytochrome in the regulation by Ire (Fig. 3). The results are consistent with our earlier results (Kiran et al., 2006). However, in comparison with the results obtained with the prototype construct, both the mutants Ire-Pmec 9G and Ire-Pmec 9C showed significantly lower responses toward light treatment. For Ire-Pmec 9G, the activation of transcription was 2.2-, 1.6-, and 1.1-fold, and for Ire-Pmec 9C, it was 1.8-, 1.2-, and 1.8-fold in red, far-red, and blue light, respectively (in comparison with dark), which is significantly lower than the prototype (Fig. 3). The results indicate that the phytochrome-mediated
activation of transcription in the constructs lre-Pmec 9G and lre-Pmec 9C promoter constructs at different concentrations. As expected, after 2 d in continuous light, 10 mM calcium activated transcription from the prototype construct lre-Pmec by 2-fold, indicating a requirement for Ca\(^{2+}\) for lre-mediated transcription (Fig. 4A). The expression was inhibited by EGTA, indicative of a requirement for Ca\(^{2+}\) ions for activation. In contrast to this, lre-Pmec 9G and lre-Pmec 9C promoters did not show any significant difference in the presence of calcium and EGTA. However, a general decline was observed in all three promoters in the presence of 100 mM calcium, which could be indicative of a general cytotoxic effect of calcium at this concentration (Fig. 4A).

In addition to the role of light in regulating the expression of light-dependent promoters, metabolizable sugars also regulate the expression of promoters by a negative feedback regulation, leading to repression of the light-dependent promoters (Krapp et al., 1991; Sheen, 1994). The lre used in this study contains a sugar response element (S-box) that represses transcription in the presence of 25 to 50 mM Glc (Acevedo-Hernandez et al., 2005). In order to see the effect of sugars at different concentrations on the expression of lre-Pmec, lre-Pmec 9G, and lre-Pmec 9C promoter constructs, we developed several transgenic lines. Seedlings harboring the lre-Pmec construct exhibited a decline in GUS activity at 50 mM Glc. In contrast to this, seedlings with lre-Pmec 9G and lre-Pmec 9C did not show a significant difference in GUS activity with respect to control at any concentration of Glc (Fig. 4B).

The 9G-Dependent Transcriptional Enhancement Synergizes with Specific Upstream Activator Sequences

To evaluate if the synergy in augmentation of transcription from the 9G core promoter was general or

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**Figure 2.** Mapping of point of transcription initiation from lre-Pmec, lre-Pmec 9G, and lre-Pmec 9C. Shown is primer extension analysis conducted with \(^{32}\)P-labeled primer (from +94 to +1 of the gusA gene) using RNA ligation-mediated RACE. The lanes C, G, T, and A represent sequencing ladders. NC represents the nontemplate control, and the lanes lre-Pmec, lre-Pmec 9G, and lre-Pmec 9C represent primer-extended products of representative transgenic lines.

**Figure 3.** Effect of the quality of light on TATA-box-dependent gene expression. GUS activities are shown in seedlings of transgenic lines of lre-Pmec (white bars), lre-Pmec 9G (checked bars), and lre-Pmec 9C (hatched bars) grown in dark for 9 d and then exposed to different light conditions (i.e. 16 h of white light/8 h of dark [WL], continuous red [RL], far-red [FR], or blue [BL] light, and continuous dark [black bars]) for 2 d. Intensities of the WL, RL, FR, and BL irradiations were 80, 10, 0.86, and 15 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), respectively. MU, Methylumbelliferone.
specific to certain upstream activator sequences, more combinations were made. 9G was introduced in a number of promoters, such as the constitutive synthetic promoter $P_{cec}$ ($P_{cec}$ 9G; Sawant et al., 2001), the chimeric seed-specific legumin promoter $P_{1306}$ ($leg-P_{mec}$ 9G; Chaturvedi et al., 2007), the chimeric pathogen-inducible promoter $PR-1a$ ($PR-1a-P_{mec}$ 9G; Lodhi et al., 2008), and the native Arabidopsis $rbcS$ promoter ($AtrbcS$-9G). Several transgenic tobacco lines were obtained and were analyzed for their GUS activity. In contrast to the results obtained with $P_{mec}$ or $lre-P_{mec}$, the transgenic lines of $P_{cec}$ 9G mutant showed at least 4-fold reduction in GUS activity (Fig. 5A). $AtrbcS$-9G showed 30- and 11-fold reduction in GUS activity in transgenic lines when compared with the native $AtrbcS$ with its prototype TATA box in light and dark, respectively (Fig. 5B). The seed-specific chimeric legumin promoter $leg-P_{mec}$ 9G showed around 8-fold higher GUS activity in seeds of transgenic lines as compared with $leg-P_{mec}$ with the prototype TATA box (Fig. 5C). In the case of $PR-1a-P_{mec}$ 9G, a mixed response was found. The mutant promoter showed slightly higher activity without salicylic acid induction as compared with the prototype TATA box. However, when induced with salicylic acid, the expression of GUS in the mutant lines decreased in contrast to the increase in the prototype promoter (Fig. 5D). Thus, the results indicate that the TATA-box and upstream activator sequences have a stringent specific requirement for their synergy in function.

**The 9G Mutation in the TATA-Box Results in a Nucleosome-Free Region**

We next examined the effect of the 9G mutation in the TATA-box on nucleosome formation in this region. Mononucleosomal DNA template was prepared from transgenic lines of either $P_{mec}$ or $P_{mec}$ 9G showing contrasting expression patterns and used as a template in a PCR assay to determine nucleosome occupancy (Sekinger et al., 2005). Overlapping primers (Supplemental Table S1) were designed for the TATA-box regions (Supplemental Fig. S1). Semiquantitative PCR with different groups of primers showed significant protection from micrococcal nuclease (MNase) action in the case of $P_{mec}$, indicative of a typical nucleosome incorporating the TATA-box (Fig. 6). In contrast to the results obtained with the $P_{mec}$ transgenic line, the $P_{mec}$ 9G TATA region showed enhanced sensitivity to MNase and, hence, poor PCR amplification. The results indicate the presence of a nucleosome-free region around the TATA-box with the T9G mutation (Fig. 6). The results are consistent in three independent transgenic lines of $P_{mec}$ (2b, 9b, and 4b) and $P_{mec}$ 9G (12, 13, and 11) tested in this study. In order to establish the specificity, the same mononucleosomal template was
used to amplify the core promoter region of the pollen-specific promoter NTP303 (Weterings et al., 1995). The position of the nucleosome was not altered when the same groups of primers were used, indicating the specificity and the nucleosomal response (Fig. 6).

Thus, a nucleosome-depleted region around the TATA-box in Pmec 9G is probably the reason for a more open structure and accessibility of the TATA-box to other PIC components and the initiation of transcription with higher efficiency.

The TATAGATA Promoters in Arabidopsis Are Not Tightly Repressed

We examined the 50-bp region upstream of the transcription start site (Zhu et al., 1995; Gershenzon et al., 2006; Thomas and Chiang, 2006) in all the Arabidopsis genes in The Institute for Genome Research database for the presence of consensus TATA-box sequence TATAGATA. We found 57 genes containing TATAGATA sequences upstream of the transcription start site (Supplemental Table S2). However, 41 of these had more than one copy of the TATA-box within 50 bp upstream of the transcription start site. Only 16 genes had only one TATAGATA and no other TATA-like sequence in 50 bp upstream of the transcription start site. One of these, AT2G07716, is a pseudogene and was not considered for further analysis. The 15 genes containing TATAGATA have diverse cellular functions (Table I). The expression pattern of the genes containing TATAGATA was examined (except AT3G52345, which is a tRNA gene and hence not considered for the analysis) in nine different tissues (germinated seed, seedling, young rosette, developed rosette, bolting, young flower, developed flower, flowers and siliques, and matured siliques) using the Genevestigator V3 tool (Hruz et al., 2008), which takes account of 4,070 microarray experiments deposited in public databases for Arabidopsis (Supplemental Fig. S3). The genes containing TATAGATA have diverse cellular functions (Table I). The expression pattern of the genes containing TATAGATA was examined (except AT3G52345, which is a tRNA gene and hence not considered for the analysis) in nine different tissues (germinated seed, seedling, young rosette, developed rosette, bolting, young flower, developed flower, flowers and siliques, and matured siliques) using the Genevestigator V3 tool (Hruz et al., 2008), which takes account of 4,070 microarray experiments deposited in public databases for Arabidopsis (Supplemental Fig. S3). The genes containing TATAGATA showed consistently higher expression, with intensities of hybridization well above 100 (Supplemental Fig. S3B, i–xiv) in all genes except AT4G19720 (Supplemental Fig. S3Bv). In this case, although the expression is low, it is consistently low in all the tissue conditions. Thus,
The expression analysis of TATAGATA-containing genes showed that the genes are always expressed at a higher level during plant development and that none of the genes is completely repressed. The results are in contrast to some of the TATATATA-containing genes we have examined; the results for 14 TATATATA-containing genes are shown in Supplemental Figure S3D. These TATATATA-containing genes show typical tissue-specific behavior: genes are expressed during specific stages of expression and remain repressed in others. To further substantiate our results, we have checked the expression of six different tissue-specific genes (pollen specific [AT3G62230], seed specific [AT5G07190], stigma specific [ATSG23960], gynoecium specific [AT4G32551], microspore mother cell specific [AT2G45800], and sepal specific [AT1G05330]) as control genes using Genevigator software (Supplemental Fig. S3A, i–vi). These tissue-specific genes are significantly expressed at the expected stages of plant development and remain completely repressed in other tissues (intensities of hybridization below 100), thus acting as good controls to substantiate our results on the almost constitutive behavior of TATAGATA-containing genes. Furthermore, we validated the microarray results by quantifying nine TATAGATA-containing genes by real-time quantitation of their transcripts in different tissues of Arabidopsis: leaf, root, seedling, and flower (Fig. 7). The quantity of transcripts of each gene was normalized by the quantity of ubiquitin gene in the respective tissues (see “Materials and Methods”). The normalized ΔCt (threshold cycle) for the nine genes in different tissues showed that all nine genes were expressed ubiquitously at all stages (Fig. 7). Thus, the results indicate that TATAGATA may be responsible for more favorable transcriptional architecture at the minimal promoter and does not allow stringent repression.

We also identified 145 genes containing TATACATA out of which 35 genes contain TATACATA as the exclusive TATA-box in their minimal promoter region (Supplemental Table S4; i.e. within 50 bp upstream of the transcription start site). We further analyzed the expression of TATACATA-containing genes using Genevigator; the expression profiles of the representative 14 genes are presented in Supplemental Figure S3C, i to xiv. The expression profiling of the TATACATA-containing genes showed that these genes are expressed consistently highly in all the tissues, as observed in the case of TATAGATA-containing genes (Supplemental Fig. S3, compare B with C).

**DISCUSSION**

Our study shows that in spite of the prototype TATA-box being only an eight-nucleotide sequence, individual position may determine specific aspects in promoter function. Our earlier work (Kiran et al., 2006) showed that the seventh and eighth positions in the prototype core TATA-box TCACTATATATAG determine light-induced promoter expression. The presence of a G or C at these positions resulted in failure to form a light-specific transcription initiation complex. This study shows that the mutation of T at the ninth position to G or C augments promoter function in transgenic tobacco lines both in the presence and absence of *lre*. Thus, in contrast to the mutations at the seventh and eighth positions (Kiran et al., 2006), the mutations at the ninth position do not alter light-specific promoter response (Fig. 1A). The expression in both light and dark was enhanced equally as compared with the prototype promoter. The light-specific activation in transcription is affected by the intracellular level of Glc (Acevedo-Hernandez et al., 2005),
Ca\(^{2+}\) (Neuhaus et al., 1993; Wu et al., 1996), and phytochromes (Martinez-Garcia et al., 2000; Kim et al., 2002; Quail, 2002). However, in contrast to the prototype promoter with \(lre\), the expression of the TATA-box with the 9G/C mutation was not altered by Glc, Ca\(^{2+}\) ions, or phytochrome. The results suggest that the ninth position is not light specific. Instead, a G or C at the ninth position provided sequence architecture more favorable to the assembly of PIC.

Transcript mapping (Fig. 2) showed that the 9G/C TATA-box led to a shift of the transcription initiation site, suggestive of a different PIC formation. The PIC composition depends upon the TATA sequence and influences transcription in yeast (Bjornsdottir and Myers, 2008). Additional factors were required for efficient in vivo transcription in certain TATA mutations. Similarly, in yeast and human, the PIC composition or the site of PIC formation varies greatly.

### Table 1. The 15 genes with TATAGATA as the consensus TATA-box within 50 bp upstream of the transcription start site in the genome of Arabidopsis

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**Figure 7.** Quantitation of the nine TATAGATA-containing genes in different tissues of Arabidopsis (leaf [striped bars], root [crossed bars], seedling [checked bars], and flower [stippled bars]) by real-time PCR. Shown here are the \(\Delta C_t\) values normalized against ubiquitin transcript in the respective tissues.
depending upon the cellular or physiological state (Martinez, 2002; Muller and Tora, 2004; Thomas and Chiang, 2006). These influence the rate of transcription and the start site selection. In both yeast and human, the TATA-containing promoters largely have single transcription start sites (Hawkes and Roberts, 1999; David et al., 2006; Kuehner and Brow, 2006). However, a large proportion of the TATA-less genes show more than one transcription start site (Yang et al., 2007; Juven-Gershon et al., 2008). The prototype TATA-box used in this study has a plant consensus sequence (Sawant et al., 1999) that gives a single transcription start site (Fig. 2).

The 9G mutation gave transcriptional activation of the prototype minimal promoter, without (Pmec) and with lre (lre-Pmec) and legumin (leg-Pmec 9G) activators but not with the constitutive (Pece 9G), light-regulated (AtRcs-9G), and salicylic acid-induced (PR-1a-Pmec 9G) promoters. Thus, transcriptional enhancement due to the 9G mutation may not be general but specific to certain promoters, depending upon synergy with upstream activators. Functional specificity between the upstream activator sequences and minimal promoters has been reported earlier (Mencia et al., 2002). Among plant promoters, we reported specificity between the two components for stringent regulation of the PR-1a gene (Lodhi et al., 2008). Specificity in functional compatibility has been reported between upstream and core promoter sequences for transcriptional activation in yeast, Drosophila, and mammalian cells (Butler and Kadonaga, 2001, 2002; Cheng et al., 2002; Li et al., 2002; Mencia et al., 2002; Gross and Oelgeschlager, 2006; Muller et al., 2007).

Activation of transcription by the 9G mutation in the TATA-box may be due to the formation of a PIC that synergizes with specific upstream sequence-dependent activators. The 9G mutation in the TATA-box results in enhancing micrococcal nuclease sensitivity around the minimal promoter (Fig. 6), suggesting that the 9G mutation leads to either a nucleosome-free region around the TATA-box or a more open chromatin structure. We have previously reported that the prototype TATA-box in Pmec used in this study is incorporated into a distinct nucleosome (Lodhi et al., 2008). The TATATATA sequence has a dyad symmetry and possesses a high capability for nucleosome formation (Boyle et al., 2008). Pyrosequencing of nucleosomes (Owen-Hughes and Engeholm, 2007) and nucleosome mapping (Albert et al., 2007; Barks et al., 2007; Lee et al., 2007; Mavrich et al., 2008; Valouev et al., 2008; Hall et al., 2009) have also demonstrated that stretches of AT or TA favor the formation of nucleosomes over them. Thus, the 9G mutation in TATAGATA might disturb dyad symmetry and TA stretch, affecting its ability to be packaged into a nucleosome. The lack of stringent nucleosomal structure over TATAGATA may result in higher accessibility of the TATA-box region to PIC assembly. However, the synergy with certain upstream sequences, and not others, suggests that the substitution of 9T with G alters the composition or conformation of the PIC. The altered PIC may make better contacts with specific activator complexes.

Lomvardas and Thanos (2002) demonstrated that exchanging the IFNβ minimal promoter (which has a distinct nucleosome containing the TATA-box at resting state that slides after viral induction) with the IL8 minimal promoter (which does not have a nucleosome over the TATA-box) resulted in a chimeric promoter that showed higher background transcription. The transcript accumulation from the chimeric promoter further increased substantially following virus infection as compared with that from the native IFNβ promoter. Thus, removal of the repressing nucleosomal architecture by replacing it with an alternative minimal promoter (IL8) that does not form a nucleosome over the TATA-box resulted in an overall increase in promoter activity. In the case of the tightly regulated seed-specific b-phaseolin gene, the repressive state in other plant tissues is due to the presence of a nucleosome over the TATA-box (Carranco et al., 2004). In developing seeds, the nucleosome gets remodeled by the transcription factor PvALF.

Our computational analysis of the native Arabidopsis promoters showed that in Arabidopsis, around 11% of protein-coding genes (Supplemental Table S5) are actually TATA containing, as per the consensus reported by Molina and Grotewold (2005). The TATA-containing genes are majorly dominated by TATA-TATA (990 genes) and TATAAATA (867 genes); these together constitute around 57% of the total TATA-box-containing genes (1,857 out of 3,218; Supplemental Table S5). The TATA-consensus sequences TATAGATA and TATACATA are least preferred, being 0.18% and 0.47%, respectively, of total protein-coding genes (Supplemental Table S5). The genes containing either TATAGATA or TATACATA did not show tight regulation during development, as analyzed by microarray analysis (Supplemental Fig. S3, B and C) or in real-time quantitation (Fig. 7), in contrast to the genes containing TATATATA (Supplemental Fig. S3D). The results indicate that the TATA-consensus-like TATATATA may favor nucleosome structure over the TATA-box, which is responsible for tight regulation of these genes. However, absence of stringent nucleosome structure over the TATA-box as a result of T9G or T9C consensus may result in expression of these genes almost constitutively in different tissues during development. It will be interesting to map nucleosomes over the minimal promoter in the 15 Arabidopsis genes to evaluate our hypothesis further. Both TATATATA and TATACATA show activation by the lre element (Fig. 1A). However, the activation by TATAGATA is at least 2-fold higher than that by TATACATA. Our results suggest that the TATAGATA sequence may be more potent in removing chromatin-repressive structure over the TATA-box as compared with TATACATA. Thus, the promoters with TATAGATA sequences are less likely to be stringently regulated and hence may not be preferred in nature as compared with
TATACATA. However, our hypothesis requires further evaluation.

MATERIALS AND METHODS

Construction of Mutations in Pmec and Ire-Pmec

The constructs Pmec and Ire-Pmec have been described by Kiran et al. (2006). In this study, mutations in Arabidopsis (Arabidopsis thaliana) were created at the ninth nucleotide position of the TATA sequence TCATATA-TATAG to G or C in Pmec by PCR using degenerate primers. A total of 10 random clones were picked for each primer and sequenced. A 52-bp light-responsive element (lre), called CMAS (Martinez-Hernandez et al., 2002), was fused in single copy immediately upstream of Pmec 9G and Pmec 9C to obtain the respective constructs under the control of lre. These were cloned into binary vector pH101 for transformation in tobacco (Nicotiana tabacum var Petit Havana). A number of independent stable transgenic lines were generated to take into account variations due to the site of insertion.

Site-Directed Mutagenesis

Mutations at the ninth position in the TATA-box of Pmec, AtrbcS, leg-Pmec, and PR-1a-Pmec promoters were generated using P9GF (forward) and P9GR (reverse) primers for Pmec, AtrbcS 9GF (forward) and AtrbcS 9GR (reverse) primers for AtrbcS, LAP9GF (forward) and LAP9GR (reverse) primers for leg-Pmec, and PR9GF (forward) and PR9GR (reverse) primers for PR-1a-Pmec by site-directed PCR mutagenesis (QuickChange XL kit; Stratagene) according to the manufacturer’s protocol. The clones were screened by DNA sequencing for the desired mutations using T3, T7, and P-10 primers. The sequences of the primers used in the mutagenesis and sequencing are given in Supplemental Table S1.

Plant Transformation

The constructs were cloned into the binary vector pH101 (Clontech). The recombinant vector plasmids were mobilized into Agrobacterium tumefaciens strain LBA4404 by electroporation. Leaf disc explants were transformed following the protocol of Horsch et al. (1985). Several independently transformed plants were developed with each construct. Transgenic plants were grown in a 16-h-light/8-h-dark cycle. The number of independent transgenic lines tested in a given experiment are specified in the figures.

Growth Conditions and GUS Assay

After transformation, seeds of the T0 generation were collected and homozygous lines were obtained through kanamycin (300 mg L \(^{-1}\)) selection. Seeds of the homozygous lines from the T1 generation were used for detailed studies. For light versus dark experiments, seeds were grown in a 16-h-light/8-h-dark cycle. Tobacco leaf tissue was washed thoroughly with distilled water and subjected to cross-linking in the presence of 1% formaldehyde for 30 min. The leaves were then rinsed with water, ground into powder in liquid nitrogen, and suspended in homogenization buffer (1 mM hexylene glycol, 10 mM PIPES/KOH, pH 7.0, 10 mM MgCl\(_2\), 0.5% [v/v] Triton X-100, 5 mM 2-mercaptoethanol, and 0.8 mM phenylmethylsulfonylfluoride [PMSF]). The homogenized extract was filtered sequentially through four layers of muslin cloth, followed by 80-, 60-, 40-, and 20-μm nylon mesh. Nuclei were pelleted at 3,000 g for 10 min and resuspended gently with a soft paint brush in wash buffer (0.5 mM hexylene glycol, 10 mM PIPES/KOH, pH 7.0, 10 mM MgCl\(_2\), 5 mM 2-mercaptoethanol, 0.5% [v/v] Triton X-100, and 0.8 mM PMSF). Nuclei were pelleted at 3,000 g for 15 min, and the pellet was resuspended in 5% Percoll (U.S. Biologicals) buffer (0.5 mM Suc, 25 mM Tris, pH 8.0, 10 mM MgCl\(_2\), 0.8 mM PMSF, 0.3% Triton X-100, 5 mM 2-mercaptoethanol, and 5% Percoll) and loaded onto a 40% to 80% Percoll step gradient containing 2 mM Suc pad at the bottom. The nuclei were collected from the 80% Percoll-2 mM Suc interface, washed in the wash buffer without Triton X-100, and resuspended in lysis buffer (110 mM KCl, 15 mM HEPES/KOH, pH 7.5, 5 mM MgCl\(_2\), 1 mM dithiothreitol, and 5 mM ml \(^{-1}\) leupeptin). The nuclear preparation equivalent to 20 mg of nuclei was incubated with micrococcal nuclease (300 units ml \(^{-1}\); Fermentas Life Sciences) in a buffer containing 25 mM KCl, 4 mM MgCl\(_2\), 1 mM CaCl\(_2\), 50 mM Tris-Cl, pH 7.4, and 12.5% glycerol at 37°C for 10 min. The reaction was stopped by adding an equal volume of 2% SDS, 0.2 mM NaCl, 10 mM EDTA, 10 mM EGTA, and 50 mM Tris-CI, pH 8.0, and treated with proteinase K (100 μg mL \(^{-1}\); Ambion) for 1 h at 55°C. The cross-link was reversed by heating at 65°C overnight. The DNA was extracted, and the samples were incubated with RNaseA (100 μg mL \(^{-1}\); Qiagen), extracted by phenol-chloroform, and precipitated in ethanol. The DNA was separated on a 1.5% agarose gel, and fragments of an average size of 144 to 160 bp were purified.

Light, Sugar, Calcium, and EGTA Treatment

For experiments on induction by light quality, 9-d-old dark-grown seedlings were exposed to white (80 μmol m \(^{-2}\) s \(^{-1}\)), red (10 μmol m \(^{-2}\) s \(^{-1}\)), blue (15 μmol m \(^{-2}\) s \(^{-1}\)), and far-red (0.86 μmol m \(^{-2}\) s \(^{-1}\)) light for 2 d before measure-

Semiquantitative PCR Using Mononucleosome Template DNA to Detect Nucleosomes

Semiquantitative PCR was used to examine the nucleosomes on Pmec and Pmec 9G promoters. The forward primers (TCPF1, TCPF2, and TCPF4) and the reverse primers (TCPF3, TCPF4, and TCPF5) were designed to overlap the nucleosome-bound region.

Regulation of Nucleosome Formation by Consensus TATA-Box

ment of GUS activity. For different sugar treatments, seedlings were grown for 9 d in a growth room at 25°C ± 2°C under a 16-h-light/8-h-dark photoperiod with a light fluence of 80 μmol m \(^{-2}\) s \(^{-1}\) in Hoagland medium and transferred to the same medium supplemented with 0, 25, 50, 100, or 200 mM Glc for 2 d before estimating GUS activity. For calcium and EGTA treatments, seedlings were grown as described above for 9 d and transferred to Hoagland medium supplemented with different concentrations of calcium (0, 0.1, 1, 10, or 100 mM CaCl\(_2\)) as well as 10 mM EGTA for 2 d before estimating GUS activity.

RNA Extraction and Reverse Transcription

Total RNA was extracted from tobacco seedlings grown in Hoagland medium in light/dark for 11 d. Tissue was frozen in liquid N\(_2\) and ground, and RNA was extracted by Tri-Reagent (Sigma Pharmaceuticals). After DNase treatment (Ambion), the integrity of RNA was tested by electrophoresis. The first-strand cDNA was prepared from 2 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen) and an oligo(dT) primer at a temperature of 42°C according to the manufacturer’s instructions. The optimal number of cycles for semiquantitative analysis was 28. The primers utilized for the GUS gene were uidA/F and uidAR (Supplemental Table S1), which gave an amplified fragment of 1.87 kb.

RNA Ligation-Mediated RACE to Map gusA Transcripts of ire-Pmec as Well as Ire-Pmec 9G and Ire-Pmec 9C Constructs

Total RNA of ire-Pmec, Ire-Pmec 9G, and Ire-Pmec 9C light-grown transgenic seedlings was used to perform 5’ RNA ligation-mediated RACE using a kit from Ambion. Primer extension was performed as described (Kiran et al., 2006).
reverse primers (TCP81G, TCP82G, TCP83, and TCP84) were used to analyze protection of the core promoter region against micrococcal nuclease digestion. The PCR efficiency of the primers was checked using tobacco genomic DNA as template. A polen-specific promoter (NTP503) of tobacco was used as the internal control. The forward (NTP302F) and reverse (NTP302R) primers were designed for semiquantitative PCR of the core promoter region. The PCR products were visualized on a 1.5% agarose gel by ethidium bromide staining. Sequences of the primers are given in Supplemental Table S1, and the positions are shown in Supplemental Figure S1.

Sequence Analysis of Upstream Promoter Regions in Arabidopsis

The 500-bp upstream sequences of all the Arabidopsis genes were downloaded from the TAIR8 database (ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_databases/TAIR8blast/TAIR8_upstream_500_20080228). The sequences in this data set are immediately upstream of the 5′ untranslated region for those genes with annotated untranslated regions and upstream of the translational start for the remainder. This data set comprised 33,281 genes. From this data set, a new data set was made with 50 bp of sequence upstream of the 5′ end of each gene. This data set was used for the promoter analysis of all genes from Arabidopsis.

For the protein-coding genes, all the loci for coding sequences were extracted from the TAIR8 database (ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_databases/TAIR8blast/TAIR8_cDNA_20080412). From this data set, all the redundant gene loci were removed (i.e. all alternative spliced forms of the genes were removed), and this resulted in 27,379 nonredundant gene loci. These loci were used to extract the 50-bp upstream sequences from the above-mentioned gene promoter database. We examined the presence of consensus TATA-box and TATA consensus motifs (i.e. TATAGATA, TATACATA, and TATAAATA) in the 50 bp upstream using the Motif Scanner Program (Thijs et al., 2001). The nucleotide frequency matrix (NFM) for the TATA-consensus was used from the NFM discussed by Molina and Grotewold (2005). For all other motifs, pure NFM was used, giving 100% frequency for each nucleotide at its respective position.

Microarray Analysis of Genes Having TATA-Box Consensus Promoters in Arabidopsis

Using Genevestigator V3 (http://www.genevestigator.ethz.ch), we studied the expression patterns of the genes having different TATA-box consensus across all 4,070 Affymetrix arrays. The profiles for various genes were generated using the meta-profile analysis tool across different developmental stages of Arabidopsis.

Real-Time PCR for the Quantitation of Genes with TATAGATA Promoters in Arabidopsis

Total RNA was extracted from different tissues of the Arabidopsis plant (leaf, root, seedling, and flower) by the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and treated with RNase free DNase (Ambion). The first-strand cDNA was prepared from 2 μg of total RNA with SuperScript II reverse transcriptase and an oligo(dT) primer according to the manufacturer’s instructions. The primers for the real-time analysis of nine target genes and one reference ubiquitin gene (AT4G05320) were designed using Primer Express software (Applied Biosystems); sequences of the primers are given in Supplemental Table S1. Real-time PCR for nine target genes and the internal reference ubiquitin gene was carried out using an ABI Prism 7000 sequence detection system (Applied Biosystems) and SYBR Green chemistry. The Ct value for each gene and reference gene was determined using ABI Prism 7000 SDS software, and the Ct value was calculated as Ct = Ct (target gene) – Ct (ubiquitin) for each particular tissue. The experiments were carried out with at least three biological replicates and two technical replicates, and each experiment was repeated twice.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT1G05330, AT1G45800, AT2G2230, AT4G32551, AT5G07190, AT5G23960, AT1G05320, AT1G27490, AT1G12970, AT1G15960, AT1G32510, AT1G22800, AT2G0716, AT3G52345, AT3G46830, AT5G20810, AT3G47000, AT3G03626, AT4G27870, AT4G18880, AT4G19720, and AT5G04360.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Positions of the primers designed from the core promoter regions of Pmec and Pmec 9G constructs are indicated by arrowheads.

Supplemental Figure S2. The nucleotide sequence of the Pmec-gusA cassette.

Supplemental Figure S3. Microarray expression profiles of genes containing TATAGATA, TATACATA, or TATAAATA sequence along with tissue-specific genes used as a control using Genevestigator V3.

Supplemental Table S1. Primer information for site-directed mutagenesis (SDM), cloning and sequencing of constructs, detection of mononucleosomes, and semiquantitative and real-time PCR experiments.

Supplemental Table S2. List of 57 genes containing the consensus TATA-box or its variant TATAGATA present within 50 bp upstream of the transcription start site in Arabidopsis.

Supplemental Table S3. List of 145 genes containing the consensus TATA-box or its variant TATAGATA present within 50 bp upstream of the transcription start site in Arabidopsis.

Supplemental Table S4. List of 35 genes containing the TATACATA sequence present within 50 bp upstream of the transcription start site in Arabidopsis.

Supplemental Table S5. Identification of TATA motifs in ~500-bp upstream promoter sequences of protein-coding and total genes in Arabidopsis.

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