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The promoter of a gene that is expressed only in pollen interacts with ubiquitous transcription factors

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Abstract The NTP303 gene of tobacco is expressed only in bicellular pollen. The minimal pollen-specific promoter of the gene is located between -103 and -51 upstream of the transcriptional start site. Using gel mobility shift assays, we demonstrated that this minimal functional promoter interacts with a leaf nuclear GT-1 binding activity. This finding suggests that ubiquitous transcription factors are involved in the pollen-specific expression of the gene in vivo.

Key words Tobacco • Pollen • Transcriptional control • GT-1 • GT-2

Introduction

During the formation of pollen grains, specific genes are transcribed at the different stages of development (Schrauwen et al. 1990; Mascarenhas 1992). To study the functional significance of pollen-specific genes, we analyzed the NTP303 gene of Nicotiana tabacum that is transcribed only in pollen (Weterings et al. 1992). The gene is transcribed in the nucleus of the vegetative pollen cell, starting at the mid-bicellular stage and continuing until pollen maturation (Reijnen et al. 1991; Weterings et al. 1992). The gene is also transcribed as the pollen grain germinates and a pollen tube is formed (Weterings et al. 1992, 1995a). Therefore, this gene belongs to the group of late pollen genes that have been described for several plant species (reviewed in Mascarenhas 1990; McCormick 1991).

DNA sequences similar to the NTP303 gene are transcribed in pollen of monocots, such as millet and sorghum (C. Frova, personal communication), and dicots (Weterings et al. 1992), including Arabidopsis thaliana (K. Weterings, personal communication). The deduced amino acid sequence of the NTP303 gene shares 91% similarity with that of the LAT51 gene of Lycopersicon esculentum (McCormick 1991) and 64% with that of the Bp10 gene of Brassica napus (Albani et al. 1992). These genes also belong to the group of late pollen genes. The evolutionary conservation of both amino acid sequence and expression pattern suggests that NTP303 is important for pollen development, germination and pollen tube growth.

The DNA sequences in the promoter of the NTP303 gene that are required and sufficient for the correct temporal and spatial expression of a GUS reporter gene in transgenic tobacco have been located between nucleotides -103 and -51 upstream of the transcription initiation site (Weterings et al. 1995b; also see Fig. 1). This region contains two 5' AAATGA 3' sequence motifs (elements I and II) that are also found at nearly identical positions in the pollen-specific Bp10 promoter studied by Albani et al. (1992). Deletion of the nucleotides -103 to -87, or mutation of element I (positions -94 to -89), result in a significant decrease in expression, thereby identifying a novel pollen-specific cis-regulatory element. Mutation of element II (-76 to -71) has no detectable effect (Weterings et al. 1995b). The sequence from position -73 to -60 is similar to the binding site for the transcription factor GT-2, which is present in several plant tissues (Dehesh et al. 1990, 1992).

These findings suggest that ubiquitous transcription factors are important for the transcriptional activation of the NTP303 gene. In this study, we used DNA mobility shift assays to investigate whether the minimal pollen-specific NTP303 promoter can interact with ubiquitous transcription factors. Since this promoter contains a GT-2 consensus sequence, and since there are indications
that the transcription factor GT-1 is involved in expression of the pollen-specific LAT52 gene of tomato (Eyal et al. 1995), we used protein extract from leaf nuclei to test whether these and other ubiquitous factors can bind to the pollen-specific promoter.

Materials and methods

DNA fragments containing NTP303 promoter sequences were obtained by the polymerase chain reaction (PCR) as described by Weterings et al. (1995b). These fragments are named -103/-51 and -86/-51 and contain the corresponding nucleotides from the NTP303 promoter (see Fig. 1). A DNA fragment in which the 5' AATGAGTAATTAAC sequence 5' TGGGCGGTAATTAAC 3', which corresponds to box II (positions -94 to -89) has been mutated to 5' TGGTGTGGTTAATATG 3', which corresponds to box II (positions -94 to -89) has been mutated to 5' TGGGCGGTAATTAAC 3'. A1J is substituted by a CC-doublet. This mutation leads to a more than 10-fold decrease in protein binding (Green et al. 1988). We also used a mutated box II in which the GG-doublet is substituted by a CC-doublet. This mutation leads to a more than 10-fold decrease in protein binding (Green et al. 1988). The GT-2 binding site has the sequence 5' TGTGTGGTTAATATG 3', which corresponds to box II (positions 12 to 13) in the promoter of the pen rbcS-3A gene (Green et al. 1988). Also we used a mutated box II in which the GG-doublet is substituted by a CC-doublet. This mutation leads to a more than 10-fold decrease in protein binding (Green et al. 1988). The GT-2 binding site has the sequence 5' TGGGCGGTAATTAAC 3'. All binding sites were tetramers, except GT-2, which was a trimer.

DNA fragments for probe preparation were separated from the vector by restriction enzyme digestion, purified by electrophoresis in polyacrylamide gels, and labeled to a specific activity of 106 cpm per μg with [α-32P]dATP using the Klenow fragment of DNA polymerase I, all according to Sambrook et al. (1989).

Nuclei were isolated from young leaves of Nicotiana tabacum, which had been kept under standard greenhouse conditions. Isolation of nuclei and extraction of nucleic acids were as described by Manzara et al. (1991).

For DNA mobility shift assays, 2.5 μg of leaf nuclear protein was mixed with 0.05 ng [α-32P]-labeled probe DNA. Protein-DNA binding was performed in a volume of 15 μl containing 80 mM KCl, 5 mM MgCl2, 10 mM HEPES-KOH (pH 7.9), 1 mM EDTA, 0.5 mM DTT, 4% (v/v) Ficoll 400 (Pharmacia), 1 μg poly(dI-dC) (Boehringer Mannheim), and competitor DNA fragments. To identify the position of the free probe, nuclear extract was substituted by 5 μg of bovine serum albumin. After 30 min incubation on ice, samples were loaded on a 6% (v/v) polyacrylamide gel (0.5×TBE; 45 mM TRIS-borate pH 8.3, 1 mM EDTA).

Results

We used the -103/-51 DNA fragment containing the minimal NTP303 promoter (Fig. 1) as a probe in gel mobility shift assays. Because sufficient quantities of vegetative nuclei from germinating pollen could not be obtained, protein extracts from entire pollen were used. However, a correlation between the fraction of retarded probe and the amount of pollen protein could not be observed (data not shown). Therefore, it was not possible to define conditions for competition experiments with excess amounts of unlabeled DNA.

In order to investigate whether the NTP303 promoter can interact with general transcription factors, we prepared nuclear extracts from isolated leaf nuclei which provide a rich source of various transcription factors (Katagiri and Chua 1992). As shown in Fig. 2 (lane 2) the -103/-51 minimal promoter probe is retarded by a factor in leaf nuclear protein extract. This factor is present in limiting amounts, since addition of a molar excess of unlabeled -103/-51 DNA fragment efficiently reduces the binding of this factor to the probe (Fig. 2, lanes 3–5). Competition by the -104M/-51 DNA fragment is comparable to that by the wildtype -103/-51 fragment (Fig. 2, lanes 6–8). The -86/-51 DNA fragment is a less efficient competitor (Fig. 2, lanes 9–11) and, when used as a probe, it is not retarded by leaf nuclear protein(s) with the amount of protein used here (data not shown).

Identification of the DNA-binding protein(s) that bind to the minimal pollen-specific promoter is possible by using competitor DNA fragments that contain defined binding sites for known transcription factors. DNA fragments containing binding site tetramers for the transcription factors ASF-1, ASF-2 and 3AF5 did not compete for binding to the -103/-51 probe (data not shown). However, a DNA fragment containing a tetrameric GT-1 binding site (Fig. 2, lanes 12–14) was a more effective competitor than the -103/-51 fragment that was used as a probe. Also, a DNA fragment containing a trimeric GT-2 site was an effective competitor for binding to the -103/-51 probe (Fig. 3, lanes 6, 7). Competition with a trimeric GT-1 binding site fragment was equally effective (Fig. 3, lanes 4, 5). In addition, DNA fragments containing either GT-1 or GT-2 binding sites seem to compete for a common factor in the leaf nuclear protein extract (Fig. 3, lanes 8–14).

These findings suggest that a nuclear GT-1 binding activity is able to bind to the minimal pollen-specific NTP303 promoter. Further support for this conclusion comes from the observation that the interaction of GT-1
expression of the NTP30 gene, which has the same nature as the DNA fragment that contains the minimal function. We have shown in previous studies that the NTP30 gene is transcribed from the NTP30 promoter, which is located in the transcribed region of the gene. This suggests that the NTP30 gene is transcribed in a promoter-specific manner. The NTP30 gene is transcribed from the NTP30 promoter, which is located in the transcribed region of the gene. This suggests that the NTP30 gene is transcribed in a promoter-specific manner.

Discussion

negative results. The -10/-35 promoter (Fig. 2, lane 1) or with the GT-1 binding sites in which the GT-1-CTD isoelectric point, which is the minimal promoter, is located in the transcribed region of the gene. This suggests that the NTP30 gene is transcribed from the NTP30 promoter, which is located in the transcribed region of the gene. This suggests that the NTP30 gene is transcribed in a promoter-specific manner.
Fig. 3 Comparison of competition by DNA fragments containing GT-1 or GT-2 binding sites. Lanes 1–7 -103/-51 probe, lanes 8–14 GT-1 tetramer probe. F indicates the position of the free probe. Competitors are: no competitor added (lanes 1, 8), 10-fold molar excess of GT-1 tetramer (lanes 2, 9), 100-fold molar excess of GT-1 tetramer (lanes 3, 10), 10-fold molar excess of GT-1 trimer (lanes 4, 11), 100-fold molar excess of GT-1 trimer (lanes 5, 12), 10-fold molar excess of GT-2 trimer (lanes 6, 13) and 100-fold molar excess of GT-2 trimer (lanes 7, 14).

We cannot discriminate whether the GT-1 binding activity corresponds to GT-1, GT-2 or both. The NTP03 promoter contains a GT-2, but not a GT-1 consensus sequence (Fig. 1). However, this does not rule out a role for GT-1 in NTP03 expression, since DNA fragments containing trimeric binding sites for either factor are equally effective competitors for protein binding to the –103/-51 pollen promoter. The DNA sequence requirements for GT-1 binding are rather relaxed (Terzaghi and Cashmore 1995), and despite claims to the contrary (see for example Kuhn et al. 1993), it is not always possible to use gel mobility shift assays for discriminating between GT-1 and GT-2 binding (J. Memelink, personal communication; also see Fig. 3). Thus, the GT-2 consensus sequence in the minimal NTP03 promoter may serve as binding site for the GT-1 protein.

GT-1 binding sites in well-characterized promoters such as the pea rbcS-3A promoter (Green et al. 1988) are usually present in pairs (Terzaghi and Cashmore 1995). The spacing between the two sites is critical for promoter activity in vivo, but not for GT-1 binding in vitro (Gilmartin and Chua 1990). GT-1 binds to DNA as a tetramer (Lam 1995), and a single polypeptide of GT-2 seems to have two DNA-binding domains (Dehesh et al. 1992). The second binding site for GT-1 or GT-2 is within the minimal NTP03 promoter may be located upstream of the GT-2 consensus sequence. We have shown that the –86/-51 promoter fragment competes less effectively for protein binding with the –103/-51 probe than the –103/-51 fragment itself (Fig. 2). In addition, in contrast to the –103/-51 fragment, it cannot compete with the GT-1 tetramer probe (Fig. 4). The –86/-51 fragment contains the GT-2-like consensus sequence (from –73 to –60), but it lacks the sequences from –103 to –87. Therefore, it is likely that this deletion removes (or inactivated) the second binding site, which would thus have been located between –103 to –74.

The –103/-51 DNA fragment fused to the 45 CaMV 35S promoter is sufficient for normal levels of reporter gene expression in pollen of transgenic plants, but in leaves and other plant tissues there is no detectable expression (Weterings et al. 1995b). Thus, if the interaction with the ubiquitously present nuclear GT-1 binding activity is important for NTP03 transcription in pollen, it would seem that also tissue-specific transcription factors interact with the minimal promoter to bring about the observed mode of pollen-specific gene activation. A potential target for any such factor is the 5’ AAATGA 3’ element I at positions –94 to –89. Mutation of this element leads to a considerable decrease in promoter activity in vivo (Weterings et al. 1995b). However, as shown in Fig. 2, this mutation does not affect the ability of the minimal promoter to interact with a factor in leaf nuclear extract, and, as shown in this paper, this factor may cor-
respond to GT-1 or GT-2. Precise identification of the nucleotide positions that are responsible for this interaction requires a further systematic mutational analysis of the minimal NTP303 promoter. Mutations that affect GT-1 or GT-2 binding in vitro could then be tested for their in vivo effect on promoter function.

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References

Fig. 4 Gel retardation experiments using GT-1 tetramer as a probe. Lane 1 No competitor. Lanes 2–4 Competition with 10-, 50- and 100-fold molar excess of -103/-51 DNA fragment, respectively. Lanes 5–7 Competition with 10-, 50- and 100-fold molar excess of -86/-51 DNA fragment, respectively. Lanes 8–11 Competition with 10-, 50-, 100- and 200-fold molar excess of GT-1 tetramer, respectively. Lane 12 Competition with 200-fold molar excess of mutant GT-1 tetramer (M). Lane 13 Free probe (F)