Journal of Plant Biology, June 2002, 45(2) : 112-117 **SHORT COMMUNICATION**

A Novel Tapetum-Preferential Gene from *Nicotiana tabacum*

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The tapetum, a thin layer of cells in the anther, is important to the process of male gametogenesis. However, only a few of its functioning genes have been reported. Here we isolated a genomic clone, *gNtA37*, that showed tapetum-preferential expression. Transcript could be detected from the microspore mother-cell stage until the time that microspores were released. It coded a protein of 245 amino acids, with an intervening sequence of 176 nucleotides. This clone also carried a 5′ upstream region of 840 nucleotides and a 3′ downstream region of 430 nucleotides. Database searches for the open reading frame did not reveal any significant homology to previously reported nucleotide sequences or polypeptide sequences.

Keywords: Anther, genomic clone, in-situ hybridization, tapetum, tobacco

Reproductive processes in flowering plants occur in two specialized floral organs: the stamen and the pistil. The anther compartment within the stamen contains diploid cells that undergo meiosis to form haploid microspores. Specialized tapetal cells, in one or more layers, surround the anther cells during male gametogenesis, and support the development of pollen grains (Raven et al., 1986). Functions of the tapetum have been determined in research with genetically engineered plants. For example, expression of a cytotoxic structural gene (e.g., a ribonuclease gene) in the tapetal layer of transgenic tobacco plants caused male sterility, and pollen development was critically hampered (Goldberg et al., 1993).

Anther development involves two phases (Goldberg et al., 1993). In phase 1, most of the specialized cells and tissues differentiate, then meiosis of the microspore mother cells occurs and tetrads of microspores are formed. In phase 2, the microspores are released from the tetrads, and the pollen grains mature and are released. This phase is accompanied by the development and apoptosis of supporting cells. These events require coordinated activities among many proteins. The number of genes that are expressed during anther development has been estimated through RNA-excess DNA/RNA hybridization experiments to be as high as 25,000, with most of them being expressed at barely detectable levels, i.e., less than 0.001% of the total mRNA in the anther (Kamalay and Goldberg, 1980). Among these very diverse genes, approximately 10,000 are expected to be anther-specific (Kamalay and Goldberg, 1980, 1984; Koltunow et al., 1990). However, only a few of these candidate genes have been described, some of which having their activity restricted to the tapetum. These include: TA13 and TA29, encoding a glycine-rich protein, and TA32 and TA36, which encode a lipid transfer protein from tobacco (Koltunow et al., 1990); the A6 gene, showing similarity to the β-1,3-glucanase gene from *Brassica napus* and *Arabidopsis thaliana* (Hird et al., 1993); the SP11 gene, encoding a self-incompatibility protein in *Brassica campestris* (Takayama et al., 2000; Shiba et al., 2001); the MZm3-3 gene from *Zea mays* that shows cysteine residues characteristic of a lipid transfer protein (Lauga et al., 2000); a type 2 metallothionein-like gene from *Z. mays* (Charbonnel-Campaa et al., 2000); myb genes, which serve as putative regulators of phenylalanine ammonia-lyase from tobacco (Yang et al., 2001); and the MS1 gene, which probably codes for a transcription factor from *A. thaliana* (Wilson et al., 2001).

Despite its importance to the development of male gametes (pollen grains), researchers have limited understanding of the number or function of the genes in the tapetum. Therefore, our objective was to investigate a novel gene preferentially expressed in the tapetal layer of tobacco (*Nicotiana tabacum*).

MATERIALS AND METHODS

Plant Material

Tobacco plants (*N. tabacum* cv. Wisconsin 38) were

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grown in a greenhouse under 16-h days at 25 ± 5°C. We collected leaf and root samples during vegetative growth, as well as flowers at several stages of development. All samples were immediately frozen in liquid nitrogen, and stored at −70°C.

RNA Blot Hybridization

Total RNAs were extracted from the anthers, from approximately the microspore mother-cell stage to the immature pollen stage. At each stage, the frozen anthers were ground in liquid nitrogen in a mortar with an RNA extraction buffer containing guanidium thiocyanate. The RNAs were then purified via CsCl density-gradient centrifugation (Hong and Jeon, 1987). Total extracted RNAs were run on a 0.8% agarose gel with formaldehyde, and blotted onto a nylon membrane. The RNA loaded on each lane was normalized to 10 µg, which was confirmed by measurement of A_{260} and staining of the gel with methylene blue (Sambrook et al., 1989). NtA37 was labeled with [α-^{32}P]dCTP by the random priming method, using a Prime-a-Gene kit (Promega, USA). DNA blot hybridizations were carried out overnight at 65°C, in a solution of 5X SSPE, 5X Denhardt's solution, 0.1% SDS, and 100 µg/mL denatured salmon sperm DNA. Afterward, the membrane was washed with 0.2X SSPE and 0.1% SDS at 65°C, and the blot was exposed to X-ray film with two intensifying screens (DuPont, USA) for two days at −70°C (Sambrook et al., 1989).

RT-PCR

First-strand cDNA was synthesized using MMLV reverse transcriptase for RT-PCR, according to the manufacturer's instructions (Gibco, USA). PCR on the first-strand cDNA was carried out using forward and reverse primers that were complementary to the sequences of NtA37. We used 35 reaction cycles, each consisting of 30 s at 94°C, 1 min at 48°C, and 40 s at 72°C. A 1-kb DNA ladder (BRL, USA) was used for size markers (Sambrook et al., 1989).

DNA Blot Hybridization

Ten µg of genomic DNA was digested and electrophoresed on a 0.7% agarose gel, then blotted onto a nylon membrane (Sambrook et al., 1989). This blot was hybridized overnight at 65°C and washed in 0.5x SSPE at 65°C. The same procedure was carried out as with the RNA blot hybridization.

Isolation of Genomic Clones

The genomic library of *N. tabacum* cv. Xanthi, in a bacteriophage EMBL3 vector (Clontech, USA), was used for isolating a genomic clone. A cDNA insert of NtA37 (Choi and Hong, 2000) was used to synthesize a randomly primed probe labeled with [α-^{32}P]dCTP, as described by the manufacturer (Promega, USA). We hybridized the blot for the plated-out genomic library on a bacterial lawn at 65°C, in a solution of 5x SSPE, 5X Denhardt's solution, 0.1% SDS, and 100 µg/mL denatured salmon sperm DNA. Washings were done at 65°C in 2x SSPE and 0.1% SDS for 10 min, 1x SSPE and 0.1% SDS for 20 min, and 0.5x SSPE and 0.1% SDS for 10 min. Positive plaques were amplified and bacteriophage DNA was isolated using a Lambda DNA mini kit (Qiagen, Germany). Southern hybridization for the genomic clone indicated a 4-kbp fragment of BamHI/PstI double digest as a putative genomic DNA fragment for the cDNA. This DNA fragment was subcloned into the plasmid pBluescript II SK(+). Unless otherwise mentioned, all the methods described above followed standard recombinant DNA methods of Sambrook et al. (1989).

Nucleotide Sequencing and Analysis

We used a USB Sequenase 2.0 kit (U.S. Biochemicals, USA) to perform nucleotide sequencing by the dideoxy chain termination method (Sanger et al., 1977). After the amino acid sequence was deduced from the nucleotide sequence, a hydropathy plot was drawn via the DNASTAR program (Lasergene, USA). Homology with reported genes and proteins at the amino acid sequence was compared using the BLASTX program (Altschul et al., 1997).

In-Situ Hybridization

Flower buds were fixed with formaldehyde-acetic acid, dehydrated, and embedded with Paraplast (Oxford, USA), according to the methods of Cox and Goldberg (1988). Ten-micrometer sections were then mounted on Poly-Prep slides (Sigma, USA). The pGEM-4 plasmid (Promega, USA) harboring *NtA37* was linearized with EcoRI or BamHI, and sense or antisense RNA transcripts were synthesized with a DIG RNA labeling kit (Roche, Germany). The transcripts were hydrolyzed to an average length of 200 nucleotides by $Na_2CO_3/NaHCO_3$ treatment (Cox and Goldberg, 1988). We performed in-situ hybridizations at 42°C, in a solution of 4x SSC, 50% (v/v) for-

mamide, 1x Denhardt's solution, 5% (v/v) dextran sulfate, 0.5 mg/mL salmon sperm DNA, and 0.25 mg/mL yeast tRNA (McKhann and Hirsch, 1993). Signal was detected with antidigoxigenin alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrates in the DIG-Nucleic Acid Detection kit (Roche, Germany). Microscopic images were then obtained on a dark field (Model Optiphot, Nikon, Japan).

RESULTS AND DISCUSSION

The *NtA37* cDNA clone was previously isolated by EST analysis of a tobacco-anther cDNA library (Choi and Hong, 2000). Because *NtA37* did not show a meaningful level of homology with the reported database sequences for nucleotides or amino acids, we decided to follow its detailed pattern of expression throughout the stages of anther development (Fig. 1A). RT-PCR for the transcript also demonstrated its anther-specific expression (Fig. 1B). The temporal expression pattern showed that *NtA37* was turned on early in anther development, and that its expression was maintained at high levels until it rapidly declined after the microspores were released (Fig. 1C).

The *NtA37* cDNA clone coded a protein of 164 amino acids for 492 nucleotides. Because its open reading frame did not reveal a translation initiation codon (data not shown), and because the size of the mRNA for the northern band was approximately 1 kb, we regarded the *NtA37* clone as a partial cDNA clone that lacked the portion toward the 5' end.

We isolated a genomic clone that had a nucleotide sequence identical to that of the *NtA37* cDNA clone,

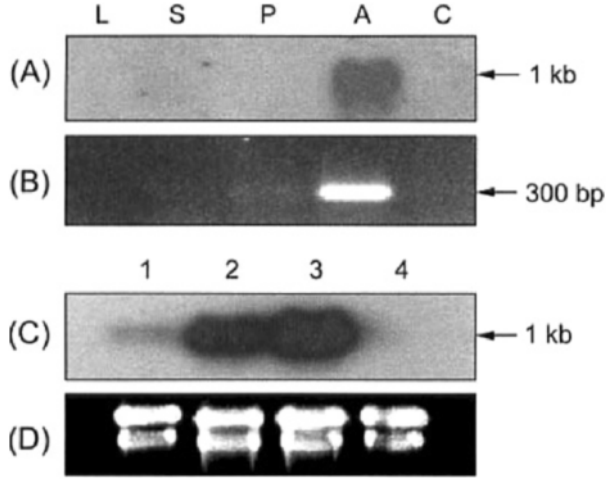

Figure 1. Expression pattern of *NtA37* in the anther of *N. tabacum*. (A) Total RNA was extracted from the leaf, sepal, petal, anther, and carpel, and was subjected to RNA blot hybridization with the ^{32}P-labeled *NtA37* clone. (B) All the RNA samples used for RNA blot in (A) were subjected to RT-PCR based on the nucleotide sequence of *NtA37*. (C) Total RNA was extracted from anthers at several developmental stages, and subjected to RNA blot hybridization with the ^{32}P-labeled *NtA37*. (D) Ethidium-bromide staining of a duplicate RNA gel of (C). L, leaf; S, sepal; P, petal; A, anther; C, carpel; 1, anther at the microspore mother-cell stage; 2, anther at the tetrad stage; 3, anther at the released-microspore stage; 4, anther at the immature pollen stage.

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1    TTCAGGAGTAAGTATATATCCTGAATTTTTGTATACTGAACTTTTGAATAGCTAATTTGA
61   AATTCAGGACTAAGTGTCCTAAATTTCTGAACTGCTAATTTAATGTTTAGGATATAAGAT
121  TCGAATTTTTGGACACGATTTTTGAACTTTAGGACACGATGTCACGAAATTTGAATTTTG
181  AGGTTTAAACTTCAGGACGTCGTGTCCTGAAATTTAAACAAATTAGTTAATCTTTAAATA
241  TATTATAAATTATAAATATATTTTAAATAACATGCTTATAACGGATGTTAGTATCACGTA
301  GCTTTTGTTGAACTCTTCGCATACATTCAAGTGTAATAACCAAGTTTATTCTTTGGGTAT
361  ATAAAGTGTTCCACTATTTAATATTCTTACAATATAATTGTAAAGTGAATTTCAAATTTT
421  CTTTTTTATTTAGTCAAAGTACTTTATACTTATGTACTTGACGTCTTAGTGATTTTTGTG
481  CTATTTTTCCCTTTAGCATAAGTACTTCAATTTCACATATTTGAACCTAAGACTCAATTT
541  TCTTTAGTAAAAAAAGAACCGAACTAAAAAAATTCTCATTTTGTCTACGAATAATTTAAC
601  TTTTCCCTTCAAGAGAATCAACAAAACATGCAAAATAACTTTTAGGTAACGTTTCTAAAC
661  TTACTGAATCATAGAAGTGTCAACTTGTAGGATTTTAGCATAACAATATGCTAATAATAT
721  TTTATGTGAAAAATTCTGTATACGCTATTGTTCGGGCTAGCGTTTTGAATCATAACGTGG
781  GCAAAGAAAATGATGCACCAATTCCAGGCCGACAAAAATTAAAGTTTCTTGAAAGAAAAA
841  ATGATACTGTATAGCTGCTCTCAACATAATAACCGAAAAAAATATATTTTTGTATGTTAT
       M  I  L  Y  S  C  S  Q  H  N  N  R  K  K  Y  I  F  V  C  Y    (20)
901  ATACAAGAAATATATAAATTTTATATACCTTTCGGCTAACGAATATAAATATTTTCAGC
       I  Q  E  I  Y  K  F  Y  I  P  F  R  L  T  N  I  N  I  F  S    (40)
961  CGCGAGCTAAGTATGATCTTTGCCGTTGTTAAAATTGgtcaaattactataaacaaagtc
       R  E  L  S  M  I  F  A  V  V  K  I                            (52)
1021 caaaagataatataattcaaccccctttatccgtaatggtgagtaaccctcacgaatta
1081 tattcaaatgggattgccgtttgtaaaatctgccttctttactctaacaagattagttca
1141 catcttttgtatgataaagttgtccataacagAAAACTCAAAACTTTGCTCTACATCCC
                                       E  N  S  K  L  C  S  T  S    (61)
1201 TCCTATATAATGTCACGTCAAGTTTATGTTTTCGTATCATTTTCTTGAAAAGATATTATA
       L  L  Y  N  V  T  S  S  L  C  F  R  I  I  F  L  K  R  Y  Y    (81)
1261 TCATCGTAGAAGAACCAAGTGGTAAGGCAACGGATTTTAGTCCGGCTAATTCCACTATGG
       I  I  V  E  E  P  S  G  K  A  T  D  F  S  P  A  N  S  T  M    (101)
1321 CTTTCTTGCAACAACTTCACCAATTCATGTTTTAGCTTTGCTTATGCTGATTTTTGCAT
       A  F  L  A  T  T  S  P  I  H  V  L  A  L  L  M  L  I  F  A    (121)
1381 CTACCAAAATGCACCATGCTCAAGGTAAATCAATTACAGGGCCATGTGTGGTCGCTTGTA
       S  T  K  M  H  H  A  Q  G  K  S  I  T  G  P  C  V  V  A  C    (141)
1441 GCAAAAAAACAATCGCTTGTGTTGTTAGATGCAGATTTGCGACAGATAAATGCTCCCAGG
       S  K  K  T  I  A  C  V  V  R  C  R  F  A  T  D  K  C  S  Q    (161)
1501 ATTGTGCGATTGATAGTATCCATTGCGTGAGTTCTTGTCTCATTCAGAACACGCCACCAC
       D  C  A  I  D  S  I  H  C  V  S  S  C  L  I  Q  N  T  P  P    (181)
1561 CAACGGCTATGATTTTGGACACGGACGACAGTCGGGACGGAGATAGCATGGAATATACCA
       P  T  A  M  I  L  D  T  D  D  S  R  D  G  D  S  M  E  Y  T    (201)
1621 ATAACTTCGATCTGAACCCCTATGTATTAGTTGAAAAATCCACTGAATGTGGACAAATAA
       N  N  F  D  L  N  P  Y  V  L  V  E  K  S  T  E  C  G  Q  I    (221)
1681 TAAACTTTAGAACCCATAAACTTCAATATATAAGTCATGAAATTCAAATCCTGAATACGC
       I  N  F  R  T  H  K  L  Q  Y  I  S  H  E  I  Q  I  L  N  T    (241)
1741 CTCTACGAATCTAAATATACTTATTATTTAGGGAGTGGGTGTGTGCTAGACACTCACAGT
       P  L  R  I  *                                                  (241)
1801 CTTTGATTCAAGATAATAAAGTTGAAAACTTTTATATATGTACTAAGCTAGCACCATCAT
1861 CAAAACTAAATAATGGTGTAGTAGTATTTTGCATTCACGGAAAATAAAATAGATGTATTT
1921 TGTATTTATCAATTTGTTGGTGAAATTATAAGTAATTACAGTTAAAAAAATAAGA      2175
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Figure 2. Nucleotide and deduced amino acid sequences of a genomic clone, *gNtA37*, for *NtA37* cDNA. Amino acid sequence is in one letter symbol. * indicates the putative termination codon. An intervening sequence is marked with a small letter. The parenthesized number indicates the number of deduced amino acids. The putative TATA boxes, CAAT box, and three polyadenylation sequences are underlined. Bold characters represent primer-binding site for RT-PCR.

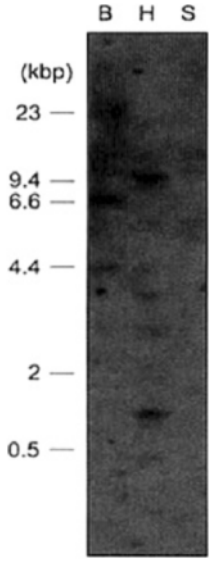

Figure 3. Genomic DNA blot analysis for *gNtA37* clone. Genomic DNA of *N. tabacum* cv. W38 was digested with BamHI (B), HindIII (H), and SacI (S). The numbers indicate the size (kbp) of lambda DNA cut with HindIII.

naming it *gNtA37*. That clone, coding a protein of 245 amino acids, covered all of *NtA37* and extended to the 5′ upstream region. An intervening sequence of 176 nucleotides was identified by comparing the nucleotide sequences between *gNtA37* and *NtA37* (Fig. 2). The genomic clone also carried a 5′ upstream region of 840 nucleotides, in which putative TATA boxes and a CAAT box could be located. However, other referred elements, such as anther box, could not be identified. In the 3′ downstream region, several polyadenylation sequences could be located. Database searches for the open reading frame did not reveal any significant homology with either the nucleotide sequences (GenBank, EMBL) or the amino acid sequences (Swiss-prot, PIR).

Genomic DNA blot analysis showed two bands of ca. 7 kbp and 4 kbp from the BamHI digest, two (ca. 10 kbp and 1 kbp) from the HindIII digest, and two (ca. 14 kbp and 6 kbp) from the SacI digest. From each digest, a few, much weaker, minor bands also could be observed. This probably indicates that *NtA37*

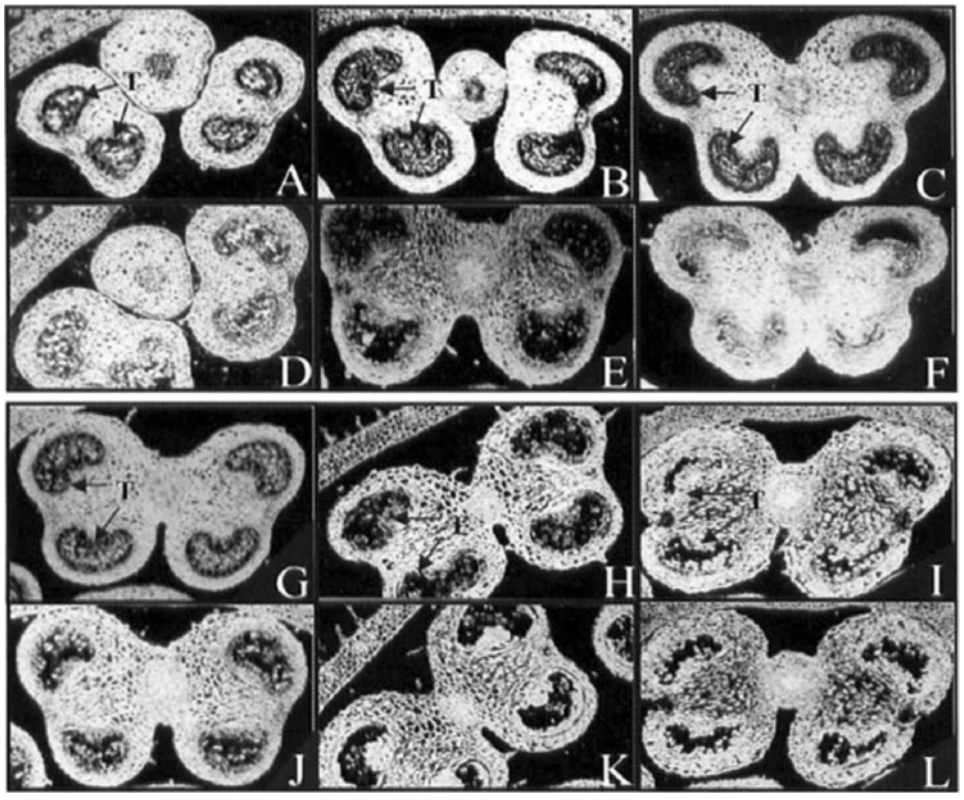

Figure 4. In-situ localization of *NtA37* transcripts in cross-sections of tobacco anther (100X). Purple-blue color shows hybridization signal. A, B, C, G, H, and I, sliced anthers hybridized by antisense *NtA37* DIG-labeled riboprobe. D, E, F, J, K, and L, sliced anthers hybridized by sense *NtA37* DIG-labeled riboprobe. A and D, anthers at the microspore mother-cell stage. B and E, anthers at the tetrad stage. C and F, anthers at the stage between tetrad and release of microspore. G and J, anthers at release d microspore stage. H, I, K, and L, anthers at the immature pollen stage.

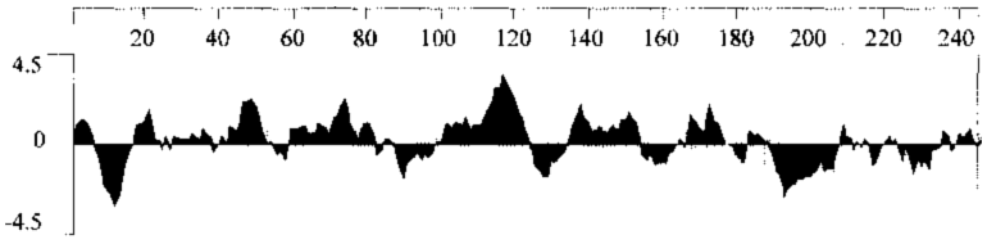

Figure 5. A hydropathy plot for the deduced amino acid sequence of *gNtA37*. Positive value on the Y-axis represents hydrophobicity. The numbers on the X-axis are position of the amino acids starting from the amino-terminus methionine.

exists in two copies over the *N. tabacum* genome (Fig. 3).

The spatial and temporal expression pattern of *NtA37* was analyzed by in-situ hybridization. Figure 4 shows the localization of *NtA37* mRNA in the transverse sections of tobacco anthers at different developmental stages. The antisense *NtA37* RNA probe produced an intense hybridization signal in the tapetum only for the major portion of its existence (Fig. 5, A-H); at the early developmental stage, an intense signal also could be observed from the vascular bundle, but it disappeared as male gametogenesis proceeded. As the microspores were released from the tetrads, the signal in the tapetum was diminished, followed by degradation of the cell layer (Fig. 5, H and I).

Properties of the *gNtA37*-encoded protein were deduced by computer analysis. The protein had a molecular mass of 27.8 kDa, with an isoelectric point of 8.17. A hydropathy plot (see Kyte and Doolittle, 1982) showed that the protein had central patches of hydrophobic regions rather than being of significant length in the amino-terminus region (Fig. 5). Those hydrophobic patches matched well with the β-strands, which were flanked by turns (data not shown), according to Chou and Fasman (1978). These calculated properties suggest that the NtA37 protein is probably cytosolic, having the characteristic structure of a β-sheet.

Among the 10,000 genes expected to be specifically expressed in the anther (Kamalay and Goldberg, 1980, 1984; Koltunow et al., 1990), only a few have been studied. Therefore, our description of the gene *gNtA37*, as reported here, can significantly aid in efforts to reveal the processes occurring in the tapetum during male gametogenesis.

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