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RESEARCH PAPER

NtWBC1, an ABC transporter gene specifically expressed in tobacco reproductive organs

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Abstract

To identify genes specifically or predominantly expressed in the stigmas/styles and to establish their possible function in the reproductive process of plants, a tobacco stigma/style cDNA library was constructed and differentially screened, resulting in the isolation of several cDNA clones. The molecular characterization of one of these clones is described here. After sequencing the cDNA and the isolated genomic clone, it was determined that the corresponding gene encodes a protein containing an ATP-binding cassette, characteristic of ABC transporters. This gene, designated as *NtWBC1* (*Nicotiana tabacum* ABC transporter of the White–Brown Complex subfamily), encodes a protein that contains the typical structure of the 'half-transporters' of the White subfamily. To establish the spatial expression pattern of the *NtWBC1* gene, northern blot and real-time RT-PCR analyses with total RNA from roots, stems, leaves, sepals, petals, stamens, stigmas/styles, ovaries, and seeds were performed. The result revealed a transcript of 2.5 kb present at high levels in stigmas and styles and a smaller transcript (2.3 kb) present at a lower level in stamens. *NtWBC1* expression is developmentally regulated in stigmas/styles, with mRNA accumulation increasing toward anthesis. *In situ* hybridization experiments demonstrated that *NtWBC1* is expressed in the stigmatic secretory zone and in anthers, at the stomium region and at the vascular bundle. *NtWBC1* is the first ABC transporter gene with specific expression in plant reproductive organs to be identified and its expression pattern suggests important role(s) in the reproductive process.

Key words: ABC transporter, developmentally regulated stamen expression, stigma-predominant expression, stigmatic secretory zone, White subfamily.

Introduction

The pistil and the stamen of the flowers are the specialized organs responsible for the reproductive processes. The pistil is generally composed of stigma, style, and ovary. The sporogenous cells of the ovary lead to the production of the female gametophyte or embryo sac that contains the egg cell. The transfer of the pollen grain (male gametophyte) from the stamen to the stigma initiates the processes that can result in fertilization. Once in the stigma, pollen–pistil

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interactions will start and decisions will be taken concerning the pollen fate. The compatible pollen will be allowed to adhere, hydrate, germinate, and penetrate into the style (Lord, 2003). Molecular studies suggest that compounds present in the exudate of the wet stigma of tobacco are responsible for the post-pollination behaviour of the pollen in the pistillate tissue (Goldman *et al.*, 1994; Cheung *et al.*, 1995). In tobacco, lipids present at the exudate seem to be an important directional cue for pollen tube growth and penetration into the stigma (Wolters-Arts *et al.*, 1998; Lord, 2003). Despite the importance of stigma/style specific proteins in the success of the pollination and fertilization processes, not many have been identified so far and little is known about their roles on these processes (Lord, 2003).

The objective of this work was to identify and characterize genes that are specifically or predominantly expressed in the stigma/style and to establish the function of their protein products in pistil development, pollination, and pollen–pistil interactions. The self-compatible *Nicotiana tabacum* species was chosen as a model system due to the size of its reproductive organs. A stigma/style cDNA library was constructed and differentially screened (Goldman *et al.*, 1992), resulting in the isolation of several cDNA clones (Goldman *et al.*, 1992, 1994, 1998). The characterization of a stigma/style predominantly expressed gene (*NtWBC1*) encoding an ABC transporter is described here.

The designation ABC transporter recognizes a highly conserved ATP-binding cassette, which is the most characteristic feature of this superfamily of proteins (Higgins, 1992; Schneider and Hunke, 1998). The ABC superfamily is a diverse group of integral membrane proteins involved in the ATP-dependent transport of solutes across biological membranes, both extra- and intracellular membranes. ABC proteins are involved in the trafficking of a great diversity of biological molecules including sugars, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, inorganic acids, and glutathione conjugates (Higgins, 1992; Theodoulou, 2000). These proteins possess a modular structure where two nucleotide-binding domains (NBDs), or ABC domains, and two transmembrane domains (TMDs) form a functional transporter. In eukaryotes, all four domains can be encoded by a single gene as one polypeptide chain, or by two genes, each producing a 'half-transporter'. Experimental evidence has demonstrated examples of 'half-transporters' that form homodimers and others that form heterodimers to produce the functional ABC transporter (Dreesen *et al.*, 1988; Liu *et al.*, 1999; Janvilisri *et al.*, 2003).

The ABC protein superfamily is very large and exists in diverse species, from eubacteria and archae to eukaryotes: yeasts, mammals, and plants. This is the largest family of proteins in many completely sequenced bacteria, for example, ABC proteins correspond to 2% of the *Escherichia coli* genome (Linton and Higgins, 1998). A complete inventory of the ABC protein genes from the *Arabidopsis thaliana* genome has established the existence of 129 polypeptides, an account that is the largest reported to date and outstrips even the human genome, predicted to encode 48 ABC proteins (Sánchez-Fernández *et al.*, 2001*a*, *b*; http://nutrigene.4t.com/humanabc.htm June/2003). The *Arabidopsis* ABC proteins fall into 13 subfamilies from which the White-Brown Complex or White subfamily is the largest one (a collection of 29 reverse orientation [NBD-TMD] 'half-transporters') (Sánchez-Fernández *et al.*, 2001*a*). The prototypes of these are the White, Scarlet, and Brown proteins of *Drosophila* that contribute to a permease complex that mediates the transport of pigment precursors responsible for eye colour. *NtWBC1* belongs to the White–Brown Complex subfamily. Despite the fact that this is the largest ABC transporter subfamily in plants, *NtWBC1* is the first plant gene of this subfamily to be characterized. The possible functions of this protein in the pollination and reproductive processes are discussed here.

Materials and methods

Identification of a stigma/style predominant cDNA clone

Construction of a tobacco stigma/style cDNA library in λgt10 and the differential screening were described elsewhere (Goldman *et al.*, 1992). The insert of the selected λgt10 cDNA clone was isolated by *Eco*RI digestion and subcloned in the *Eco*RI site of pGEM1 vector (Promega), originating the plasmid pMG13.

DNA and protein sequence analyses

The DNA sequencing was performed according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using Big-Dye chemistry (Applied Biosystems) and automated sequencer ABI3100 (Applied Biosystems). DNA and deduced amino acid sequences were analysed using freely available computer software like Phred/Phrap/Consed and tools accessible from the NCBI (http://www.ncbi.nlm.nih.gov/), Expasy (http://us.expasy.org/tools/), and Japanese Genome Net (http://www.genome.ad.jp/) sites. The sequence data reported here are available in the GenBank under accession numbers AY341251, AY341252, AY496971, and AY496972.

DNA isolation procedures

Genomic DNA was isolated from leaves of *N. tabacum*, *N. sylvestris*, and *N. tomentosiformis*, following the procedure of Dellaporta *et al.* (1983). For Southern blot analysis, the genomic DNA was digested with restriction enzymes, separated by electrophoresis on a 1% agarose gel and transferred to Hybond N^+ nylon membrane according to the manufacturer's protocol (Amersham Biosciences). Hybridization was performed with DNA probes in 6×SSC, 5×Denhardt's solution, 0.5% SDS, and 100 μg ml^{-1} denatured carrier DNA at 65 °C overnight. Filters were washed at 65 °C, once in 6×SSC, 0.5% SDS for 15 min, once in 2×SSC, 0.1% SDS for 30 min, once in 1×SSC, 0.1% SDS for 30 min, once in 0.5×SSC, 0.1% SDS for 30 min, and once in 0.1×SSC, 0.1% SDS for 30 min. Hybridized filters were exposed to Kodak X-Omat films, for the appropriate time, at −70 °C in between intensifying screens.

Radioactive labelled DNA probes were prepared from the gel-purified (Sambrook and Russell, 2001) *Eco*RI cDNA fragment of pMG13 using the Random Primers DNA Labeling System (Invitrogen).

Isolation of the NtWBC1 *gene*

A *N. tabacum* genomic library constructed in λ Charon 32 (Koltunow *et al.*, 1990) was screened using the cDNA clone pMG13 as a probe. A total of 80 000 recombinant plaques were screened and a single hybridizing clone was found. The isolated genomic clone for the *NtWBC1* gene was subcloned as two *Hin*dIII fragments (approximately 6.3 kb each) into pUC18 vector (Pharmacia) generating the plasmids pCO08 and pCO09.

RNA isolation and analyses

Tobacco plants (*N. tabacum* cv. Petit Havana SR1) were grown under standard greenhouse conditions. Roots, stems, leaves, sepals, petals, stamens, stigmas/styles, ovaries, and seeds were collected and frozen in liquid nitrogen. For the developmental expression studies, stigmas/styles were excised from flowers at stages 1–12 of the tobacco flower development as described previously (Goldberg, 1988; Koltunow *et al.*, 1990). All samples were stored at −70 °C until RNA isolation. Total RNA was extracted from the different samples essentially as described by Dean *et al.* (1985). The RNA was quantified by measuring OD at 260 and 280 nm. To verify the RNA quality, 10 μg of total RNA from each sample was fractionated in a 2.2 M formaldehyde–1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The presence of intact 28S and 18S rRNA bands was used as a criterion of RNA integrity. The intensity of the rRNA bands was used to confirm the quantification by spectrophotometry and the equalized loading of RNA in each lane of the agarose gels used for blotting.

For northern analysis, total RNA was electrophoretically separated as described above and transferred to Hybond N^+ nylon membrane according to the manufacturer's recommendations (Amersham Biosciences). Hybridization and washing conditions were the same as described above for the Southern blot experiments, except that the temperature used was 50 °C. A final wash in 0.1×SSC, 0.1% SDS for 30 min at 58 °C was performed.

For *in situ* hybridization experiments, pistils and anthers were dissected from stages 6 and 10 tobacco flowers, as described previously (Goldberg, 1988; Koltunow *et al.*, 1990). Small pieces of stigmas/styles and anthers were fixed at 40 °C for 2 d in 4% formaldehyde, 5% acetic acid, 50% ethanol, and stepwise dehydrated until 100% ethanol prior to infiltration with xylene and subsequent embedding in paraffin and sectioning. Sections were rehydrated and taken through the *in situ* hybridization procedure essentially as described by De Almeida-Engler *et al.* (2001). The *Xba*I-*Eco*RI fragment (pPT3 subclone) from the pMG13 cDNA was used for generating the single-stranded ^{35}S-RNA probes. This 520 bp fragment does not include the conserved ATP-binding cassette. Hybridization was done at 42 °C in 2.25×SSPE (1×SSPE: 0.18 M NaCl, 0.001 M $NaPO_4$ at pH 7.7, 0.001 M EDTA), 50% formamide, and washed at 42 °C in 0.1×SSC, 50% formamide. Sections were exposed for an appropriate period of time and photographed under dark-field microscopy.

The RACE experiment for the identification of the 3′ end of the anther transcript was performed using the primer CL3 (5′-ATCATCTTATTGTCTTTCTCATGC-3′) and the kit Gene Racer™ (Invitrogen) following the manufacturer's instructions.

Real-time RT-PCR reactions

All the PCR and RT-PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, USA). TaqMan EZ RT-PCR kit (Applied Biosystems, USA) was used for RT-PCR reactions. The thermal cycling conditions comprised an initial step at 50 °C for 2 min, followed by 30 min at 60 °C for reverse transcription, 95 °C for 5 min, and 40 cycles at 94 °C for 20 s and 60 °C for 1 min. The TaqMan PCR Reagent kit was used for the PCRs. The thermal cycling conditions comprised an initial step at 50 °C for 2 min, followed by 10 min at 95 °C, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. In all experiments appropriate negative controls containing no template DNA or RNA were subjected to the same procedure to exclude or detect any possible contamination or carryover. Each sample was repeated at least twice. The results were normalized to the amount of total RNA in each sample (200 ng). In real-time PCR analysis, quantification is based on the threshold cycle, which is inversely proportional to the logarithm of the initial copy number (Tyagi *et al.*, 1998). Real-time PCR reactions were performed with each primer pair and its TaqMan probe, using known amounts of target obtained by serial dilutions of genomic DNA as template over several orders of magnitude (1 pg to 100 ng). A linear relationship was obtained by plotting the threshold cycle against the logarithm of the known amount of initial template. The equation of the line that best fits the data was determined by regression analysis. The R^2 value was calculated for each data set to estimate the accuracy of the real-time PCR with TaqMan as a quantification method. The amounts of target copies contained in an unknown sample were determined by extrapolating from the linear regression of the standard curve (correlation coefficient of 0.99 or greater) obtained for each primer TaqMan set (Freeman *et al.*, 1999). The following primers and probes (Applied Biosystems, USA) were used in this work: for NtWBC1fw, 5′-ATCTCACGTAGCCGGAGCA-3′; NtWBC1rv, 5′-TTTGTTCTGGTGGACGGGAT-3′; NtWBC1probe, VIC-5′-CCGCAGCCCCGCTGAGGA-3′-TAMRA (VIC, trademark product from Applied Biosystems; TAMRA, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine).

Results

Sequence analysis of a stigma/style-predominant cDNA clone

To identify genes specifically or predominantly expressed in tobacco pistils, a stigma/style cDNA library was previously constructed and a differential screening against seedling cDNA probes was performed (Goldman *et al.*, 1992). This procedure has been successful and several cDNA clones have been isolated and characterized (Goldman *et al.*, 1992, 1994, 1998). The molecular characterization of a novel stigma/style cDNA clone (pMG13) is shown here.

The pMG13 clone contains a cDNA of 1801 bp that corresponds to the 5′ end of an incomplete cDNA that misses approximately 700 bp of the 3′ end (based on the transcript size). The coding region seems to be complete at the 5′ end and it was possible to identify a putative initial methionine. The pMG13 cDNA insert was used as a probe in the screening of a genomic library and resulted in the isolation of a clone containing the entire coding region and almost 6 kb of sequence upstream of the putative translation initiation codon. An additional stigma/style cDNA clone corresponding to the *NtWBC1* gene was identified in the TOBEST (Tobacco EST project in progress in the authors' laboratory) database and has allowed the definition of the polyadenylation site. The cDNA and genomic clones were sequenced, demonstrating that both cDNAs are 100% identical to the genomic clone and that the *NtWBC1* gene contains no intron. The identified open reading frame encodes a polypeptide with 730 amino acids, which has no predicted signal peptide.

A search of the protein database using the deduced amino acid sequence and the BLAST algorithm (Altschul *et al.*, 1990) has revealed similarity to ABC transporters. The designation ABC transporters recognizes a highly conserved ATP-binding cassette, which is the most characteristic feature of this superfamily of proteins. The ATP-binding cassette includes the Walker A and B motifs (Walker *et al.*, 1982), as well as the ABC signature (Bairoch, 1992), that can be recognized in the sequence of this protein. The NtWBC1 sequence contains six putative transmembrane segments as identified by the computer program TMHMM (tool available at Expasy). In the NtWBC1 sequence, the ATP-binding cassette is localized at the N-terminus and the hydrophobic region, containing the transmembrane domain, is at the C-terminus, which is typical of the ABC transporters of the White subfamily (Sánchez-Fernández *et al.*, 2001*a*).

Based on the BlastP program, the highest similarities obtained were to ABC transporters from the *Arabidopsis* genome. The nearest equivalent to NtWBC1 in *Arabidopsis* is At3g55090 (67% identity and 76% similarity at the amino acid level), closely followed by At3g53510, At2g39350, At2g37360, At5g13580, and At3g55110. These transporters have been sequenced and annotated during the *Arabidopsis* Genome Initiative (2000), but have not yet been characterized. The NtWBC1 putative homologue in the *Arabidopsis* genome (At3g55090) has no intron (http://www.arabidopsis.org./servlets/TairObject?id=39599&type=locus) as well as that determined here for NtWBC1 in the *N. tabacum* genome. There are also significant similarities to an ABC transporter of *Oryza sativa* (55% identity and 66% similarity at the amino acid level), to the White protein from *Drosophila melanogaster* (28% identity and 45% similarity), and the ABCG2 proteins from *Mus musculus* (27% identity and 44% similarity), and from humans (26% identity and 43% similarity). The animal sequences are smaller than the plant sequences and the alignment of all these ABC transporters is shown in Fig. 1. Among these sequences, the overall structural arrangement (NBD-TMD) is conserved and despite the amino acid differences, the localization of the transmembrane segments was also preserved during evolution. All of these sequences have at least six transmembrane segments and contain a putative di-leucine motif near the first transmembrane segment, as first observed by Schmitz *et al.* (2001) for some human and *Drosophila* ABC transporters of the White subfamily. In addition, in all these sequences the highly conserved 'SGG(Q)' ABC signature has been replaced by 'SGGE', in which the negatively charged glutamic acid substitutes the uncharged polar glutamine.

The N. tabacum *genome contains two copies of* NtWBC1 *sequence*

To investigate the number of sequences similar to *NtWBC1* in the tobacco genome, a Southern blot analysis was performed of genomic DNA digested with *Bam*HI, *Eco*RI, and *Hin*dIII and probed with pMG13 cDNA insert. After washing at high stringency, allowing 5% mismatch (0.1×SSC, 0.1% SDS, 65 °C) a few bands could be visualized in each of the digestions (Fig. 2A). The stronger bands present in each of the *Eco*RI (4.6 kb) and *Hin*dIII (6.3 kb) digestions perfectly correspond in size to bands produced by digestions of the genomic clone obtained in this study and, therefore, contain the *NtWBC1* genomic sequence. To elucidate the origin of the bands, an additional Southern blot with genomic DNA extracted from *N. tabacum*, *N. sylvestris*, and *N. tomentosiformis* was performed. As it is shown in Fig. 2B, each of the bands identified in the *Eco*RI digestion of *N. tabacum* DNA has its correspondent in the *N. sylvestris* or *N. tomentosiformis* genome. Similar results were obtained for the *Hin*dIII digestion (data not shown). In both cases, the *Eco*RI (Fig. 2B) and the *Hin*dIII digestions, the stronger band always correlates with a band of the *N. sylvestris* genome. It is accepted that *N. tabacum* is an allotetraploid species, originated by the hybridization of the *N. sylvestris* (as female parent) and *N. tomentosiformis* (as male parent) ancestors (van Buuren *et al.*, 1992). The results indicate that *NtWBC1* existed as a single copy gene in each of the ancestral genomes and currently has two copies in the *N. tabacum* genome.

NtWBC1 *gene has a stigma/style-predominant expression pattern*

In order to determine the expression pattern of the *NtWBC1* gene, a northern analysis with total RNA extracted from roots, stems, leaves, sepals, petals, stamens, stigmas/styles, ovaries, and seeds was performed. NtWBC1 probe elicited very strong hybridization to a transcript of approximately 2.5 kb from stigmas/styles. This transcript is weakly detectable in sepals, petals, and ovaries. This probe also produced hybridization to a smaller transcript in stamens (2.3 kb). After high stringency wash (0.1×SSC, 0.1% SDS at 58 °C for 30 min) only the transcripts of the stigmas/styles and stamens are clearly visible (Fig. 3A). Real-time RT-PCR with fluorescent TaqMan probes was used to quantify the expression level of the *NtWBC1* gene in the different vegetative and reproductive organs. The primers and fluorescent probe used are presented in the Materials and methods. As shown in Fig. 3B, *NtWBC1* is specifically expressed in the reproductive organs and predominantly in the stigmas/styles. Its transcript level is 1.5 times higher in stigmas/styles than in stamens (anthers and filaments). *NtWBC1* is also expressed at much lower levels in other floral organs: 6.2-, 12.7- and 17.0-times lower in sepals, petals, and ovaries, respectively, than in stigmas/styles. Taken together, this study's results show that *NtWBC1* is a novel stigma/style predominantly expressed gene, encoding an ABC transporter not characterized previously.

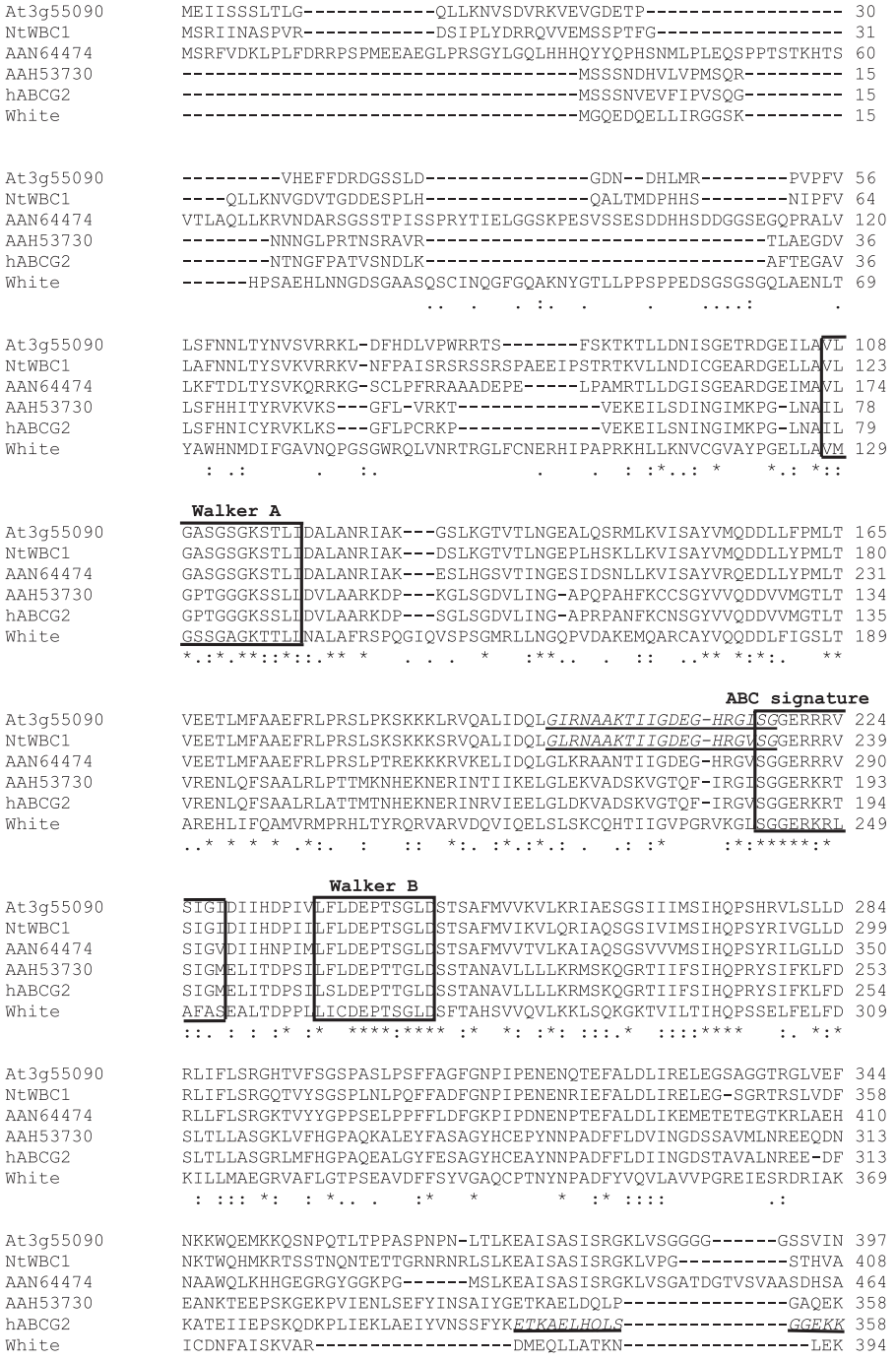

Fig. 1. Alignment of NtWBC1 amino acid sequence and the ABC transporters belonging to the White subfamily from several distantly related species, including plants (dicot and monocot) and animals (insect and mammals). The alignment was produced by Clustal W analysis (Thompson *et al.*, 1994) with default parameters using the following sequences: At3g55090 (*Arabidopsis thaliana*); NtWBC1 (*Nicotiana tabacum* sequence described in this paper); AAN64474 (accession number from *Oryza sativa*); AAH53730 (accession number of ABCG2 from *Mus musculus*); hABCG2 (accession number NM 004827 from *Homo sapiens*), and White (accession number P10090 from *Drosophila melanogaster*). The Walker A, Walker B, and ABC signatures are boxed. The transmembrane segments are double underlined and the putative di-leucine motif is shown in bold and underlined. The START motif is in italics and bold underlined. The asterisk means that the residues in that column are identical in all sequences in the alignment; the colon means that conserved substitutions have been observed; the full point means that semi-conserved substitutions are observed. Gaps were introduced to achieve maximum similarity between the sequences and are shown as dashes.

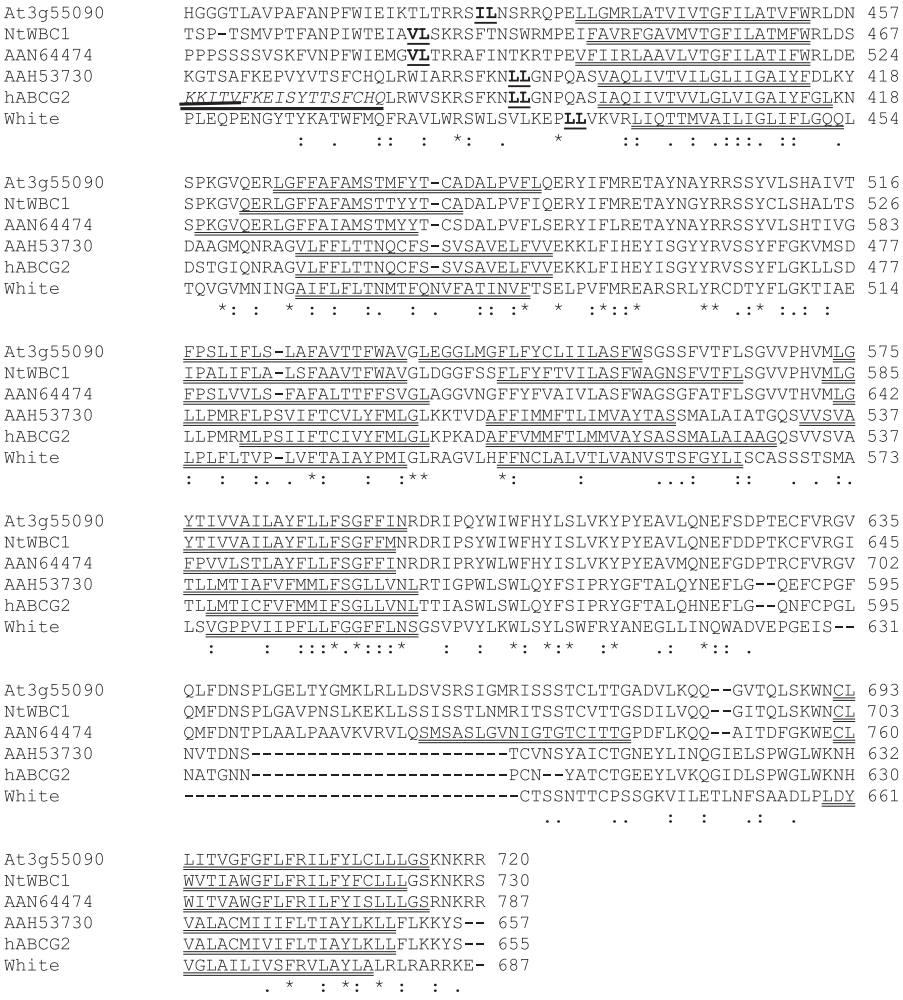

Fig. 1. (continued)

NtWBC1 *gene expression is developmentally regulated*

The expression pattern of the *NtWBC1* gene was studied based on northern blot and real-time RT-PCR experiments using total RNA isolated from stigmas/styles at the different stages of tobacco flower development as described by Goldberg (1988) and Koltunow *et al.* (1990). At stage 1, anthers, and pistil are fully differentiated and the calyx encloses the corolla, anthers and pistil. At stage 12, the corolla limb is fully expanded and deep pink, the anthers have dehisced, the stigma is covered with a sticky exudate, and pollination occurs. At stages 6–7, the pistil is already capable of sustaining pollen tube growth and fertilization can occur, although theses processes are still inefficient. From stages 6–7 to stages 11–12, the pistil is preparing itself for pollination and successful fertilization. A detailed examination of the expression of *NtWBC1* during flower development is shown in Fig. 4. The results obtained by northern blot and by real-time RT-PCR indicate that *NtWBC1* gene expression increases during development toward anthesis and reaches the higher levels in the stages prior pollination (stages 8–11), when stigmas/styles are in preparation for competent receptivity. The maximum transcript level was detected at stage 10 (11.4- and 3.2-times higher than at stages 4 and 6, respectively). *NtWBC1* expression decreases in open flowers at stage 12, in which most of the pistils have been already pollinated. At stage 12 the *NtWBC1* transcript has reached a level similar to what it was found in stage 6 stigmas/styles. The stigma/style-specific gene encoding PELPIII, a protein important for the pollination process (De Graaf *et al.*, 2003), has a comparable expression pattern (Goldman *et al.*, 1992). The results suggest that *NtWBC1* may play an important role in reproductive physiological processes in the stigma and style.

NtWBC1 *mRNA accumulates in specific tissues of the stigma and anther*

To determine more precisely in which cell types the *NtWBC1* gene is expressed, *in situ* localization of the mRNA was performed. An *NtWBC1* antisense RNA probe radioactively labelled was hybridized to longitudinal sections of stigmas/styles and transverse sections of anthers

(Fig. 5). In stage 6 stigmas/styles the hybridization signal is restricted to the stigmatic secretory zone (Fig. 5A). In stage 10 stigmas/styles the localization of the mRNA remains the same (data not shown). At stage 6 anthers, the *NtWBC1* transcript could be detected in the stomium region (Fig. 5C, E). However, at stage 10–11, the anthers start dehiscence along the stomium region and the *NtWBC1* hybridization signal is no longer detected (Fig. 5F). On the other hand, at these later stages a hybridization signal in the vascular bundle could be seen (Fig. 5F, H). This signal was not detected at stage 6 anthers. Taken together, the results demonstrated that *NtWBC1* expression is developmentally regulated both in stigmas/styles (different transcript levels) as well as in anthers (expression in different tissues).

In the pistil, *NtWBC1* expression is confined to the stigmatic secretory zone, the first tissue to get in contact with the pollen grains and in which the pollen tube grows toward the ovary. This result suggests a possible function in pollen–pistil interactions. In the anther, the expression on the stomium region suggests that *NtWBC1* may also have a role in anther dehiscence.

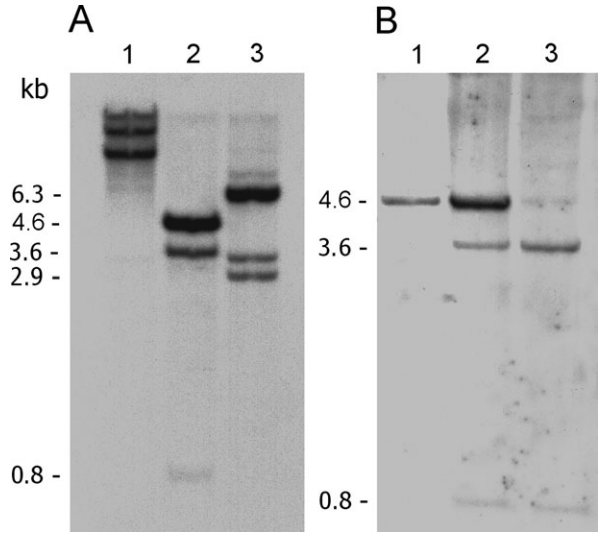

Fig. 2. Southern blot analysis of the *NtWBC1* gene. (A) *N. tabacum* genomic DNA was digested with the indicated restriction enzymes and hybridized with the pMG13 cDNA clone as a probe: (1) *Bam*HI; (2) *Eco*RI; and (3) *Hin*dIII. (B) Genomic DNA of (1) *N. sylvestris*; (2) *N. tabacum*; and (3) *N. tomentosiformis* was digested with *Eco*RI restriction enzyme and hybridized as above.

Characterization of the NtWBC1 *genomic and anther cDNA sequences*

To try to understand the size difference between the *NtWBC1* transcripts of stigmas/styles and anthers, a 3′ RACE was performed using anther RNA. The amplification resulted in a single band of approximately 850 bp that was cloned and sequenced. The comparison with the genomic and stigma/style cDNA clones has demonstrated that the anther sequence is almost identical to the other sequences (796/798 – 99.7%). In addition, it was established that the polyadenylation in anthers occurs 13 nucleotides downstream of the polyadenylation site in stigmas/styles. Therefore, the explanation for the anther transcript being approximately 200 bp smaller than the stigma transcript is not the site of polyadenylation.

To identify the potential *cis*-acting elements present in the *NtWBC1* promoter region, analyses of the 2.7 kb region (The complete *NtWBC1* DNA sequence is deposited in the Genbank database under accession number AY341251) upstream of the initial methionine (+1) were performed in the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE) as well as in the Transfac program (www.genome.ad.jp). The *NtWBC1* 5′ flanking sequence contains a putative TATA box (TATATAAA) localized at −191 nucleotides (nt) of the 5′ end of the pMG13 cDNA clone and −283 nt

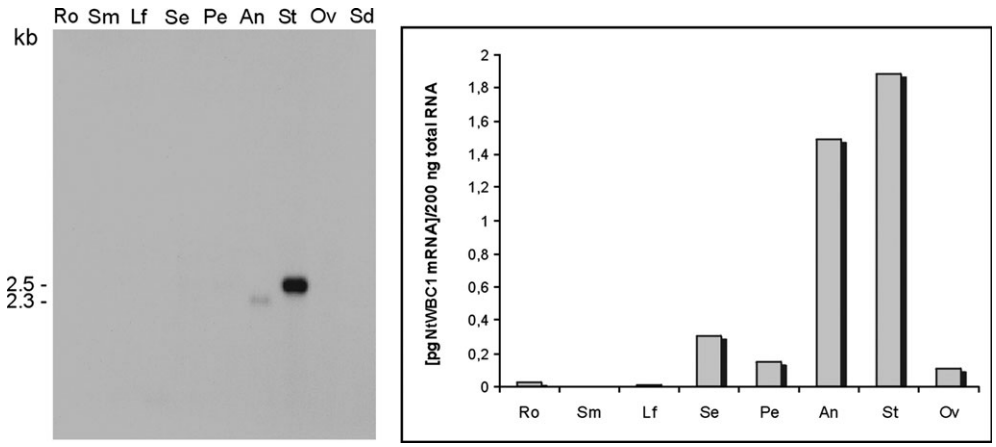

Fig. 3. Accumulation of the *NtWBC1* mRNA in the different vegetative and reproductive organs of *N. tabacum.* (A) Northern blot analysis of total RNA (10 μg) extracted from each indicated organ. (B) Real-time RT-PCR experiment performed with 200 ng of total RNA of each indicated organ. Ro, roots; Sm, stems; Lf, leaves; Se, sepals; Pe, petals; An, anthers and filaments (stamens); St, stigmas and styles; Ov, ovaries; Sd, seeds. The northern blot and the real-time RT-PCR experiments were performed with different RNA samples made of organs pooled in several developmental stages and represent independent results.

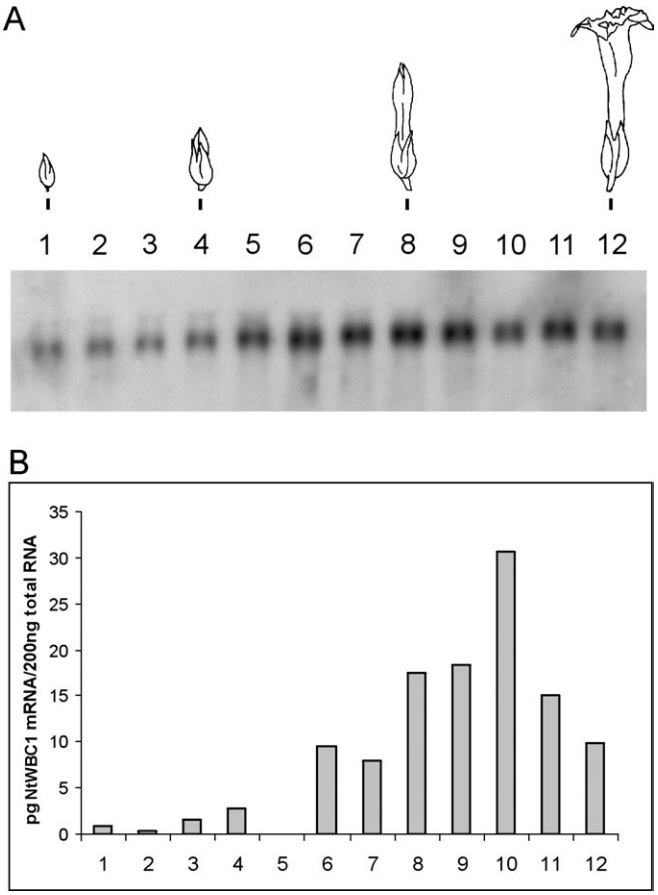

Fig. 4. Accumulation of NtWBC1 mRNA during tobacco flower development. (A) Northern blot analysis of total RNA (10 μg) extracted from stigmas and styles of flowers from stages 1–12 (Goldberg, 1988; Koltunow *et al.*, 1990). (B) Real-time RT-PCR experiment performed with 200 ng of total RNA of each indicated developmental stage. The northern blot and the real-time RT-PCR experiments were performed with different RNA samples and represent independent results. At least four attempts were made to amplify NtWBC1 from stage 5 RNA sample without result.

from the first ATG. An additional putative TATA box (TATATATAA) is situated at −12 nt of the initial methionine and could be responsible for the transcription initiation in stamens, in which a smaller mRNA was found. The analysis has also revealed some interesting *cis*-acting regulatory DNA elements. A putative AGAMOUS binding sequence (TTTTCCAAATTGGTTGAT) is positioned 502 nt upstream of the first ATG. Regulation by the AGAMOUS transcription factor is expected for a gene expressed at stamens and pistils and could explain the spatial expression pattern observed for the *NtWBC1* gene.

Among several other possible *cis*-acting elements identified, one was particularly striking. Two putative motifs (AATC(T/A)CG(T/A)GAT) for SREBP-1 (sterol regulatory element-binding protein 1) have been found around position −416 nt and are juxtaposed, one in each DNA strand. SREBPs are a family of transcription factors identified in mammals that regulate genes involved in lipid metabolism (Yang *et al.*, 2003). The presence of the SREBP-1 motif in the *NtWBC1* promoter region raises the possibility that its protein product is somehow involved in the lipid metabolism in specific cells of the plant reproductive organs.

Discussion

NtWBC1 *encodes an ABC transporter specific of reproductive organs*

To study the processes of pistil development, pollination, and pollen–pistil interactions at the molecular level, a gene (*NtWBC1*) predominantly expressed in the pistil and specifically expressed in the flower reproductive organs was identified and characterized. This gene encodes a protein containing an ATP-binding cassette with strong similarity to several ABC transporters. The ABC protein superfamily is probably the most diverse family of proteins, but the conservation of the NBD has allowed the identification of members of this superfamily in the EST databases and genomes sequenced. Based on their domain organization and sequence similarity, they have been organized into subfamilies. The 'half-transporters', with an NBD-TMD organization and sequence similarity to the *Drosophila* White protein, have been grouped in one subfamily. In the *Drosophila* genome this subfamily is composed of 15 members (Dean *et al.*, 2001) from which the White, Brown, and Scarlet proteins are the best characterized ones. In the human genome, the proteins of this group are organized in the ABCG subfamily, composed of five members (Dean *et al.*, 2001), including the well-studied genes *ABCG1*, *ABCG2*, *ABCG5*, and *ABCG8*. In the *Arabidopsis* genome, this is the largest subfamily containing 29 members and has been denominated the White subfamily (Sánchez-Fernández *et al.*, 2001*a*). NtWBC1 has strong similarity to and the same structural organization as the At3g055090 protein, human ABCG2, and White and, therefore, belongs to the White subfamily. *NtWBC1* is the first plant gene of this subfamily to be characterized and to have its temporal and spatial expression pattern determined.

As no ABC transporter has yet been shown to function with fewer than the four core domains (two NBDs and two TMDs), it is assumed that the four core domains form the basic unit required to mediate solute translocation (Higgins, 1992; Schmitt and Tampé, 2002). NtWBC1 is a 'half-transporter' and should interact with another polypeptide chain to form either a homodimer or a heterodimer with an as yet unidentified ABC transporter protein of the White subfamily to establish a functional transporter in the reproductive organs. Both forms are conceivable for NtWBC1 since the two cases have been described within the White subfamily, for example, in *Drosophila*, the White protein works as a heterodimer interacting with Brown or Scarlet (Dreesen *et al.*, 1988; Ewart *et al.*, 1994); in humans, ABCG5 and ABCG8 most likely cooperate as

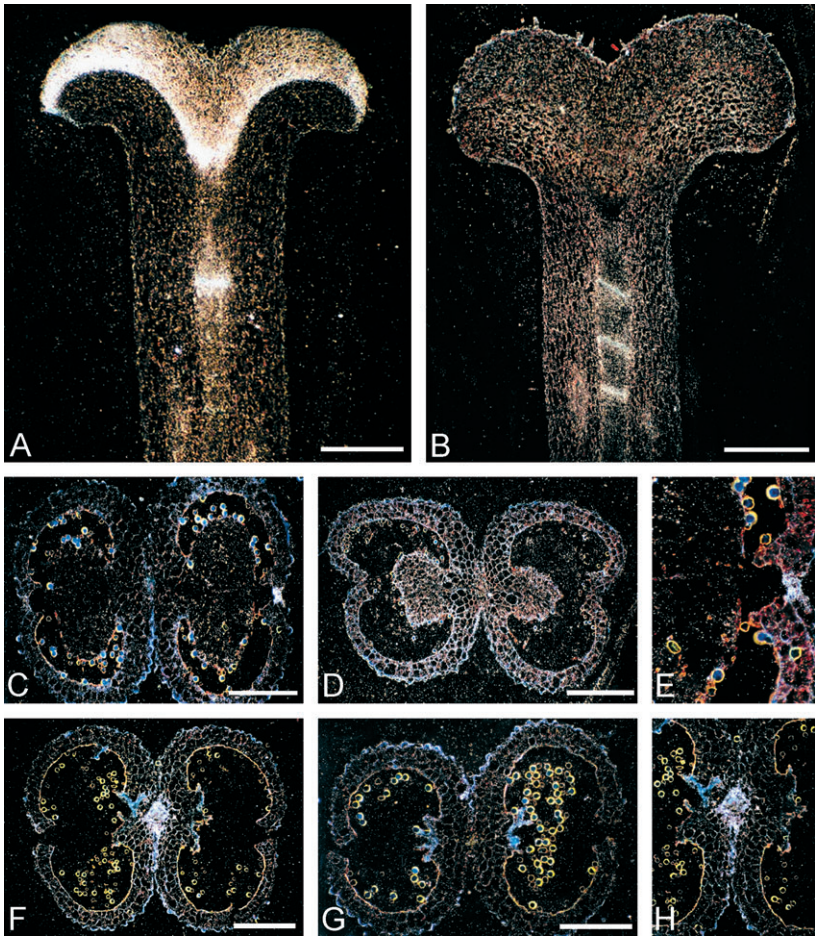

Fig. 5. Localization of NtWBC1 mRNA in the reproductive organs of *N. tabacum*. Stigmas/styles and anthers from the indicated developmental stages were fixed, embedded in paraffin, sliced and hybridized with single-stranded ^{35}S-RNA probes, as outlined in the Materials and methods. In the photographs taken by dark-field microscopy, the white grains represent regions containing RNA/RNA hybridization. (A) *In situ* hybridization of *NtWBC1* antisense RNA probe in a longitudinal section of a stage 6 stigma/style. (B) *In situ* hybridization of *NtWBC1* sense RNA probe, used as a negative control, in a longitudinal section of a stage 6 stigma/style. (C) *In situ* hybridization of *NtWBC1* antisense RNA probe in a transverse section of a stage 6 anther. (D) *In situ* hybridization of *NtWBC1* sense RNA probe, used as a negative control, in a transverse section of a stage 6 anther. (E) Amplification (4×) of photograph shown in (C). (F) *In situ* hybridization of *NtWBC1* antisense RNA probe in a transverse section of a stage 10–11 anther. (G) *In situ* hybridization of *NtWBC1* sense RNA probe, used as a negative control, in a transverse section of a stage 10–11 anther. (I) Amplification (1.5×) of photograph shown in (F). The bar in each photograph represents 0.5 mm.

heterodimers (Schmitz *et al.*, 2001; Yu *et al.*, 2002) whereas ABCG2 probably acts as homodimer (Schmitz *et al.*, 2001; Janvilisri *et al.*, 2003). At least three new clusters encoding ABC transporters of the White subfamily, expressed in stigmas/styles, have already been identified (I daSilva and MHS Goldman, unpublished results) in the TOBEST database (http://143.107.203.68/Mhelena/default.html). They are candidates for interacting with the NtWBC1 protein and form a functional transporter.

The *NtWBC1* gene is exclusively expressed in the reproductive organs (stigmas and stamens), with a predominant expression in the pistil (stigmas). It is interesting to note that the transcript detected in stamens is smaller than the one present in stigmas (Fig. 3). Differences in transcript sizes are generally a consequence of alternative initiation sites, alternative splicing or alternative polyadenylation signals. The possibility of alternative splicing in *NtWBC1* can be ruled out since the analysis of the genomic clone has demonstrated that in *N. tabacum* this gene contains no intron. Alternative polyadenylation sites occur for the *NtWBC1* gene in the reproductive organs, but the anther 3′ UTR is 13 nucleotides longer and, therefore, can not account for the size difference observed in northern blot experiments. The use of alternative polyadenylation signals has been reported for another ABC transporter, the Brown gene of *Drosophila*, in which there are two transcripts (2.8 and 3.0 kb), both encoding the same predicted protein of 675 amino acids (Dreesen *et al.*, 1988). In humans, various transcripts of *ABCG1* have been detected in different cells arising from alternative splicing events or the use of different transcription initiation sites (Lorkowski *et al.*, 2001). The production of more than one transcript size, arising from the use of different transcription initiation sites, also occurs with the human *ABCG2* (Allikmets *et al.*,

1998). The use of alternative initiation sites is a possible explanation for the transcripts of the *NtWBC1* gene and could produce different amino acid sequences in the N-terminal portion of the NtWBC1 protein in the female and male reproductive organs. The analysis of the *NtWBC1* genomic 5′ flanking region and the identification of a second putative TATA box in the 5′ UTR of the stigma mRNA contributes to this hypothesis. In this case, the stamen transcript would encode a protein that starts at the second methionine of the NtWBC1 sequence (MSSPTFG...) presented in Fig. 1 and be an equivalent of the aligned animal sequences, without an N-terminal extension of 24 amino acids. However, at this moment it can not be ruled out the possibility that the two transcripts are identical at the 5′ end and have different sizes of poly(A) tails.

According to the prediction of localization for the NtWBC1 protein by the PSORT program (Nakai and Horton, 1999; at Expasy), NtWBC1 is likely to exist on the plasma membrane, both for the stigmas/styles and stamens polypeptide sequences. The same localization was previously assumed for the White protein, in which this ABC transporter would facilitate the uptake of pigment precursors. However, the White protein was not found in the plasma membrane as expected. Experiments of immunogold labelling and electron microscopy have detected the White and Scarlet proteins in intracellular membranes surrounding the pigment granules (Mackenzie *et al.*, 2000). Schmitz *et al.* (2001) have suggested that the *Drosophila* White, Scarlet, and Brown and the human ABC transporters *ABCG1*, *ABCG5*, and *ABCG8* are located in the membranes of specialized intracellular compartments associated with the *trans*-Golgi network. The same authors have also proposed that the presence of a di-leucine signalling motif in the N-terminal region of these ABC transporters may be involved in the targeting mechanisms of the protein localization. The NtWBC1 protein sequence contains a putative di-leucine signaling motif just prior to the first predicted transmembrane segment as well as the homologous sequences from *Arabidopsis*, *Oryza sativa*, *Mus musculus*, human *ABCG2*, and the White from *Drosophila* (Fig. 1). In the case of the human ABCG2, there is experimental data showing that the majority of this protein is in the plasma membrane, however, ABCG2 was also found within intracellular membranes (Rocchi *et al.*, 2000). It remains to be established the subcellular localization of NtWBC1 (JB de Molfetta and MHS Goldman, unpublished results).

Possible roles of NtWBC1 protein in plant reproduction

To approach the question of NtWBC1 function in the pistil, its temporal and spatial expression patterns were analysed. NtWBC1 is developmentally regulated in stigmas/styles, with a higher transcript level in stages 9 and 10. This higher expression level occurs in parallel with the most intensive production of exudate 2–3 d prior to anthesis. The beginning of exudate secretion coincides with the appearance of the droplet-like lipids in the stigmatic secretory zone (Cresti *et al.*, 1986). In addition, *NtWBC1* mRNA is specifically localized to the cells of the stigmatic secretory zone. These are the cells responsible for the exudate production and that are in direct contact with the pollen tubes growing toward the ovary. The results suggest that the NtWBC1 protein product may be involved in exudate production or some other reproductive physiological process performed by these cells.

A novel stigma-predominantly expressed gene encoding an ABC transporter was identified. Until 1992 there was only one plant ABC transporter identified (Dudler and Hertig, 1992). Although the number of plant ABC transporter genes studied has been increasing rapidly, the role of most plant ABC transporter has not yet been clarified. To develop new ideas about the function of NtWBC1, a detailed analysis of the NtWBC1 protein sequence (http://motif.genome.ad.jp/motif-bin/motif_markseq_blocks) was performed. The analysis has unravelled the presence of a putative START (Steroidogenic acute regulatory protein signature) domain also recognizable at the *Arabidopsis* At3g55090 protein and human ABCG2 (Fig. 1). The START domain is a lipid-binding motif, which is primarily involved in eukaryotic signalling mediated by lipid binding (Ponting and Aravind, 1999). Representatives of the START domain family have been shown to bind different ligands such as sterols (StAR protein) and phosphatidylcholine (PC-TP). In plants, the START domain has already been found in proteins such as Glabra2, an homeodomain protein involved in regulating trichome morphogenesis and root-hair development (Rerie *et al.*, 1994). The finding that a putative lipid-binding START domain is present in the NtWBC1 protein could suggest that this protein may participate in the translocation of lipids within the cell or to the plasma membrane. The fact that the human ABCG2 has recently been shown to transport lipids (Janvilisri *et al.*, 2003) is consistent with this prediction.

There is evidence for the role of lipids in pollen–pistil interaction in both dry and wet stigmas. In *Arabidopsis*, a species with dry stigma, the *cer* and *fiddlehead* mutants have demonstrated that altered lipids affect pollen hydration and germination (Fiebig *et al.*, 2000; Pruitt *et al.*, 2000). In species with wet stigmas like tobacco, the lipids present in the exudate represent a directional cue for pollen tube growth (Wolters-Arts *et al.*, 1998). How are these lipids produced and released to the exudate? It is possible that NtWBC1 protein is involved in some step of this process. Further functional analysis is required to establish the role of NtWBC1 and its correlation to plant reproduction mechanisms like exudate production and anther dehiscence.

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Note added

During the revision process of the present article a paper describing the characterization of an ABC transporter of the White subfamily in *Gossypium hirsutum* was published (Zhu *et al.*, 2003).

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