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A gene similar to bacterial translocase I (mra Y) identified by cDNA-AFLP is expressed during flower bud development of *Arabidopsis thaliana*

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Abstract

The cDNA-amplified fragment length polymorphism approach was used to identify genes expressed differentially during late flower bud development in *Arabidopsis thaliana*. A cDNA corresponding to the *attrans* 11 gene was isolated and encoded a protein similar to bacterial UDP-*N*-acetylmuramoyl-pentapeptide-transferase (translocase I), an enzyme implicated in peptidoglycan biosynthesis. The expression of *attrans* 11 in tissues involved in view of flower development and plant reproduction are discussed.

Keywords: Flower development; cDNA-AFLP; Glycosyl transferase 4; Bacterial *mra Y* gene; Plant reproduction; *Arabidopsis thaliana*

1. Introduction

Flower organogenesis is one of the most interesting processes in plant development with homeotic genes regulating floral whorl identity [1–3]. The second stage of floral development involves a series of complex events, such as differentiation of the floral organs and maturation of the gametes.

The development of the male gametophyte (pollen grain) occurs in two phases. In phase I, microspore mother cells give rise to four microspores that form the tetrad. In phase II, microspores are released from the tetrad and undergo two successive mitoses which form three-celled mature pollen grains [4,5]. Pollen is covered by a complex structure in which the exine layer, composed of sporopolenin, lies closest to pollen protoplasm. The outermost layer of the pollen cell wall, known as tryphine, derives from anther tapetum. This secretory tissue undergoes programmed cell death (PCD) just before the onset of microspore mitosis [6,7]. The contents of tapetum are released into the loculus and attaches to the exine. The pollen cell wall plays an important role in pollen–stigma interactions by allowing pollen tube development within the style transmitting tissue [8].

The female gametophyte (embryo sac) is derived from a megaspore mother cell and undergoes meiosis that gives rise to four megaspores. After PCD of three megaspores, the remaining megaspore undergoes three mitotic divisions followed by cellularization to form a seven-celled embryo sac. The embryo sac matures inside the ovule located in the ovary [4]. An ovule tissue (integument) encases the embryo sac but leaves a small pore (micropyle) through which the pollen tube can adhere to the embryo sac and deliver sperm cells during fertilization. The ovary gives rise to the fruit in which embryo development occurs [9,10].

Molecular characterization of the stages of flower development has been approached by isolating inflorescence-specific genes through cDNA library screening or subtractive hybridization [11–13]. These techniques have the disadvantage of requiring a large amount of biological material. RNA fingerprinting methods require small quantities of starting material and are

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therefore better suited for isolating genes that are differentially expressed in small organs. Of these methods, differential display [14] and cDNA-AFLP [15] are the most used. The latter is an adaptation of the original AFLP [16]. The ligation of adapters at the extremities of cDNA restriction fragments allows highly stringent PCR conditions and results in matches that are more specific than in differential display. We have used cDNA-AFLP to isolate novel genes for the study of plant–microbe interactions [17].

In the present report, we describe the identification, cloning and expression of a gene highly similar to the bacterial *mra Y* gene that encodes UDP-*N*-acetylmuramoyl-pentapeptide-transferase, also known as translocase I [18–20]. This enzyme is involved in the biosynthesis of bacterial cell wall peptidoglycan, but has not yet been identified in plants or in other eucaryote. Real time PCR showed that this gene is most expressed in flowers and siliques. Results obtained in situ indicated that this gene is expressed throughout the development of tapetum and ovule in the late stages of flower formation. The possible roles of this gene during late flower bud development are discussed.

2. Material and methods

2.1. Plant material

Arabidopsis thaliana ecotypes Columbia and Landsberg erecta were grown in a growth cabinet at 22 °C with 15 h of light/day at an light intensity of ±45 μmol m^2/s. For execute the cDNA-AFLP experiments, Columbia ecotype flower buds were collected from plants 50 days-old and separated into two groups, according to their developmental phase: Phase 1 (P1)—flower buds <1.0 mm (microspore mother cell meiosis, stages 1–7 [21]) and Phase 2 (P2)—flower buds >1.0 mm (pollen grain maturation, stages 8–12 [21]). This correlation between *A. thaliana* flower tissue differentiation and pollen development was defined by Alves-Ferreira et al. [22]. Flower buds were frozen in liquid nitrogen and transferred to −70 °C. Roots, inflorescence stems, leaves, inflorescences and siliques of the Landsberg erecta ecotype were collected from plants 50 days-old, frozen in nitrogen liquid and stored at −70 °C until RNA extraction.

2.2. cDNA-AFLP

2.2.1. mRNA isolation and cDNA synthesis

Total RNA was extracted from frozen flower buds as described by Ragueh et al. [23] with slight modifications. Polyadenylated RNA was isolated from 150 μg of total RNA using poly-d[T]25 oligonucleotides coupled to paramagnetic beads (Dynal A.S. Oslo, Norway). cDNAs were synthesized from 1 μg of poly(A)+RNA using a cDNA synthesis module kit (RPN1256, Amersham), then extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and resuspended in sterile water (20 μl).

2.2.2. AFLP and PAGE analysis

cDNA (250 ng) was digested with restriction endonucleases *Mse*I and *Eco*RI and the fragments were linked to *Mse*I and *Eco*RI adapters, as described in a CORE AFLP kit (GIBCO-BRL). The primers utilized in cDNA-AFLP were radioactively labeled with ^{33}Pγ-ATP as described by Bachem et al. [15]. Twenty-eight pre-amplification cycles were run (94 °C, 30 s, 60 °C, 1 min, 72 °C, 1 min) using 1/10 of the template volume and primers corresponding to *Mse*I and *Eco*RI adapters without extension. The reaction products were examined in 1% agarose gels and then diluted 10× with TE buffer. Five microliters of these dilutions were used for selective amplifications (42 cycles, including 14 touchdown cycles in which the annealing temperature was reduced from 65 to 56 °C in 0.7 °C steps and maintained for 28 cycles). Six primer combinations were used in this assay: (A) *Eco*RI–AAG and *Mse*I-0, (B) *Eco*RI–AAG and *Mse*I-A, (C) *Eco*RI–AAG and *Mse*I-T, (D) *Eco*RI–AGC and *Mse*I-0, (E) *Eco*RI–AGC and *Mse*I-A, and (F) *Eco*RI–AGC and *Mse*I-T. The samples were denatured in 50% formamide at 95 °C, and separated in 5% polyacrylamide gels according to standard protocols [24]. After electrophoresis, the gels were dried onto 3MM Whatman paper (Whatman, Maidstone, UK) in a gel dryer. The cDNA-AFLP patterns were detected by exposure to Kodak BIOMAX film at −70 °C.

2.2.3. TDF isolation, reamplification and cloning

The fragments identified as being differentially expressed (transcript-derived fragments (TDFs)) were excised from the radioactive gel, soaked in water, purified by glycogen precipitation as described by Reuber and Ausubel [25], and reamplified. The primer combinations were the same as those used in the selective amplifications, but the PCR conditions were different with 36 cycles including touchdown cycles (the annealing temperature was reduced from 65 to 59 °C in 1 °C steps and maintained for 30 cycles). The reamplified TDFs were cloned in the plasmid pCRII® (Invitrogen) using a TA cloning kit (Invitrogen)®.

2.3. Rapid amplification of cDNA ends PCR (RACE PCR) and amplification of Attrans 11 cDNA

Total RNA was extracted from inflorescences of the *A. thaliana* L. erecta ecotype according to Ragueh et al. [23]. A SMART RACE PCR kit (Clontech) was used for the 5′ and 3′ RACE reactions. The primers used were

attrans RACE 5′ (5′-GCAGCCATTGCAGCCAAAGCTCCACCTA-3′) and *attrans* RACE 3′ (5′-GCATCAGTTTCCATGGGAGATACAGG-3′). The RACE products were cloned in the plasmid pCRII® and sequenced. The sequences of 5′ and 3′ RACE were aligned using the program CLUSTAL W 1.8 (BCM Search launcher). To amplify full-length *Attrans* 11 cDNA, primers were designed for the 5′and 3′ ends of the open reading frame derived from the 5′ RACE and 3′ RACE assembly sequences. The primers used were For 120 CD (5′-ATGCCCTTCTTCACATCGGCGATTC-3′) and Rev 120 CD (5′-TGCAGAAATAAGACCTATGTAAGCTG-3′). The amplification protocol consisted of one cycle at 94 °C for 1 min, 35 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1.5 min and a final extension at 72 °C for 5 min. The PCR product was cloned in the plasmid pCRII® and sequenced.

2.4. Real time PCR

The *Attrans* 11 cDNA sequence was used to design primer sets to amplify a 150-bp product. The primers used were: 5′-TGGAGCTTGTTTTGGGTTTC-3′ and 5′-CGACACCAGACGATATGAA-3′. The expression levels of an *A. thaliana* constitutive gene (At1g10740) were used to normalize the mRNA sources. The primers used were: 5′-CCTAATGGGAAACGCAGGT-3′ and 5′-AAGAGCATCGGGTGGATTC-3′. First-strand cDNA was primed with random hexamers, using MultiScribe reverse transcriptase as described by the manufacturer (PE Biosystems, Foster City, CA). The reactions were analyzed with an ABI 5700 sequence detection system using SYBR Green chemistry (PE Biosystems). The PCR conditions were 95 °C for 30 s and 60 °C for 1 min, for 40 cycles. Each reaction was run with two different samples, each time in triplicate.

2.5. In situ hybridization

In situ hybridization was done using *Attrans* 11 cDNA cloned in the plasmid pCRII®. The Plasmids were linearized with appropriate enzymes and used as templates for transcription in vitro. Antisense and sense probes were synthesized using a digoxigenin SP6/T7 labeling kit (Boehringer Mannheim) and subsequently cleaved by alkaline hydrolysis. Flower buds were fixed in 4% paraformaldehyde/4% DMSO, then treated with proteinase K (10 μg/ml) for 30 min at 37 °C, dehydrated at 4 °C, before embedding in paraplast (Sigma). For hybridization, tissue 8 μm thick were positioned on slides and incubated with a solution containing 50% formamide, 0.3 M NaCl, 12 mM Tris pH 7.5, 1.25 × Denhardt's solution, 6 mM EDTA, 12.5% dextran sulfate, and 1.25 mg/ml tRNA. After hybridization, the slides were washed twice for 1 h in 2 × SSC at 55 °C, then treated with RNase solution (20 μg/ml RNAse, 0.5 M NaCl, 10 mM Tris pH 8, 1 mM EDTA) at 37 °C for 30 min and washed again in 2 × SSC at 55 °C for 60 min. Hybrids were detected using digoxigenin nucleic acid detection kits (Boehringer Mannheim), according to the manufacturer's instructions. The substrates used were NBT (nitro blue tetrazolium salt) and 5-bromo-4 chloro-3 indolyl phosphate. The sections were dehydrated, washed twice in Histoclear and then mounted in Cytoseal 60 mounting medium (Stephens Scientific).

2.6. DNA sequencing, nucleotide and protein sequence analysis

Sequencing was done on a Perkin–Elmer Applied Biosystems system (ABI Prism 370, 373 and 377). Databases searches were done using the BLAST (NCBI, National Center for Biotechnology Service) and MIPS *A. thaliana* group (MIPS, Munich Information Center for Protein Sequences) network services. The programs CLUSTAL W 1.8 (BCM Search Launcher) and GENE DOC BOXSHADE [26] were used for alignment analysis. A dendogram tree was constructed using the MEGA analysis platform [27]. Protein prediction was done using SAPS [28], PSORT [29], TMPRED (BCM Search Launcher), PFAM [30] and the hydrophobicity profiles of Weizmann Institute programs (http://bioinformatics.weizmann.ac.il/hydroph).

The sequence of *Attrans* 11 can be found in the GenBank database under accession number AY130289.

3. Results

Flower buds of the two phases of anther development, P1 (Flower buds <1.0 mm) and P2 (flower buds >1 mm) were used for poly (A)+RNA isolation and cDNA-AFLP. Using six primers combinations, 20 TDFs present predominantly in late flower bud development were detected. The fragments were excised from dried polyacrylamide gels and reamplified using the same cDNA-AFLP primers. Fig. 1 shows the differential RNA fingerprint obtained. Eight TDFs were cloned and sequenced, since they presented the most differential patterns of expression among the 20 TDFs.

Database searches revealed that seven of these TDFs displayed sequence homology with genes found in the data banks of the *A. thaliana* genome project, including disease resistance gene (At1g53360), chaperonin cpn60 (At1g55490), RING finger protein (At3g60220), putative F-box gene (At5g56810), putative ZINC finger gene (At5g46650), and a hypothetical gene (At4g13500). One TDF showed sequence homology with 5′ non-translated region of a phytocystatin (At4g16500). Further experiments are necessary to determine the expression pattern of these genes during flower development.

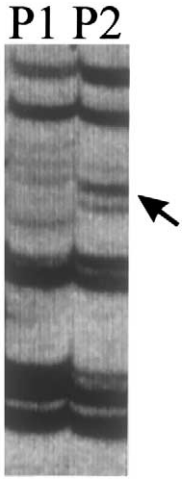

Fig. 1. cDNA-AFLP autoradiography showing the TDF pattern of *A. thaliana* flower buds at the microspore mother cell meiosis stage (P1) and pollen maturation stage (P2). The arrow indicates TDF B2.

The sequence of TDF B2 was identical to 145 bp of the last exon and 55 bp of the 3′ non-translated region of the putative gene T9A21.120 from Contig myd32 of the *A. thaliana* Genomic Bank. This sequence predicts a protein similar to the bacterial protein UDP-*N*-acetylmuramoyl-pentapeptide-transferase (also translocase I–mra Y). This protein is a transmembrane enzyme involved in the biosynthesis of bacterial cell wall peptidoglycan and acts by transferring UDP-*N*-acetyl muramoyl-pentapeptide to bactoprenol, a cytoplasmic membrane carrier lipid. Subsequently, another transferase adds *N*-acetylglucosamine (GlNAc) to the oligossacharide chain linked to the lipid carrier, which is then flipped to the periplasmic space where peptidoglycan synthesis occurs. Considering the similarity between T9A21.120 and bacterial translocases, this putative gene was named as *Attrans* 11 (at-*A. thaliana*, Trans 11-translocase).

The 5′ and 3′ RACE amplification strategy was successful in providing the complete sequence of *Attrans* 11 cDNA. The overlap sequence of 5′ and 3′ RACE had 57 bp. The assembly of these overlapping sequences produced an open reading frame of 978 bp, giving rise to a hypothetical protein of 326 amino acids. Based on this consensus sequence, primers were constructed to isolate complete *Attrans* 11 cDNA from a pool of flower bud cDNA. The sequence of this cDNA and its putative derived protein are shown in Fig. 2. Comparison between the *Arabidopsis* genome project annotation of this gene and our data revealed several differences in the intron/exon organization (data not shown).

The sequences of some bacterial translocases I were aligned with ATTRANS 11 (Fig. 3) and this alignment was used to construct a phylogenetic tree (Fig. 4). The resulting tree indicated that ATTRANS 11 had a high

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ATGCCCTTCTTCACATCGGCGATTCTAGTTACCTTAGCTGGTTATATT
 M  P  F  F  T  S  A  I  L  V  T  L  A  G  Y  I
TTTGTTCCGCTTCTAGACAGACTGAGAGTGCATGAGCCAATTAGGACA
 F  V  P  L  L  D  R  L  R  V  H  E  P  I  R  T
TTTGGGCCAGTTCCACATAACCGCAGACCAACAATCCCGACAATGGGT
 F  G  P  V  P  H  N  R  R  P  T  I  P  T  M  G
GGGTTGTTCTTTGTTCCAATTGGTGTTGTTGTTGCAATAGCCTTGACT
 G  L  F  F  V  P  I  G  V  V  V  A  I  A  L  T
AAAGTTTCATCCATCGAAGTCTTGGGAGCAGCAGCCGCAACTGTAGCA
 K  V  S  S  I  E  V  L  G  A  A  A  A  T  V  A
TTTGCAGCCATTGGGCTAATTGATGACTCCTTAAGCCTCTACAGTGAG
 F  A  A  I  G  L  I  D  D  S  L  S  L  Y  S  E
AATAATAATGGTTTATCTGCAAAGATACAACTTCTTTTGGAGGCAGCT
 N  N  N  G  L  S  A  K  I  Q  L  L  L  E  A  A
GTTGGGACTTGCTTTGCGTTTTGGTTGGAGACTGCAAGCTTATCATCT
 V  G  T  C  F  A  F  W  L  E  T  A  S  L  S  S
CCTTATGGCATGAAAATGTTGGTCCCCTTGCCTTCACCATTAGGTCTT
 P  Y  G  M  K  M  L  V  P  L  P  S  P  L  G  L
GTTTTCTTGGGAAAACTTTACCTACTGTTGACATCGTTTTACTTTGTT
 V  F  L  G  K  L  Y  L  L  L  T  S  F  Y  F  V
TCCATGGGAAACTTAGTCAAAGCAACCGATGGTCTCGATGGATTGGCG
 S  M  G  N  L  V  K  A  T  D  G  L  D  G  L  A
GGAGGTATTGCTGCTTTGTGTTTTGTTGCAATGGCAATAGCAGTTCTT
 G  G  I  A  A  L  C  F  V  A  M  A  I  A  V  L
CCTATTTGCTCTGATCTCTCTGTATTTGGAGCTTCGATGGCTGGAGCT
 P  I  C  S  D  L  S  V  F  G  A  S  M  A  G  A
TGTTTTGGGTTTCTGCTTCACAATCGATACAGAGCATCAGTTTCCATG
 C  F  G  F  L  L  H  N  R  Y  R  A  S  V  S  M
GGAGATACAGGATCCTTGGCTCTAGGTGGAGCTTTGGCTGCAATGGCT
 G  D  T  G  S  L  A  L  G  G  A  L  A  A  M  A
GCTTGTTCAGGAATGTTCTTCCCGTTGTTCATATCGTCTGGTGTCGCA
 A  C  S  G  M  F  F  P  L  F  I  S  S  G  V  A
GTTTTGGAAGCTTCTTCTGTCATTATACAGGTCGTGTATTACTCGCCA
 V  L  E  A  S  S  V  I  I  Q  V  V  Y  Y  S  P
ACTAAGCGTTTAAAAGGAAAAGGGCGTCGGATATTCAAGACTGTCCCG
 T  K  R  L  K  G  K  G  R  R  I  F  K  T  V  P
TTTCATCATCACCTTAGGCTAAACGGTTTAAAGGAGCCAATGATAGTA
 F  H  H  H  L  R  L  N  G  L  K  E  P  M  I  V
ACGATGGCATATGTAATATCCTCTTTGCTCTCTCTTTCAGCAGCTTAC
 T  M  A  Y  V  I  S  S  L  L  S  L  S  A  A  Y
ATAGGTCTTATTTCTGCATAA
 I  G  L  I  S  A  *
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Fig. 2. cDNA and amino acid sequence of *attrans* 11.: Putative transmembrane regions of AtTrans I are underlined. *Stop codon.

identity with bacterial translocases I, especially that of the cyanobacterium *Synechocystis* sp. strain PCC6803.

To find protein motifs in ATTRANS 11, we used the PFAM program that assembles protein families according to consensus domains. Like the bacterial translocases I, ATTRANS 11 contained a glycosyl transferase 4 domain, characteristic of UDP-GlcNAc/MurNAc:Polyisoprenol-*P* GlcNAc/MurNAc-1-*P*-transferase family [32,33].

Based on prediction analysis the estimated molecular weight of ATTRANS 11 was 34 kDa. The putative protein has a neutral charge, eight probable transmembrane regions (Figs. 2 and 5), and a hydrophobicity pattern very similar to the *E. coli* protein mra Y (Fig. 5). Prediction of protein localization suggested three possible sites of anchorage: the endoplasmic reticulum membrane, the plasma membrane and the Golgi body.

To determine the expression pattern of *Attrans* 11, total RNA isolated from stems, siliques, roots, leaves and inflorescences were used in real time PCR semi-quantitative assays. Table 1 shows that *Attrans* I amplifies around 17 cycles in flower and siliques, and

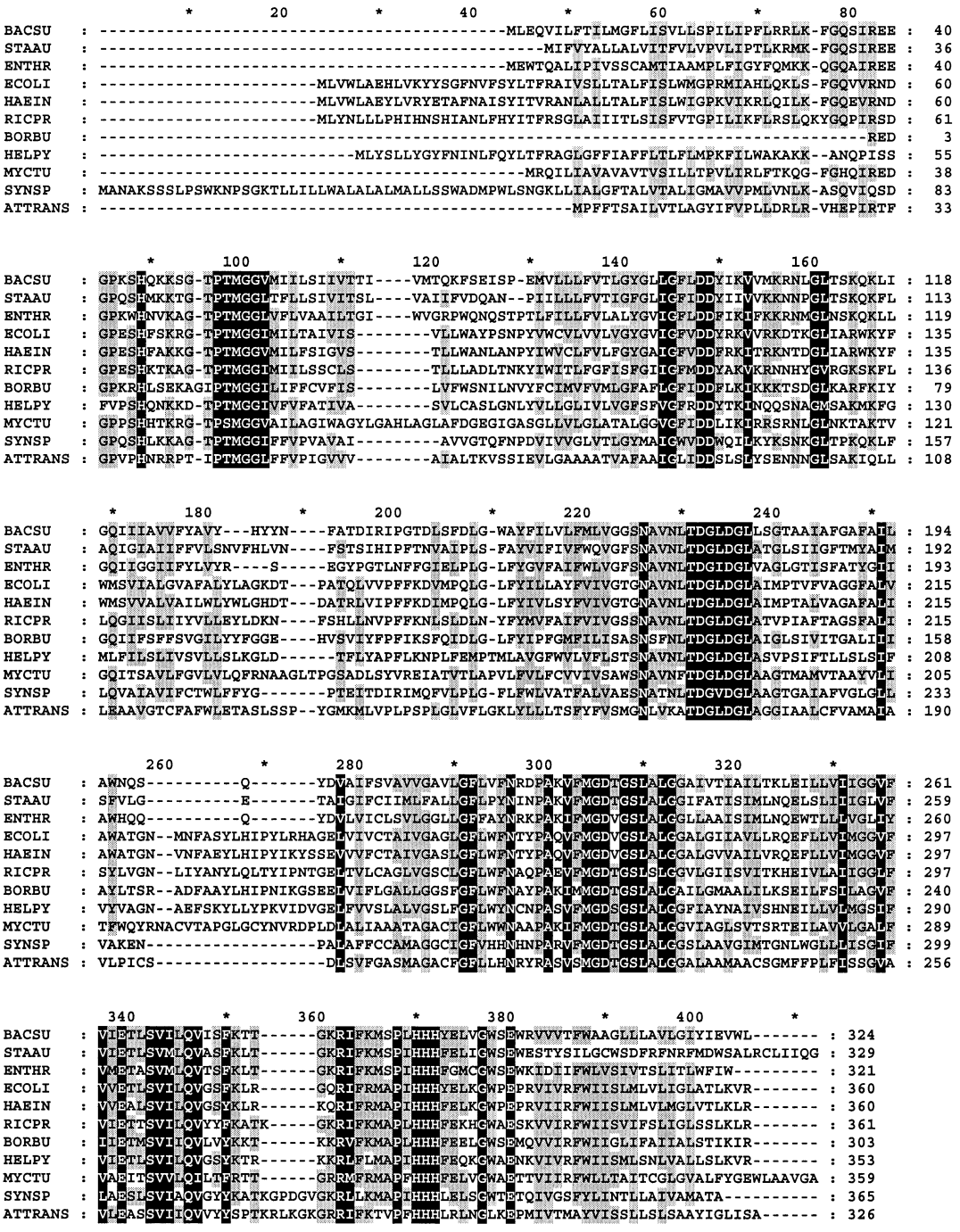

Fig. 3. Alignment of translocases I: ECOLI, *Escherichia coli* (P15876); HAEIN, *Haemophylus influenzae* (P45062); HELPY, *Helicobacter pylori* (O25325); RICPR, *Rickettsia prowazekii* (Q9ZCW0); STAAU, *Staphylococcus aureus* (O07322); BORBU, *Borrelia burgdorferi* (Q44766); BACSU, *Bacillus subtilis* (Q03521); ENTHR, *Entherococcus hirae* (O07668); MYCTU, *Mycobacterium tuberculosis* (O06221); SYNSP, *Synechocystis* sp. (D64005); *attrans* (AY130289), *A. thaliana* translocase 11. Alignment was constructed using CLUSTAL W 1.81 and edited with the GENE DOC program. GENE DOC produces a display that emphasizes the degree of conservation in each column in the alignment. This focuses attention on which amino acid residue are least tolerant of change, or where a change in the sequence is most likely to change the structure or function of the protein. Black shade: 100% of conservation. Gray shade: 60% conservation or greater.

around 18 cycles in roots, leafs and stems. According to real time PCR parameters, this difference in one cycle number indicates that *Attrans* 11 is about 1.9 times more expressed in flowers and siliques than in other organs. The expression of *Attrans* 11 in flowers was also confirmed by northern blot assay (data not shown). The tissue-specific expression of *Attrans* 11 was analyzed by RNA in situ hybridization of *A. thaliana* flower buds.

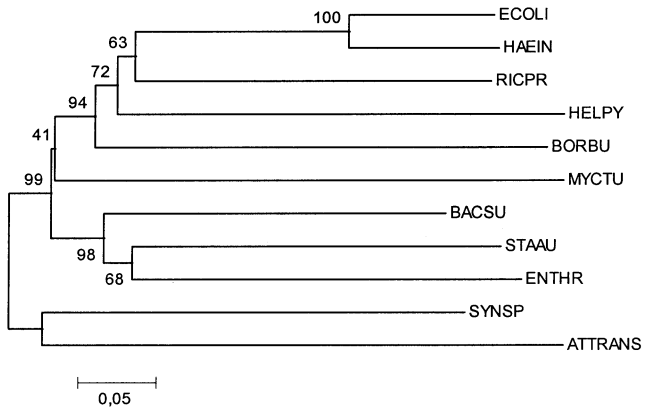

Fig. 4. Phylogenetic unrooted tree of translocases I. The tree was constructed with MEGA software using neighbor joining and *P*-distance parameters. Bootstrap values are shown on the tree.

Labeled antisense RNA hybridized only in tapetum and ovule inner integument (Fig. 6A, C). The sense RNA of *Attrans* 11 did not hybridize with any inflorescence tissue (Fig. 6B, D).

Table 1
Real time semi-quantitative PCR analysis of the *attrans* 11 gene using RNA from different organs of *A. thaliana*

Organ	Sample	C_T[a]	Average C_T	Lipase correction ΔC_T[b]
Inflorescence (I)	(I1)	17.19		
	(I2)	17.35	17.3	17.3
	(I3)	17.36		
Stem (S)	(S1)	18.64		
	(S2)	18.49	18.49	18.33
	(S3)	18.34		
Leaf (L)	(L1)	18.9		
	(L2)	18.72	18.77	18.38
	(L3)	18.71		
Silique (S)	(F1)	17.98		
	(F2)	17.86	18.02	17.39
	(F3)	18.22		
Root (R)	(R1)	19.91		
	(R2)	20.3	20.12	18.42
	(R3)	20.16		

[a] C_T is the cycle number at which normalized product fluorescence exceeds a threshold within the exponential range of the amplification reaction as determined for primer set used.

[b] ΔC_T is the difference in C_T values between the control and the average C_T after correction for differences in C_T in lipase amplifications. Lipase correction ΔC_T^b is C_T after correction for differences in the lipase cycle numbers between samples.

4. Discussion

4.1. ATTRANS 11 may be involved in glycosylation

ATTRANS 11 contains a glycosyl transferase 4 domain that is characteristic of UDP-GlcNAc/MurNAc:Polyisoprenol-*P* GlcNAc/MurNAc-1-*P*-transferase family [31,32], which also includes UDP-*N*-acetylglucosamine:dolichol phosphate *N*-acetylglucosamine-1-*P* transferase (GPT) proteins involved in the eucaryotic glycosylation pathway. The proteins of this family share some similarities: they are transmembrane

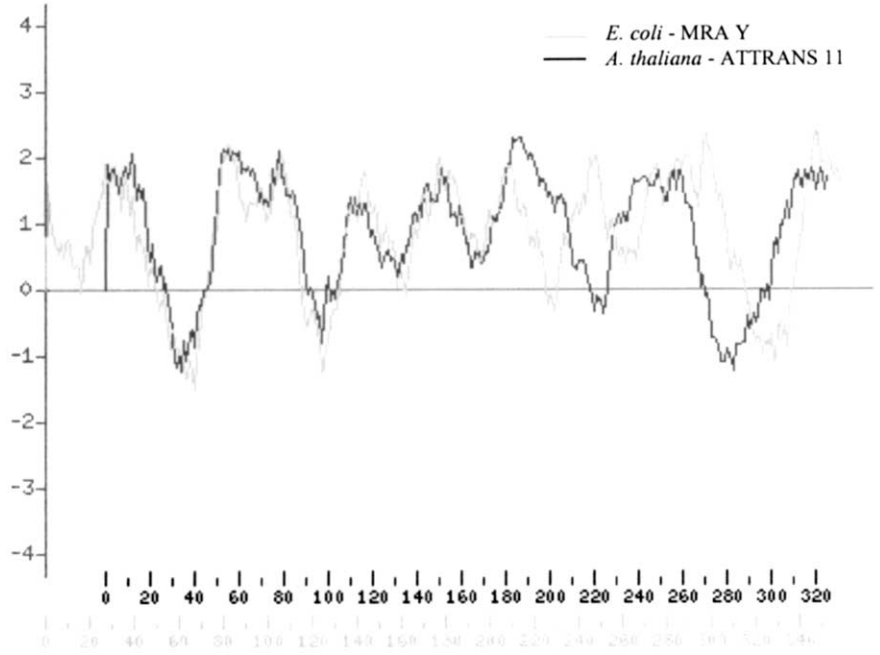

Fig. 5. Comparison between the hydrophobicity patterns of *E. coli* mra Y protein (gray) and ATTRANS 11 (black). Hydrophobicity patterns were determined using Weizmann Institute programs.

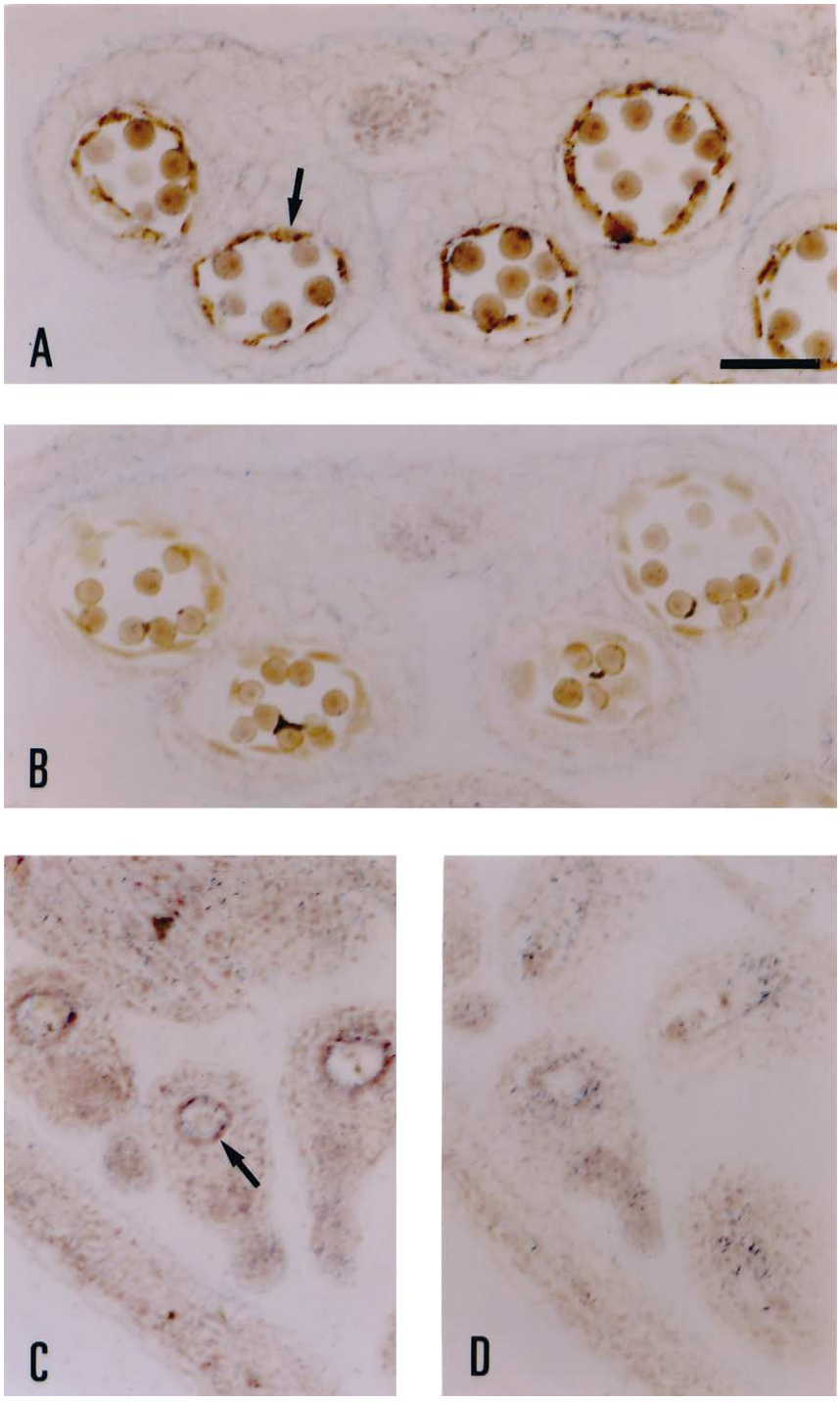

Fig. 6. *Attrans* I In situ hybridization in *A. thaliana* flower bud transversal sections (A and B) and longitudinal sections (C and D). Cross Sections were probed with *attrans* 11 antissense mRNA (A and C) and *attrans* 11 sense mRNA (B and D). Arrows indicate the presence of *attrans* 11 RNA/RNA hybrids in tapetum (A) and ovule inner integument (C). Photographs were taken using bright field optics. Scale bar, 50 μm (A, B, C, D).

proteins with several membrane spanning domains, they catalyze reactions among the same class of substrates (complex oligosaccharides linked to UDP and polyisoprenol lipid carriers anchored to membranes), they contain six conserved domains, and are inhibited by the antibiotic tunicamycin [33]. This antibiotic inhibits peptidoglycan biosynthesis in bacteria and glycosilation in eucaryotes, with subsequent blockade of transport of some proteins in the latter.

Koizumi et al. [33] cloned an *A. thaliana* gene that encodes for a GPT. Genes encoding GPT proteins have been isolated from *Saccharomyces cerevisae* [34] and mouse [35]. This enzyme catalyzes the first step of glycan biosynthesis by transferring the olygosaccharide GlNAc

linked to UDP (UDP–GlNAc) to a lipid carrier (dolichol) anchored in the ER membrane. Subsequently, another GlNAc and five mannose residues are added to the glycan attached to the lipid carrier. Thereafter, the lipid-linked oligosaccharide flips to the luminal side of the ER and four residues of mannose and three residues of glucose are linked to the glycan chain. Finally, the complete glycan is added to an asparagine residue in the consensus sequence Asn–X–Thr/Ser of the target protein. Further glycan modifications occur in the Golgi body. Glycosylation serves to label target proteins and direct them to their final destination. Glycans can also induce correct folding of the protein and aid in water-trapping and cell–cell adhesion [36].

The ATTRANS 11 characteristics cited above (a glycosil transferase 4 domain, several transmembrane domains and possible localization in the endoplasmic reticulum membrane) suggest that this protein participates in glycosylation events. The isolation of this gene, never identified before in eucaryotes, could give new insights to glycosilation events in plants.

4.2. ATTRANS 11 may participate in A. thaliana reproduction

Real time PCR assays indicate that *Attrans* 11 is predominantly expressed in flowers and siliques. In situ hybridization experiments revealed that the highest ATTRANS 11 expression was in flowers and siliques, organs related with plant reproduction, result that is in agreement with real time PCR assays. The ATTRANS 11 characteristics cited above suggest the participation of glycosylation processes in tapetum, ovule cells and siliques. Interestingly, anther tapetum and inner integument are believed to nourish microspores and the embryo sac, respectively. A possible correlation between our results and a function for ATTRANS 11 in gamete nourishment remains to be determined.

The presence of glycoproteins during flower development, such as the proteoglycan arabinogalactan proteins (AGPs), has been reported. These molecules consist mainly (90%) of carbohydrates, have a peptide core rich in hydroxyproline and occur in various plant organs and cell types [37,38]. The expression of AGPs in pollen tubes, stigma and style is well documented [39,40], as is their occurrence in tapetum [41,42], pollen [43], ovules [41] and embryos [41]. The identity of AGPs is not determined by the core peptide, but by the synthesis of complex carbohydrate chains, which may involve numerous glycosyltransferases [38]. The observation that each cell type contains a series of AGPs and that these molecules are stage-regulated indicates a possible tissue and developmental regulation of the enzymes involved in AGP synthesis. These arguments support the hypothesis that ATTRANS 11 can participate in the biosynthesis of specific AGPs that reach peak expression during late flower bud development. Additional experiments are in progress to elucidate the function of ATTRANS 11.

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References

[1] M. Ng, M.F. Yanofsky, Three ways to learn the ABCs, Curr. Opin. Plant Biol. 3 (2000) 47–52.
[2] J.K. Okamuro, B.G.W. Den Boer, K.D. Jofuku, Regulation of *Arabidopsis* flower development, Plant Cell 5 (1993) 1183–1193.
[3] E.S. Coen, E.M. Meyerowitz, The war of the whorls: genetic interactions controlling flower development, Nature 353 (1991) 31–37.
[4] R.E. Pruitt, M. Hulskamp, From pollination to fertilization in *Arabidopsis*, in: E.M. Meyerowitz, C.R. Sommervile (Eds.), Arabidopsis, Cold Spring Harbor Laboratory Press, 1994, pp. 467–483.
[5] S. McCormick, Male gametophyte development, Plant Cell 5 (1993) 1265–1275.
[6] M. Wang, S. Hoekstra, S. van Bergen, G.E.M. Lamers, B. Oppedijk, M. van der Heijden, W. de Priester, R. Schillperoort, Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L, Plant Mol. Biol. 39 (1999) 489–501.
[7] A. Papini, S. Mosti, L. Brighigna, Programmed cell death events during tapetum development of angiosperms, Protoplasma 207 (1999) 213–221.
[8] G.M. Zinkl, L.K. Wilheimi, D. Preuss, Sticking together: cell adhesion interactions in *Arabidopsis* reproduction, J. Plant Res. 111 (1998) 299–305.
[9] C.S. Gasser, K. Robinson-Beers, Pistil development, Plant Cell 5 (1993) 1231–1239.
[10] L. Reiser, R.L. Fischer, The ovule and the embryo sac, Plant Cell 5 (1993) 1291–1301.
[11] I. Atassonov, C. Masuyata, H. Tanaka, J. Kataoka, S. Kuwata, Comparative study of screening with subtracted probe and differential screening isolation of flower-specific cDNAs clones from *Nicotiana sylvestris*, Plant Sci. 118 (1996) 185–195.
[12] S. Utsugi, W. Sakamoto, Y. Ogura, M. Minoru, F. Motoyoshi, Isolation and characterization of cDNA clones corresponding to the genes expressed preferentially in floral organs of *Arabdopsis thaliana*, Plant Mol. Biol. 32 (1996) 759–765.
[13] P. Rubinelli, Y. Hu, H. Ma, Identification, sequence analysis and expression studies of novel anther-specific genes of *Arabidopsis thaliana*, Plant Mol. Biol. 37 (1998) 607–619.
[14] P. Liang, A.B. Pardee, Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction, Science 257 (1992) 967–971.
[15] C.W.B. Bachem, R.S. van der Hoeven, S.M. de Bruijn, D. Vreugdenhill, M. Zabeau, G.F. Visser, Visualization of differential gene expression using a novel method of RNA fingerprint-

ing based on AFLP: analysis of gene expression during potato tuber development, Plant J. 9 (1996) 745–753.
[16] P. Vos, R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Fritjers, J. Pot, J. Peleman, M. Kuiper, M. Zabeau, AFLP: a new technique for DNA fingerprinting, Nucl. Acids Res. 23 (1995) 4407–4414.
[17] J.L. Simões-Araújo, R.L. Rodrigues, L.B. Gerhardt, J.M. Mondego, M. Alves-Ferreira, N.G. Rumjanek, M. Margis-Pinheiro, Identification of differentially expressed genes by cDNA-AFLP technique during heat stress in cowpea nodules, FEBS Lett. 27 (2002) 44–50.
[18] M. Ikeda, M. Wachi, H.K. Jung, F. Ishino, M. Matsuhashi, The *Escherichia coli mra Y* gene encoding UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-*N*-acetylmuramoyl-pentapeptide transferase, J. Bacteriol. 173 (1991) 1021–1026.
[19] P.E. Brandish, M.K. Burnham, J.T. Lonsdale, R. Southgate, M. Inukai, T.D.H. Bugg, Slow binding inhibition of phospho-*N*-acetylmuramyl-pentapeptide-translocase (*Escherichia coli*) by mureidomycin A, J. Biol. Chem. 271 (1996) 7609–7614.
[20] D.D. Boyle, W.D. Donache, *mra Y* is an essential gene for cell growth in *Escherichia coli*, J. Bacteriol. 180 (1998) 6429–6432.
[21] D.R. Smyth, J.L. Bowman, E.M. Meyerowitz, Early flower development in *Arabidopsis*, Plant Cell 2 (1990) 755–767.
[22] M. Alves-Ferreira, J.A. Engler, F.C. Miguens, M. van Montagu, G. Engler, D.E. de Oliveira, Oleosin gene expression coincides with lipid accumulation in the plastids and cytoplasmatic bodies, Plant Physiol. Biochem. 35 (1997) 729–739.
[23] F. Ragueh, N. Lescure, D. Roby, Y. Marco, Gene expression in *Nicotiana tabacum* in response to compatible and incompatible isolates of *Pseudomonas solanacearum*, Physiol. Mol. Plant Pathol. 35 (1989) 23–33.
[24] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, second ed., Cold Spring Harbor Press, New York, 1989.
[25] T.L. Reuber, F.M. Ausubel, Differential display RNA methods, in: D.W. Galbraith, H.J. Bohnert, D.P. Bourque (Eds.), Methods in Plant Cell Biology, Academic Press, 1995, pp. 432–440.
[26] K.B. Nicholas, H.B. Nicholas Jr., Gene doc—a tool for editing and annotating multiple sequence alignments. Distributed by the author. Available from http://www.psc.edu/biomed/genedoc(1997).
[27] S. Kumar, K. Tamura, M. Nei, MEGA: Molecular Evolutionary Genetics Analysis, Version 1.02, Pennsylvania State University, University Park, PA, 1993.
[28] V. Brendel, P. Bucher, I. Nourbakhsh, B.E. Blaisdell, S. Karlin, Methods and algorithms for statistical analysis of protein sequences, Proc. Natl. Acad. Sci. USA 89 (1992) 2002–2006.
[29] P. Horton, K. Nakai, A probabilistic classification system for predicting the cellular localization sites of proteins, Intell. Syst. Mol. Biol. 4 (1996) 109–115.
[30] A. Bateman, E. Birney, R. Durbin, S.R. Eddy, K.L. Howe, E.L. Sonnhammer, The Pfam protein families database, Nucl. Acids Res. 28 (2000) 263–266.
[31] M.A. Lehrman, A family of UDP-GlcNAc/MurNAc: polyisoprenol-*P* GlcNAc/MurNAc-1-*P*-transferases, Glycobiology 4 (1994) 768–771.
[32] A.R. Dal Nogare, N. Dan, M.A. Lehrman, Conserved sequences in enzymes of UDP-GlcNAc/MurNAc family are essential in hamster UDP-GlcNAc: dolichol-*P* GlcNAc-1-*P* transferase, Glycobiology 8 (1998) 625–632.
[33] N. Koizumi, T. Ujino, H. Sano, M.J. Chrispeels, Overexpression of a gene that encodes the first enzyme in the biosynthesis of asparagine-linked glycans makes plants resistant to tunicamycin and obviates the tunicamycin-induced unfolded protein response, Plant Physiol. 121 (1999) 353–361.
[34] K.O. Hartog, B. Bishop, Genomic sequence coding for tunycamicin resistance in yeast, Nucl. Acids Res. 15 (1987) 3627.
[35] B. Rajput, N. Muniappa, L. Schantz, L.L. Naylor, P.A. Lalley, I.K. Vijay, Mouse UDP-GlNAc:dolichol 1-phosphate transferase: molecular cloning of the cDNA, generation of anti-peptide antibodies, and chromosomal localization, Biochem. J. 285 (1992) 985–992.
[36] P. Lerouge, M. Cabanes-Macheteau, C. Rayon, A.-C. Fischette-Lainé, V. Gomord, L. Faye, *N*-glycoprotein biosynthesis in plants: recent developments and future trends, Plant Mol. Biol. 38 (1998) 31–48.
[37] M. Kreuger, G.-J. van Holst, Arabinogalactan proteins and plant differentiation, Plant Mol. Biol. 30 (1996) 1077–1086.
[38] A. Majewska-Sawka, A. Nothnagel, The multiples roles of arabinogalactan proteins in plant development, Plant Physiol. 122 (2000) 3–9.
[39] E. Lord, Adhesion and cell movement during pollination: cherchez la femme, Trends Plant Sci. 5 (2000) 368–372.
[40] A.Y. Cheung, H.-M. Wu, Arabinogalactans proteins in plant sexual reproduction, Protoplasma 208 (1999) 87–98.
[41] R.I. Pennell, L. Janiche, P. Kjellbom, G.N. Scofield, J.M. Peart, K. Roberts, Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers, Plant Cell 3 (1991) 1317–1326.
[42] C.P. Scutt, P.M. Gilmartin, The *Men-10* cDNA encodes a novel form of proline-rich protein expressed in the tapetum of dioecius *Silene latifolia*, Sex Plant Reprod. 11 (1998) 236–240.
[43] C. Ferguson, A. Bacic, M.A. Anderson, S.M. Read, Subcellular distribution of arabinogalactan proteins in pollen grains and tubes as revealed with a monoclonal antibody raised against stylar arabinogalactan proteins, Protoplasma 206 (1999) 105–117.