DNA Sequence and Expression Analysis of Root-Knot Nematode–Elicited Giant Cell Transcripts

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Fifty-eight cDNA clones isolated from a library of transcripts exhibiting up regulation in tomato root giant cells induced by infection with the parasitic nematode *Meloidogyne incognita* were characterized. A survey of plant tissues identified 31 transcripts present in tissues other than root, including actively dividing and expanding tissues and mature leaf tissues. The identities of approximately 20% of the giant cell transcripts were inferred from DNA sequence data; they include sequences encoding a plasmalemma proton ATPase, a putative Myb-type transcription factor, and the largest subunit of RNA polymerase II.

Sedentary endoparasitic nematodes form complex and intimate associations with their host plants. Root-knot nematodes (*Meloidogyne* spp.) are economically the most important and also the most sophisticated of these pathogens. The worms hatch as second-stage larvae and invade the root in the zone of elongation. They migrate intercellularly, first to the root apex and then to the developing vascular cylinder (Wyss *et al.* 1992), where permanent feeding sites are established. In response to repeated stimulation from the parasite, cells in the root stele are directed to reactivate DNA synthesis and nuclear division (but not cell division) and also to assume a new differentiated fate termed a "giant cell" (Bird 1961). These multinucleate cells become large and avacuolate and undergo extensive remodeling of the cell wall, adopting characteristics of transfer cells (Bird 1961; Jones and Northcote 1972). They are metabolically active (Bird 1971) and serve as the obligate nutritive source for the developing nematode, in which the locomotive musculature ultimately atrophies. Giant cell formation, coupled with limited proliferation of nearby pericycle and cortical cells, results in the characteristic root-knot gall.

Light and electron microscope studies, reviewed by Endo (1987), have shown that the parasite's hollow, retractable feeding stylet traverses the plant cell wall through a "feeding plug" that remains after stylet withdrawal. The plasmalemma is pushed away from the wall by the stylet, the tip of which appears closely associated in the host cytoplasm with a "feeding tube" surrounded by rough endoplasmic reticulum. Feeding tubes are evident in the host cell after stylet withdrawal (Mankau and Linford 1960). Recent work on the ring nematode, *Criconemella xenoplax,* has shown that the stylet orifice is open to the host cytoplasm, with the plasmalemma connected to the stylet in a manner resembling a patch-clamp electrode (Hussey *et al.* 1992a). Callose is deposited between the stylet and the invaginated membrane (Hussey *et al.* 1992b) and is the only obvious sign of plant defense in the susceptible host.

Little is known of the molecular mechanisms underlying the induction and maintenance of giant cells and concomitant feeding by *Meloidogyne* spp. Giant cells are not transformed *per se;* their formation and maintenance require repeated stimulation from the nematode (Bird 1962). The nature of the stimulus is unknown, but possibly it involves a component or components of the material originating in the dorsal or subventral esophageal glands and secreted through the feeding stylet; disease-inducing secretion have been reviewed by Hussey (1989). These secretions may also contribute to the feeding plug or the feeding tube; it is not known whether these are of host or parasite origin. Similarly, the host target for the presumed nematode ligand or ligands is arcane, as is the manner in which this signal is transduced to elicit karyokinesis uncoupled from cytokinesis and subsequent giant cell differentiation.

Using a differential screening approach, Gurr *et al.* (1991) identified a gene in potato with expression "correlated with events in the immediate vicinity of" the potato cyst nematode, *Globodera rostochiensis,* but the nature of this gene was not revealed. By studying the expression of a GUS reporter fused to the promoter of the TobRB7 gene (Conkling *et al.* 1990), which encodes a presumed water channel expressed during early root development, Opperman *et al.* (1994) showed that transcription of this gene is reactivated in tobacco giant cells induced by *Meloidogyne incognita.*

To obtain a more comprehensive view of transcriptional changes associated with nematode infection, we used a subtractive approach to construct a cDNA library of transcripts exhibiting up-regulated expression in giant cells (Wilson *et al.* 1994). Briefly, cDNAs of giant cell transcripts were directionally cloned in a phagemid vector, and the resultant single-stranded recombinant DNA was annealed with driver cDNA from uninfected root tissue. Unannealed DNA was transformed into an *Escherichia coli* host, and 287 recombinants were recovered. By genomic Southern blotting, we confirmed that the clones were of plant (and not nematode) origin and demonstrated that our bank included the presumed tomato homologue of the TobRB7 gene. Because this gene is known to be expressed in giant cells but not in uninfected root cells at the same developmental age (Conkling *et al.* 1990; Opperman *et al.* 1994), its presence in our library is consistent with our clones encoding genes with authentically up-regulated expression in giant cells.

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Table 1. DNA sequence and RNA dot blot analysis of giant cell cDNA clones

Clone DB#	GenBank accession no.[a]	Identity[b]	Tissue source of RNA[c]					
			Gall	Driver[d]	Leaf	Hyp.[e]	Apex[f]	Root[g]
101	L24001	Pioneer	−	−	−	−	−	−
102	L24002	Pioneer	−	−	−	−	−	−
103	L23860	16-kD E_2 enzyme	−	−	−	+	+	−
104			+	−	−	+	+	+
113	L24003	Pioneer	−	−	−	−	−	+
114	L24004	Pioneer	+	−	+	+	+	−
115	L24005	Pioneer	+	−	+	+	+	−
117	L24006	Heptamer repeat	−	−	−	+	+	+
118	L24013	Pioneer	−	−	−	−	−	−
125	L24007	Pioneer	−	−	−	+	+	−
137	L24008	Pioneer	−	−	−	+	+	−
139	L24010	Pioneer	+	−	+	−	−	+
140	L24014	Pioneer	+	−	+	−	−	+
141	L24009	Pioneer	+	−	−	+	+	−
142	L24011	Anti-Ef-3	−	−	−	−	+	−
149	L24012	Pioneer						
161	L24015	Pioneer	−	−	−	−	−	−
163	L24017	16-kD E_2 enzyme	−	−	−	+	+	−
164	L24016	Pioneer	−	−	−	−	−	+
165	L24018	Pioneer	−	−	+	−	−	−
166	L24019	Pioneer	−	−	+	−	+	−
173	L26982	Pioneer	+	+	+	−	−	+
197	L24020	Pioneer	−	−	−	−	−	−
198	L24021	Pioneer						
199	L26983	Anti-Ala tRNA synthase	−	−	−	−	+	−
201	L24022	Pioneer						
203	L24023	Pioneer	−	−	−	−	−	−
205	L24024	Pioneer	−	−	−	−	+	−
207	L24025	Pioneer	−	−	−	−	−	−
208	L24060	Pioneer	−	−	−	−	−	−
209	L24026	Pioneer						
210	L24027	Pioneer	−	−	−	−	+	−
212	L24028	Pioneer	−	−	−	−	+	−
214	L24029	Pioneer	−	−	−	−	−	−
215	L24061	Zn-finger domain	−	−	−	−	+	−
216	L24030	Pioneer	−	−	−	−	−	−
217	L24031	Laminin B receptor	+	−	+	−	−	+
218	L24032	Pioneer						
220	L24033	Anti-PEP-carboxylase	−	−	−	−	−	−
221	L24054	Pioneer	−	−	+	−	−	−
222	L24055	Pioneer	−	−	−	−	−	−
223	L24056	Pioneer	−	−	−	−	−	−
224	L24057	Pioneer	−	−	−	−	−	−
226	L24058	Proton ATPase	−	−	−	+	−	−
239	L24059	eF IIe 5′ untranslated region	−	−	−	+	+	−
240	L24062	Pioneer	−	−	−	+	+	−
241	L24063	Pioneer						
244	L24064	Pioneer	−	−	+	−	+	−
263	L24065	Pioneer	−	−	−	+	+	+
264	L24066	Pioneer	−	−	−	+	+	−
265	L24067	Anti-Tnt1-94 transposon	−	−	−	+	+	+
266	L24068	Pioneer						
275	L24069	Pioneer	−	−	−	+	+	−
279	L24110	Pioneer	−	−	−	−	−	+
280	L24111	Myb DNA-binding site	−	−	−	+	+	−
288	L24107	Pioneer						
289	L24108	Pioneer						
291	L24109	Pioneer						

[a] No entry indicates that the clone was unsequenceable.
[b] Putative identity of partial cDNA clones based on DNA or deduced amino acid homology with sequences in GenBank or PIR. Criteria for assigning identity are discussed in the text. Clones with no meaningful homology are termed pioneers. No entry indicates that the clone was unsequenceable.
[c] RNA samples from the tissues indicated were probed with antisense probes from the cDNA clones: + indicates a signal was detected; − indicates no signal was detected; no entry indicates that the clone was not tested.
[d] RNA isolated from cultured uninfected roots and used as driver for the subtractive cloning.
[e] Hypocotyls.
[f] Cotyledons plus apex.
[g] Primary root from young seedlings.

Here we present an analysis of clones in this bank. As was previously speculated (Bird 1992), we found that genes expressed in giant cells include genes normally expressed only in nonroot tissues, including leaf and apex tissues. On the basis of partial DNA sequences, tentative identity can be assigned to some of these transcripts.

RESULTS AND DISCUSSION

Expression profiles.

Dot blots of RNA samples isolated from tomato tissues (galls and whole mature roots from tissue culture, mature leaves from greenhouse-grown plants, and cotyledons, hypocotyls, and roots from 1-wk-old seedlings) were hybridized with probes synthesized from randomly selected giant cell cDNA clones. The presence of equal amounts of target RNA in each dot was confirmed by probing with ribosomal sequences (not shown). Results of 48 experiments are summarized in Table 1. Fourteen probes failed to produce a detectable hybridization signal to any of the RNA samples, suggesting that these cDNAs represent low abundance messages. The remaining cDNA clones hybridized to various subsets of the RNA samples. Representative profiles are shown in Figure 1. Note that the exposure times for the different filters varied significantly. This variation is indicative of differences in transcript abundance, because the specific activities and amount of probe used in each experiment was essentially the same.

Only one clone (DB#173) produced a signal in the RNA from mature roots. This is important because mature root tissue was the source of the RNA used as driver in the construction of the subtractive library, and it provides strong evidence that the subtraction was effective. Eleven of the cDNA clones detected transcripts in seedling root RNA (Table 1) and presumably encode functions associated with young, expanding roots. Three of these appeared to be root-specific. In a survey of randomly chosen cloned root mRNAs, Evans *et al.* (1988) were unable to identify any as being root-specific, suggesting that this class of message might be rare in healthy roots. It will be interesting to determine the function of these transcripts in our bank.

Despite the fact that only cDNA synthesized from giant cell mRNA was exposed to the vector (Wilson *et al.* 1994), thereby ensuring that all clones in our bank must encode giant cell transcripts, most probes also failed to detect transcripts in gall RNA. Although this is partly due to the small size of the giant cells in relation to the total mass of the gall (it was not technically feasible to collect the numbers of giant cells required for isolation of sufficient RNA for blot analysis), it also suggests that the clones in our bank do not encode abundant transcripts. Ultimately, the (elevated) presence in giant cells of transcripts defined by each clone in our bank will need to be confirmed. To date we have tested only the DB#103 gene by *in situ* hybridization; its transcripts clearly are present in giant cells but are absent from adjacent cells (D. McK. Bird and M. A. Wilson, unpublished).

Although different clones reveal a range of expression patterns, many detected transcripts in RNA isolated from hypocotyls or from cotyledon plus apex tissue, generally at levels much higher than in uninfected seedling root tissue. Ten cDNA clones detected a signal in leaf RNA. Overall, these results give a picture of giant cells sharing transcripts with actively dividing and expanding tissues and also nonroot tissues.

DNA sequencing.

Partial cDNA sequence of 57 cDNA clones is presented in Table 1 as GenBank accession numbers (Bilofsky and Burks

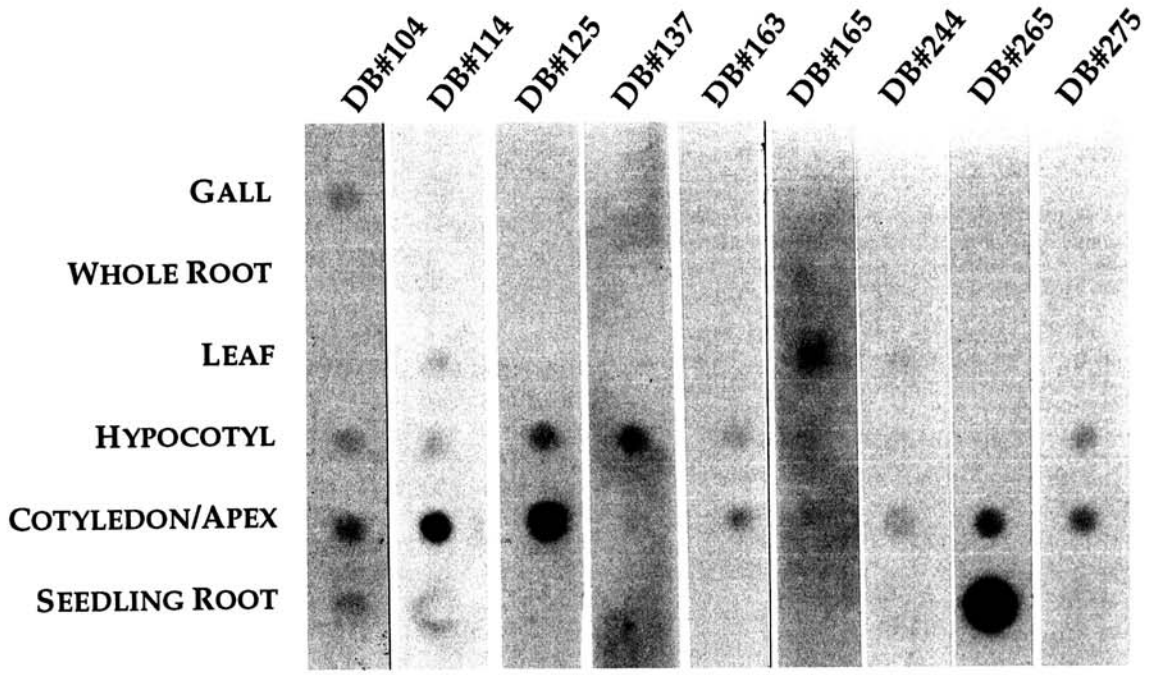

Fig. 1. RNA dot blot analysis of giant cell subtracted cDNA clones. Total RNA (5 μg) isolated from various tomato tissues was blotted onto nitrocellulose and hybridized with primer-extended ^{32}P-labeled antisense probes. Exposure times: DB#114, 35 min; DB#104, 3.5 hr; DB#125, DB#137, and DB#265, 20 hr; DB#244 and DB#275, 66 hr; DB#163 and DB#165, 112 hr.

1988). Reflective of the directional nature of the cloning, each sequence began with a poly-T tract (ranging in length from four to 75 residues), corresponding to the poly-A tail of the transcript. Each sequence was read only as far as the first ambiguity, including potential compressions. Thus, although the sequences represent readings from one strand only, we believe the degree of accuracy to be high. The presence of a long oligomeric tail rendered some clones unsequenceable. Four independent rRNA clones from the same bank were also determined. The sequences (not shown) corresponded exactly to those published for tomato (Kiss *et al.* 1989). This also suggests that the number of artifacts introduced by polymerase chain reaction in our bank might be low.

Sequences were compared to others in public-domain DNA and protein data banks by means of the BlastN and BlastX algorithms (Altschul *et al.* 1990). With some exceptions noted below, only database matches involving homology with the correct strand and in the correct part of the matching gene (i.e., the 3′ end) were considered, and a score of at least 100 was chosen as indicating a potentially valid homology. Most of the cDNA sequences failed these tests; they are listed as "pioneers" in Table 1. However, some homologies are striking and have a high degree of likelihood of being valid. By isolating and characterizing full-length cDNA clones, including *in situ* localization to giant cells, the identity of the DB#103 transcript was confirmed as encoding an E_2 enzyme,

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 K PSSDS
YK PSYDNS
YKKPSYDSG
YK PSYDNG
YKKPSYDSG
YK PSYDS
YK PSYDN
YK PSYDS
YK PSYDN
YK PSYDRL*

TS PSYSP
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Fig. 2. Carboxyl terminus domain of the deduced DB#117 gene product, aligned to show the heptamer repeat. The asterisk represents the carboxyl terminus. In bold below is the canonical heptamer repeat for the carboxyl terminus domain of the largest subunit of RNA polymerase II.

a key component of the protein ubiquitination pathway (Bird and Wilson, unpublished). The DB#163 cDNA is identical with the DB#103 sequence but has an additional 19 residues immediately before the poly-A tail. Because the gene encoding these transcripts, *LeUBC10,* appears to be unique, the different 3′ ends might arise by differential RNA processing.

Computer translation of the DB#117 cDNA revealed a hypothetical protein with multiple contiguous repeats of a seven amino acid motif, shown aligned in Figure 2, and terminating with a stop codon 47 bp 5′ from the poly-A tail. This motif is strikingly similar to the motif TSPSYSP, a structure diagnostic for the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II. *Arabidopsis* has 40 copies of this repeat (Dietrich *et al.* 1990), and *C. elegans* has 42 (Bird and Riddle 1989). Although not previously cloned from tomato, it seems likely that DB#117 encodes this gene. It is not surprising that an RNA polymerase II subunit might be up-regulated in giant cells.

The conservative substitution of lysine for serine has been observed (although not to this degree) at certain positions in the *Arabidopsis* and soybean proteins (Dietrich *et al.* 1990). The aspartic acid to serine also is conservative, but the tyrosine to threonine is novel. These changes certainly would alter the degree of phosphorylation and shape of the CTD and thus presumably its function. It is significant to note that *Arabidopsis* employs alternate splicing to generate multiple CTDs, and soybean actually has multiple genes encoding this protein. Southern blotting (not shown) suggests that the DB#117 region is unique.

BlastX analysis revealed significant homologies between the inferred DB#280 product and different members of the *myb* gene family (Fig. 3). Homology is highest with the DNA binding domain of the petunia *myb Ph3* gene product, although multiple alignment with Mybs from other plants and vertebrates revealed that this homology extends further. As nuclear transcription regulators, members of the Myb family play pivotal roles in the regulation of cellular proliferation (Calabretta and Nicolaides 1992). Through interactions with other trans-activators (e.g., members of the Ets family) Myb proteins effect growth control and oncogenesis (Wasylyk *et al.* 1993).

The *Arabidopsis* gene *GL1,* required for the initiation of trichome development, encodes a Myb-type protein (Marks *et al.* 1991). It is likely that other plant developmental processes are mediated by Myb; it is an intriguing possibility that giant

DB#280:		**QLQAKHGNKWRKIAAEVPGRTAKRLGKWWEVSKRRQQREQ**
Petunia Ph3	[Z13998]	+L AK GNKW ++AA++PGRT + +W +R+QR
Moss	[S24244]	L A GN+W +IAA++PGRT + +W +++ R Q
Snapdragon 315	[JQ0961]	+L A GN+W KIA +PGRT + +W +++
Barley HV33	[P20027]	GN+W +IA+ +PGRT + +W +++ R+Q
Snapdragon 308	[JQ0960]	+L + GNKW IA +PGRT + +W RR+
***Arabidopsis* MYB1**	[D10936]	A HGNKW IA +PGRT + W + RR+
Maize Zm.P1	[P27898]	+L A GN+W IA+ +PGRT + +W RQ
Drosophila	[P04197]	Q + GN+W KIA +PGRT + W + RR+
Human	[M13666]	Q + GN+W +IA +PGRT + W + RR+ ++

Fig. 3. The DB#280 gene encodes a Myb-like DNA binding domain. The deduced DB#280 gene sequence (bold) aligned with Myb sequences from petunia (Avila *et al.* 1993), moss (Leech *et al.* 1993), snapdragon 315 and 308 genes (Jackson *et al.* 1991), barley (Marocco *et al.* 1989), *Arabidopsis* (Shinozaki *et al.* 1992), maize (Grotewold *et al.* 1991), *Drosophila* (Slamon *et al.* 1986), and human (Katzen *et al.* 1985). Database accession numbers are in brackets. Identical amino acids are indicated; + represents conservative substitutions. None of the sequences have gaps.

cell formation is one of these processes, involving either transcriptional activation or repression. In *Arabidopsis* (Marks *et al.* 1991) and in maize and barley (Marocco *et al.* 1989), the *myb* genes exist in a multigene family. The part of *myb* spanned by DB#280 appears unique in the tomato genome (not shown).

A BlastN search revealed striking homology between the DB#226 sequence and the 3′ end of the *pma4*-encoded isoform (Moriau *et al.* 1993) of a plasmalemma H^+-ATPase from *Nicotiana plumbaginifolia* (Fig. 4). The DB#226 gene appears to be different from those encoding two previously cloned tomato isoforms (Ewing *et al.* 1990). The high degree of homology between the 3′ untranslated regions (UTRs) of these genes (the tobacco stop codon, and presumably also the DB#226 terminator, is a further 60 residues 5′) might indicate a biological role for this part of the sequence.

On the basis of measurements of transmembrane potentials following various chemical treatments, it has been postulated that giant cells possess proton efflux pumps (Jones *et al.* 1975). Using an *in vivo* fluorescence assay, Dorhout *et al.* (1992) demonstrated ATP-dependent acidulation of the walls of giant cells, but not of other cells at the infection site. It seems likely that the DB#226 gene product is responsible for this activity. A range of functions have been ascribed to proton-ATPases. Changes in intracellular pH can have global influences on physiological functions, including regulation of transcription and translation. Proton-ATPases clearly play a major role in cells undergoing intense solute transport; it has also been proposed that they mediate acidification of the cell wall to facilitate cell expansion (Serrano 1989). Cell expansion and solute flux clearly are features of giant cells.

Other sequences in the giant cell cDNA bank, including a number of the pioneer clones, encode recognizable structural motifs. For example, part of the DB#215 sequence specifies a zinc finger domain. Additional homologies have been observed in the UTRs of some genes. The 3′ UTR of DB#239 contains a $(GCC)_5$ element previously noted in the 5′ UTR of human general transcription factor TFIIE (Sumimoto *et al.* 1991); sequences such as these may play a role in translational regulation.

Four clones in the cDNA bank (DB#142, DB#199, DB#220, and DB#265) have high degrees of homology with sequences in GenBank (Blast scores of 246, 499, 806, and 173, respectively) but to the antisense strand. These sequences terminate with a poly-A tail, suggesting that they do not represent artifactually cloned sequences. Significantly, when used as probes in the dot blot assay, DB#142, DB#199, and DB#265 detected transcripts in plant tissues (Table 1). The DB#265 transcript, which encodes a sequence with antisense homology to the tobacco retrovirus-like transposon Tnt 1-94 (Grandbastien *et al.* 1989), and also the *Arabidopsis* copia-like element (Voytas and Ausubel 1989) appear to be particularly abundant in seedling root tissue (Fig. 1). It is an intriguing possibility that these antisense transcripts play a role in giant cells, and perhaps also in normal plant cells. Further analysis of these and other genes with up-regulated expression in giant cells will likely prove interesting.

MATERIALS AND METHODS

Construction of a subtracted giant cell cDNA bank has been described elsewhere (Wilson *et al.* 1994). Inserts were oriented such that single-stranded DNA produced following rescue with M13K07 correspond to the message-sense strand. ssDNA template was annealed with universal primer, and half of each reaction subjected to sequencing by the dideoxy chain termination method (Sanger *et al.* 1977). DNA sequences were manipulated with GCG software (Devereux *et al.* 1984) and analyzed by Blast algorithms (Altschul *et al.* 1990).

Total RNA was isolated from plant tissues by the guanidinium/acid phenol method (Chomczynski and Sacchi 1987), and 5-μg aliquots were heat-denatured and dot-blotted onto nitrocellulose membranes. Hybridization probes were prepared by primer extension of half of the annealed sequencing DNA template in the presence of α-[^{32}P]-dCTP. The specific activity of these probes typically was 2×10^9 dpm/μg. Filters were probed under standard conditions (Wahl *et al.* 1979).

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Tobacco:  TGACCAGATAAAAACAAATTTGTCTGATAAAGGGGGAAAACTTTTATCTTCTGTGATCTTCCCCCCATC...
DB#226:   ATAAAACAAAAAAACATGTCTTTTAATATAAAGGGGGAAAACATTGTCTTGTGTTGTCTTTCCCCCCTCTCA

Tobacco:  TATTTTTACTTAACTTCGTTATGTATTTTGATTTTGAAGCGCCGCCCATTGAAAGGGAGGGATTGTGTCCAA
DB#226:   TCTATTTCTTTAACTTCGTTATG...TTATATCTTGAAGCACCACCCATTGAA....AGGGATTGTGTCCAT

Tobacco:  GTTTTTTCCAACATTTTAATGGTGAAGTGACATCTCCTTGTAACAACACTACTACTCTTCCAAATTCACCTC
DB#226:   GTTTTTTCCAAAATTTTAATGGTGAAGTGACATTTCCTTGTAACAACAGT..TTTTCTTACAATTTCTTCAC

Tobacco:  CTCTTTCTTTTTTCCTTGTTTTTCATTTGATGAGN(24)-polyA
DB#226:   CTCTTTTTTTCTTTCTT-polyA
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Fig. 4. Alignment of the DB#226 sequence with the 3′ untranslated region of the *pma-4* gene from tobacco (*Nicotiana plumbaginifolia*) (Moriau *et al.* 1993), which encodes a plasmalemma proton ATPase.

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