A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass

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A novel stylar-specific glycosylated protein, sp41, was characterized. Sp41 constitutes >12% of the transmitting tract tissue soluble proteins and is mainly localized in the extracellular matrix. Two cDNA clones corresponding to sp41 mRNA were isolated and sequenced. The deduced sequences are, respectively, 80% and 49% homologous to acidic and basic pathogen-induced (1→3)-β-glucanases of the leaf. Thus a subfamily of (1→3)-β-glucanase pathogenesis-related (PR) proteins constitutes one of the major stylar matrix proteins. The accumulation of sp41 transcripts in normally developing and elicitor-treated styles and leaves was followed using an RNase protection assay. During development sp41 transcript accumulation starts well after carpel differentiation. It is first detected in styles at 8 days before anthesis. The maximal level of accumulation is reached during anthesis. Elicitor-treated styles do not accumulate the leaf-type (1→3)-β-glucanase transcript, although they retain the capacity to synthesize leaf-type pathogenesis-related proteins such as the pathogen-induced acidic chitinase. The developmental regulation of sp41 expression points to a role for them in the normal processes of flowering and reproductive physiology.

Key words: glucanase/glycosylated polypeptide/pathogenesis-related/style cDNA

Introduction

The secretory matrix of the stylar transmitting tract of angiosperms has been described as a nutrient medium that pollen tubes traverse on their way towards the ovules. In closed-type styles, the elongated sausage-like cells of the tract secrete a proteinacious fluid which forms a matrix. The matrix is further surrounded by a cortex of less permeable cells. The resulting structure could act as a pollen tube guide. A recent development, however, indicates that a passive role for the style in pollen tube growth is simplistic. It has been shown that inert latex particles move, in a directed manner, towards the ovules through transmitting tract matrix in the gynoecia of widely divergent species (Sanders and Lord, 1989). The translocation rate of the beads through the tract tissue was, in fact, similar to those of growing pollen tubes. The agent responsible for bead movement in the style is not known. However, movement of cells during embryogenesis in animals, is thought to be facilitated, at least in part, by an extracellular matrix composed of glycoproteins and proteoglycans provided by the surrounding cells (Newman et al., 1985; Dufour et al., 1988). Sanders and Lord (1989) suggest that stylar matrix proteins may play a similar role in bead movement.

The S-glycoproteins are the only molecularly characterized matrix proteins to date and they are limited to those species that exhibit gametophyte self-incompatibility (Cornish et al., 1988; Broothaerts et al., 1989). Attempts to identify additional biochemical components of the style matrix have revealed a complex array of arabinogalactan proteoglycans (Clarke et al., 1979; Fincher et al., 1983), glycoproteins and lipopolysaccharides (Heslop-Harrison, 1983). During an investigation of pathogenesis-related (PR) proteins in tobacco plants, we found that PR protein expression was regulated in a developmental and spatially unique manner in flower parts (Lotan et al., 1989). Antiserum to PR-2,N,O, which are (1→3)-β-glucanases that accumulate in pathogen stressed leaves (Kaufmann et al., 1987), reacted with a 41 kd stylar glycosylated polypeptide (sp41). Here we characterize sp41 and find it to be a heavily glycosylated (1→3)-β-glucanase, comprising >12% of the total soluble transmitting tract proteins and 20% of the total soluble matrix proteins. The massive extracellular accumulation of sp41 is suggested to be a significant factor in the physiochemical properties of the transmitting tract matrix.

Results

Purification of sp41 and partial polypeptide sequencing

Based on the physical properties of sp41 a procedure for the purification of the protein was established and is detailed in Materials and methods. Figure 1A illustrates the results of the major isolation steps, ion-exchange chromatography (Figure 1A, lane 2) followed by Con A affinity chromatography (Figure 1A, lane 3). Reverse-phase chromatography of the Con A bound fractions yielded one major protein peak that was submitted to Edman degradation and found to be blocked at the N-terminus. Therefore, the polypeptide was cleaved by CNBr, and the products were refractionated on a hydrophobic column and sequenced. The sequences obtained from two of the fragments were MENVYNAL and MRYN (M is conjectured). By comparison with partial sequence data available on PR proteins from tobacco leaves (Van den Bulcke et al., 1989), sp41 was tentatively identified as being closely related to the leaf acidic-type glucanases.

Sp41 is a major stylar polypeptide localized chiefly in the extracellular matrix

The availability of purified sp41 polypeptide enabled direct measurement of the relative proportion of sp41 among the total style proteins. Styles were microdissected under a dissecting microscope and transmitting tract tissue was removed. The soluble extracts of this tissue were compared immunologically with various amounts of purified sp41
Sp41 is encoded by two distinct mRNAs

Oligodeoxynucleotides were used to screen a stylar-specific cDNA library in order further to characterize sp41 as to amino acid sequence. Probes were prepared which are homologous to the partial amino acid sequence determined for sp41, and to the sequence of areas around putative active sites described for glucanases (Fincher et al., 1986). Fifteen hybridizing plaques were identified, subcloned and either partially or fully sequenced. Two similar but non-identical groups of clones emerged. The existence of two classes suggests a gene family expressing two transcripts represented by the cDNAs sp41a and sp41b (Figure 3A). At the nucleic acid level the transcripts show 94% identity in the coding region, while at the amino acid level they exhibit 95% identity and 97% similarity (i.e. conservative amino acid differences). The partial amino acid sequences determined above for the sp41 CNBr fragments completely matched the decoded sequences of sp41a cDNA. There are three and four potential glycosylation sites (Kornfeld and Kornfeld, 1985) in sp41b and sp41a, respectively. This is consistent with the multiple SDS–PAGE bands detected after partial digest of sp41 with endoglycosidase F (Lotan et al., 1989). The glycosylated nature of sp41 does not seem to influence the biochemical activity of the polypeptide, which we have established as an endo-type glucanase (E.C. 3.2.1.39; N.Ori, unpublished).

A hydrophathy plot of the deduced amino acid sequence of sp41a cDNA (Figure 3B) shows an amino terminus containing a very hydrophobic central core, characteristic of a signal peptide (Von Heijne, 1985). We applied the rules for protein processing of putative signal peptides and deduced the presence of a cleavage site between amino acids alanine 33 and glutamine 34. Cleavage at this point results in colinearity with the vacuolar (1–3)-β-glucanase form (Figure 4). It is also consistent with the finding that the mature sp41 protein was N-terminally blocked. N-terminal glutamines are often blocked by enzymatic or spontaneous conversion to a non-cleavable pyroglutamyl residue (Abraham and Podel, 1981). Sp41a and sp41b cDNA contain unusually long 5′ untranslated sequences of at least 162 and 143 bases, respectively (Figure 3A). Among possible secondary structures of these leader sequences are hairpin structures of −10.3 and −8.9 kcal respectively. This element and an AUG codon found at position 6 (in sp41a), which is followed by a translational stop three codons downstream, is rarely found in eukaryotic leader sequences and could indicate a role in regulation of sp41 translation.

Sequence comparison between sp41 and other (1–3)-β-glucanases are provided in Figure 4. The sp41 sequence shows a 47% identity with the monocot (1–3,1–4)-β-glucanase, 47% with the monocot (1–3)-β-glucanase and 49% identity with the dicot basic, vacuolar, (1–3)-β-glucanase. Comparison with the dicot acidic extracellular glucanases shows two levels of homology: 40% to PR(35)
and a higher homology of ~82% to PR(36) and PR(37). The strong similarity to the latter PR proteins agrees with the serological cross-reactivity of sp41 with the acidic pathogen-induced glucanases (Lotan et al., 1989).

**RNase protection assay to detect expression of (1→3)-β-glucanase transcripts**

Expression of stylar transcripts were studied using a 32P-radiolabelled transcript complementary to stylar glucanase mRNA. The radiolabelled probe was synthesized in vitro using the sp41b cDNA clone as template. The probe (Figure 5A, lane 1) consisted of a 379 base antisense fragment of the 3' end of the cDNA from the AvaII site at position 912 and an additional 60 bases of vector poly linker. It is thus expected that the 439 base long probe will protect 379 bases when hybridized to the cDNA that was digested with EcoRI, which removed the poly linker region (Figure 5A and B, lane 2). We also would expect to observe a fragment of this size when the antisense probe is hybridized to its in vivo cognate transcript. Indeed a 379 base long protected fragment was seen when the probe was hybridized to total stylar RNA (Figure 5B, lane 1). In contrast, when the sp41b probe was hybridized to sp41a cDNA digested with EcoRI, four protected fragments were obtained of 300, 190, 140 and 120 bases (Figure 5B, lane 3). These fragments presumably resulted from full or partial RNase digestion at non-homologous regions. Identical size fragments are observed when the probe is hybridized to total stylar RNA (Figure 5B, lane 1). The relative intensity of the fragments in this case differs from that obtained with cDNA (Figure 5B, lanes 2 and 3). This is probably due to the differences in intrinsic duplex stability between RNA–RNA and RNA–DNA hybrids, which could modulate the RNase digestion reaction kinetics. As all protected fragment sizes are accounted for by the two clones they probably represent the major, if not only, expressed glucanase transcripts in the style. This observation is consistent with the fact that only two different cDNAs were isolated.

The RNase protection assay, as carried out, was sensitive to the concentration of substrate RNA. This is seen by comparing lanes 3–5 in Figure 5A. Densitometric scanning of the data in Figure 5 shows that the 300 base fragment is present at 1.5 times the intensity of the 379 base fragment. After adjusting for the size dependent intensity difference, due to the use of a uniformly labelled probe, we conclude that in total stylar RNA the transcripts corresponding to sp41a cDNA are 1.9 times more abundant than those of sp41b.

**Developmental dependence of sp41 transcript and polypeptide expression**

The induction of stylar (1→3)-β-glucanase mRNA accumulation in developing styles was examined using the RNase protection assay. As shown in Figure 6, (1→3)-β-glucanase mRNA was readily detected in styles of ~5 mm length, 6 days before anthesis (Figure 6, lane –6). However, transcripts were barely detectable (even in overexposed gels) when styles are ~1 mm size, 8 days before anthesis (Figure 6, micrograph and lane –8). In newly differentiated flower buds, which contain pistil primordia, no transcript was
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Amino acid and nucleotide sequencing was performed to establish the original protein sequence for the sp4la gene (Nori et al., 1998). The amino acid sequence of sp4la is shown in Fig. 3. The amino acid sequence was determined by automated Edman degradation of the polypeptide. The amino acid sequence was then compared to the nucleotide sequence of sp4la. The results of the comparison are shown in Table 1. The amino acid sequence of sp4la is highly conserved, with only a few amino acid changes.

The hydrophobicity of the sp4la polypeptide was also determined. The hydrophobicity plot is shown in Fig. 3B. The hydrophobicity of the sp4la polypeptide is low, indicating that it is primarily hydrophilic.

The results of the amino acid and nucleotide sequencing and hydrophobicity analysis of sp4la indicate that the gene is likely to encode a hydrophilic protein. The protein may play a role in the defense of tobacco against pathogens.

TMV and elicitor induced accumulation of (1-3)-β-glucanase transcripts in the leaf and style

The data in Fig. 4 indicates that the two cDNAs isolated from sp4la are closely related to the pathogen-activated acidic-form (1-3)-β-glucanases found in leaves. Therefore, we expected the sp4la probe to detect those transcripts. When the probe was hybridized to RNA extracted from non-infected leaves no transcript was detected (Fig. 8A, lane 4), but two protected fragments of 230 and 240 bases were seen after inoculation with TMV (Fig. 8A, lane 5). The transcripts probably represent the leaf pathogen-induced acidic (1-3)-β-glucanases. In contrast, transcripts of style-form (1-3)-β-glucanases were not detected in leaves. However, when the gel was exposed for a longer period, fainter bands that comigrated with style-form protected fragments, were visible in both infected and non-infected leaves (Fig. 8B, arrows). Analysis of the signal intensity of the protected fragments in the different lanes revealed that the amount of style transcript in leaves is constitutively expressed at a level that is at least 500 times lower than that found in styles.

Pathogenesis-related (PR) proteins can be induced in tobacco leaves by a variety of pathogens and abiotic elicitors (Van Loon, 1985; Bol and Van Kan, 1988; Lotan and Fluur, 1990). We have shown that PR proteins accumulate in flowers in a developmentally specific manner, yet retain their responsiveness to elicitors of pathogenesis-related proteins. For example, when the elicitor tunicamycin was applied to styles, PR proteins such as PR-1, chitinase and (1-3)-β-glucanase were detected on immunoblots (Lotan et al., 1989). We therefore examined tunicamycin-treated leaves and styles at the protein and transcript level. Tunicamycin elicited the chitinase PR proteins in tobacco leaves as well as in styles (Figure 9). Since the same treatment elicited leaf-type (1-3)-β-glucanase transcripts (Figure 8A, lane 6) and proteins (Figure 7, lane L) in leaves, we expected to find leaf specific (1-3)-β-glucanase transcripts in elicitor-treated styles. However, only sp4la transcripts appeared, (Figure 8A, lane 3). Thus, despite the elicitor-induced accumulation of
chitinase and PR-1 in styles (Figure 9 and Lotan et al., 1989), the leaf specific glucanase transcripts do not accumulate in this tissue.

Discussion

The composite data regarding (1-3)-β-glucanase genes in tobacco (Kauffman et al., 1987; Van Loon et al., 1987; Shinshi et al., 1988; Van den Bulcke et al., 1989, and this work) depict a multi-genic family that contains at least three subgroups, each of which includes more than one gene. The groups are: 1, the leaf acidic polypeptides which are localized extracellularly and are induced by pathogens; 2, the leaf basic polypeptides that are located in the vacuoles and are maintained constitutively but are also induced to a higher level by pathogens; 3, the flower polypeptides, located mainly in the stylar matrix whose accumulation is developmentally regulated.

Comparison of the two sp41 cDNA clones with other tobacco (1-3)-β-glucanases and monocot (1-3, 1-4)-β-glucanases revealed a high level of sequence homology which indicates conservation in enzyme structure. At the functional level, substrate binding studies of cellulase from egg white lysozyme (Blake et al., 1967) have pointed to tryptophyl residues interacting with the glucan substrate. It can be seen that tryptophan residues at position 106 and 271 are conserved in both monocot and dicot (1-3)-β-glucanases as well as (1-3, 1-4)-β-glucanase (Figure 4). These residues may be candidates for hydrogen bond formation with the substrate. Also, based on crystallographic examination of egg white lysozyme, both glutamic and aspartic acid residues have been implicated in the hydrolysis of glycosidic bonds (Blake et al., 1967), observations that have been extended to β-glucan endohydrolases as well (Hej et al., 1989b).

Fig. 5. RNase protection assay of style RNA. The 32P-labelled 439 base antisense fragment of RNA transcribed from sp41b cDNA was used as probe in each case. (A) Lanes: M, radiolabelled DNA mol. wt markers; lane 1, non-digested probe subjected to the full procedure except for the RNase digestion step; lane 2, 0.1 µg of EcoRI-digested DNA of sp41b cDNA clone; lanes 3, 4 and 5, 0.1 µg, 1 µg and 5 µg, respectively, of total RNA from styles. The autoradiograph was exposed for 1 h. (B) Lane 1, 5 µg of total RNA from styles, lane 2, 0.1 µg of EcoRI-digested DNA of sp41b cDNA clone; lane 3, 0.1 µg of EcoRI-digested DNA of sp41a cDNA clone. Autoradiograph was exposed for 6 h.
residues are found to be conserved near the carboxyl end of all the glucanases. Finally, the two types of glucanase classes in Figure 4 show substrate specificity for either (1-3)-linked or (1-3,1-4)-linked β-glucan. In a few locations marked by asterisks in Figure 4, we observe that the (1-3,1-4)-β-glucanase differs in residues found to be conserved in all (1-3)-β-glucanases. These positions should provide clues as to the evolution of the distinct substrate specificities of these closely related polypeptides.

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were shown to be more promiscuous in their expression. The Kunitz trypsin inhibitor genes are highly expressed during soybean embryogenesis and were detected at a 1000-fold lower level in leaves (Jofuku and Goldberg, 1989).

Patatin, the major storage protein present in potato tubers, was detected in leaves under certain physiological conditions (Rocha-Sosa et al., 1989). It will be of interest to subject other supposedly tissue-specific plant genes to the type of high-sensitivity transcript analysis used here.

The results shown in our study emphasize that different homologous genes, with distinct induction pathways, are involved in the (1→3)-β-glucanase pathogen response of the plant on the one hand (Lotan and Fluhr, 1990), and in the developmental appearance of (1→3)-β-glucanases in flowers on the other. They raise the question of whether other flower PR proteins, for example the endochitinase detected in ovaries and anthers (Lotan et al., 1989), are also diverged subclasses of their leaf homologues. The function of (1→3)-β-glucanases in the style is not known. If their function were solely in defence against pathogens we would expect concomitant appearance of the other PR proteins as is manifested in leaf defence. Despite their solitary appearance during development in the style a defence function may be pertinent. However, the highly glycosylated nature of the (1→3)-β-glucanases together with their massive accumulation in styles should have a dramatic influence on the physiochemical properties of the transmitting tract matrix.

If we add to these properties the possibility that the (1→3)-β-glucanase interact with constituents in the pollen tube, especially the pollen cell wall (1→3)-β-glucan (callose), then sp41 is an excellent candidate to facilitate phase matrix-driven translocation.

Materials and methods

Protein gels and immunoblotting

Tissue samples were ground in PBS buffer. Treatment of leaves or flowers with TMV or elicitors was as described by Lotan et al. (1989). Protein extracts were loaded on 12.5% denaturing SDS-PAGE (Laemmli, 1970).

The gels were transferred to nitrocellulose sheets and reacted with antibodies as described by Lotan et al. (1989). Gels were quantified by scanning with a 300A computing densitometer (Molecular Dynamics) or with a 620 video densitometer (BioRad).

Immunohistochemistry

Freshly cut styles before pollination were prefixed for 24 h in 3.5% glutaraldehyde, 3% paraformaldehyde, 2.5% dimethyl sulphoxide, 1% Acrolein (Fluka AG) in 0.1 M cacodylate buffer at pH 7.2. They were washed twice in the same buffer then infiltrated with 10% gelatin at 37°C for 30 min, cooled on ice for 5 min then crosslinked with 2% glutaraldehyde for 2 h. Ultracryomicrotomy (Tokuyasu, 1973), immunolabelling (Roth, 1983) and postembedding (Keller et al., 1984) were performed as follows: the plant tissue was immersed in a solution of 2.3 M sucrose, 150 mM NaCl, 3 mM KCl and 10 mM Na2HPO4, at pH 7.4 and subsequently frozen in liquid nitrogen and cut in a Reichardt ultratcut equipped with a FC-4 cryochamber. Affinity-purified primary antibody was prepared on a column containing immobilized sp41 protein according to Axén et al. (1967). Ultra thin sections were labelled with affinity-purified sp41 antibody, and sections were then treated with immunogold (15 nm) goat anti-rabbit IgG; electron micrographs were made with a Phillips 410 operated at 80 kV.

Purification of the 41 kd polypeptide and amino acid sequencing

Styles (7 g) were ground in liquid nitrogen with 0.5 g polyvinyl polypyrrolidone and 100 mg of ascorbic acid. The cold powder was mixed with 10 ml of 20 mM Tris pH 7.4, 1 mM diethiothreitol and 1 mM phenylmethylsulphonyl fluoride and stranded through four layers of gauze and two layers of Miracloth (Calbiochem). The style extract was centrifuged at 10 000 r.p.m. (Sorvall T-34) for 10 min. The supernatant was strained again through four layers of gauze and two layers of Miracloth, and loaded on a G-25 Sephadex column (2.5 cm × 30 cm, Pharmacia) pre-equilibrated with 20 mM Tris-HCl pH 8. The protein-containing fractions were collected (90 ml) and loaded on a 3 ml QAE A-25 (Pharmacia) anion exchange column pre-equilibrated with 20 mM Tris—HCl pH 8. The flow-through from the QAE A-25 column was concentrated 10-fold on an Amicon YM-10 membrane and the pH adjusted to 9.5 with 20 mM Na2HPO4. The concentrated (1 mg/ml protein) was loaded on an anion exchange column (Mono-Q, HR 5/5, Pharmacia) and fractionated using a NaCl gradient from 0 to 0.5 M, containing 20 mM piperazine. Enzymatically active fractions were collected and adjusted to 20 mM Tris—HCl pH 7.4, 100 mM NaCl, 1 mM MnCl2, 1 mM MgCl2 (Con A binding buffer). The combined fractions were applied to a FPLC Con A affinity column (Altech, Dur, 300 Angstrom, 50 × 4.6 mm). Adsorbed proteins were released in a gradient (0-0.2 M) of methyl-α-D-mannopyranoside in Con A buffer. The major protein fraction contained all the glucanase activity. For amino acid sequencing the purified enzyme was loaded on a hydrophobic Protein Plus HPLC column (4.6 mm × 25 cm, Zorrox biosizers, Dupont) and fractionated in an acetonitrile gradient containing 0.1% trifluoroacetic acid. The major peak was cleaved with cyanogen bromide according to Gross and Wiskop (1962) and peptides were separated on the Protein Prep HPLC column as detailed above. Amino acid sequencing was carried out on an Applied Biosystems model 475A.

Plant total and poly(A)+ RNA isolation and construction of cDNA libraries

Total RNA isolation was achieved using the phenol—SDS method (Ausubel et al., 1989). Poly(A)+ RNA was isolated from total plant RNA by using an oligo(dT) cellulose column (Biolabs), as described by Aviv and Leder (1972). First and second strand cDNAs were synthesized using the materials and protocols in the 'cDNA synthesis system plus' kit (Amersham). Two libraries were constructed: one was primed with oligo(dT) primer and the other with random primer. EcoRI methylase and EcoRI linkers (Biolabs) were ligated to the cDNA and digested according to Wu et al. (1987). cDNA was sized on agarose gels and ligated to lig11 dephosphorylated arms and packaged with packaging extract (Stratagene). cDNA libraries were screened with three oligodeoxynucleotides. The sequences of the oligonucleotides were: 5′GCA/AGTT/AGTTACA/C/GTAC/AGTG/TTC/TTCTCA3′ corresponding to the amino acid sequence MN/VYNAL, and 5′ACATGCGGAAATATGCTTTCC3′ and 5′GATGGCCAGCACCTCGGACA3′ corresponding to consensus regions established by comparison of published sequences of (1→3)-β-glucanases from the widely divergent species, tobacco and barley (Fincher et al., 1986). The cDNA clones isolated from the cDNA library were subcloned into BlueScript plasmid (Stratagene) at the EcoRI site. The plasmids were grown on bacterial strain XL1-blue (Stratagene). Double and single strand DNA was isolated according to Stratagene protocols. The sequence shown in Figure 3 was determined on both strands using 'Sequenase' (USB) enzymes and protocols.

Preparation of radioactive RNA probe

Sp41 in BlueScript vector was linearized by digestion with AvaII (439 bases probe). Proteinase K (5 µg, Boehringer) was added to the restriction reaction and incubated at 37°C for 30 min. The DNA was extracted twice with phenol/chloroform, precipitated and resuspended in water. Radiolabelled libraries were subcloned into BlueScript plasmid (Stratagene) at the EcoRI site. The plasmids were grown on bacterial strain X1L-blue (Stratagene). Double and single strand DNA was isolated according to Stratagene protocols. The sequence shown in Figure 3 was determined on both strands using 'Sequenase' (USB) enzymes and protocols.

Radioactive RNA probe (100 000 c.p.m.) was added to each RNA sample. The reaction was incubated and resuspended in 20 µl of hybridization buffer (80% formamide, 0.04 M PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA). Samples were denatured by 30 s incubation at 90°C. Hybridization was performed for 2 h at 50°C and then the temperature was lowered over a period of 5 h to room temperature. Non-hybridized probe was removed by addition of 30 µl of RNase A 5 µg/ml (I. A. 100 mM Tris pH 7.4, 40 µg/ml RNase A) and 1 µl of RNase T1 (USB, 0.7 mg/ml, 267 U/mg). The reactions were incubated at 33°C for 30 min,
then brought to 0.125 μg/ml proteinase K, 0.5% SDS and 20 μg/reaction glycogen, and further incubated at 37°C for 15 min. Following precipitation and washing as before, the protected fragments were resuspended in RNA loading buffer. The samples were heated to 90°C for 30 s and fractionated on a 4% or 6% denaturing polyacrylamide urea gel at 25 mA (not more than 1000 V). The gel was fixed in 10% acetic acid and 15% ethanol for 15 min, dried and exposed.

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References


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The nucleotide sequence data reported here will appear in the EMBL Nucleotide Sequence Database under the accession numbers X54430 (Sp41a) and X54431 (Sp41b).