Antiserum against a Stigma-exudate Protein of Tobacco, SE32, which was Identical with PPAL, a β-Expansin-like Protein Specific to Stigma, Cross-reacted with another Stigma-exudate Protein, SE35

Tsutomu Kuboyama*1, Kaoru T. Yoshida2) and Genkichi Takeda3)

1) Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
2) Laboratory of Conservation Ecology, Department of Ecosystem Studies, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
3) Laboratory of Plant Breeding, Faculty of Agriculture, Tamagawa University, Machida, Tokyo 194-8610, Japan

In the first step of pollination in tobacco, pollen grains attach to the stigma exudate, which we previously found to contain two major stigma-exudate proteins, SE32 (32 kDa) and SE35 (35 kDa) (Kuboyama et al. 1997). In the present study, we determined each N-terminal amino acid sequence. The N-terminal amino acid sequence of SE32 was identical to that of PPAL, a stigma specific protein which was similar to grass pollen allergens and β-expansin. However, no sequences identical to the N-terminal amino acid sequence of SE35 were found in the protein databases. An antiserum raised against SE32 was found to react with both SE32 and SE35 in SDS-gel blot analysis. SE32 was detected only in the stigma, while SE35 in both the stigma and the style. These two proteins were not detected in other floral or vegetative organs. Immunohistochemical analysis showed that the antigens of the anti-SE32 antiserum were localized in the extracellular space of transmitting tissue. Accumulation of SE32 started in the completely elongated bud and increased with flower maturation. However, accumulation of SE35 was already detected in the young bud. The antigenicity of SE35 to anti-SE32 antiserum showed structural similarity between SE32 and SE35, and SE35 might belong to the same protein family as SE32. SE32 and SE35 might soften transmitting tissue of the stigma and style, and they might play a role in the elongation of the transmitting-tissue cells or the penetration of the pollen tube into the tissue.

Key Words: Nicotiana tabacum, expansin, pollen-tube pathway, PPAL, stigma exudate, stigma secretion, transmitting tissue.

Introduction

In tobacco, the pollen grain germinates on the stigma exudate and the pollen tube elongates into the extracellular matrix (ECM) of the transmitting tissue. Cytology, biochemistry and observation of stigma-less plants have suggested that the stigma exudate and the ECM of the stylar transmitting tissue are important for proper elongation of pollen tubes (Cheung et al. 1995, Cheung 1995, Goldman et al. 1994). Several cDNAs for proteins that are secreted in the ECM of the pollen-tube pathway of tobacco have been cloned. Some ECM proteins belong to arabinogalactan proteins (AGPs) or extensin-like proteins. They are highly glycosylated and have a relatively large (> 45 kDa) molecular mass (for a review see Cheung and Wu 1999). Transmitting tissue-specific (TTS) protein, an AGP, has been reported to promote pollen-tube elongation (Cheung et al. 1995). Some other ECM proteins are defense-related proteins, such as β-1, 3-glucanase, proteinase inhibitor and thaumatin-like protein (Ori et al. 1990, Adkinson et al. 1993, Kuboyama 1998). In potato and tomato, basic chitinase is abundantly secreted in ECM of transmitting tissue (Wemmer et al. 1994, Harikrishna et al. 1996). In general, defense-related proteins are induced after infection with a pathogen, but these pistil-specific proteins are synthesized along with the organ development and pathogen infection is not necessary for their induction. A stigma-specific gene (STIG1) and a pistil-specific allergen-like protein (PPAL) genes, which do not belong to the above-mentioned categories, are specifically expressed in the stigma, but not in the style (Goldman et al. 1994, Feron et al. 1998). The DNA sequence of the STIG1 gene did not display any significant homology with any gene sequences in the database (Goldman et al. 1994). On the other hand, the PPAL gene shares homology to class I pollen allergens of grass pollen and to β-expansins (Feron et al. 1998).

We previously analyzed proteins of the stigma-exudate of tobacco by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and detected more than 10 polypeptides (Kuboyama et al. 1997). Among them, a 32 kDa protein (SE32) and a 35 kDa protein (SE35) had some charac-
teristics distinct from the others; e.g., they have almost the same isoelectric point (pI) and are the most acidic of the proteins detected by 2D PAGE (pI = 4.5). In addition, they did not bind to concanavalin A (Con A), although most of the stigma exudate proteins bind to Con A (Kuboyama et al. 1997). In the present study, we determined the N-terminal amino acid sequence of SE32 and SE35, and raised antiserum against SE32 to investigate its temporal and spatial distribution. The anti-SE32 antiserum unexpectedly cross-reacted with SE35. Therefore, we simultaneously investigated the distribution of SE35 to determine the relationship between SE32 and SE35.

Materials and Methods

Plant materials

The seed of *N. tabacum* L. cv Hicks 2, *N. rustica* L. cv Rustica, *N. repanda* Willdenow ex Lehmann, *N. trigonophylla* Dunal, *N. glauca* Graham, *N. longiflora* Cavanilles and *N. sylvestris* Spagazzini & Comes were provided by Japan Tobacco Inc., Iwata, Japan. Plants were grown in a greenhouse.

*N-terminal amino acid sequencing of SE32 and SE35*

The flowers of *N. tabacum* were emasculated one day before anthesis and used for collection of protein at anthesis. The stigmas and the most apical part of styles were dissected from the flowers, rapidly frozen in liquid N₂ and stored at −80°C until use. Protein extraction, and 2-D PAGE were performed as described previously for SE39b (Kuboyama et al. 1997), because SE32 and SE35 were in the same fraction as SE39b in the acetone precipitation. After 2D PAGE, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane filter (Millipore, Bedford, MA.) by the method of Hirano and Watanabe (1990) and detected by staining with Coomassie Brilliant Blue (CBB) (Hirano et al. 1991). The SE32 spot on the PVDF membrane was cut out and applied to a gas-phase protein sequencer (A491 model; Perkin Elmer, Foster City, CA).

*Protein gel blot analysis*

After 2D PAGE, the protein spot of SE32 was stained with CBB and cut out. Then, the protein was electroeluted from the gels as described by Hirano et al. (1991). The recovered protein was mixed with Freund’s complete adjuvant (WAKO, Osaka, Japan) and injected into a mouse to obtain antiserum. The protein in the stigma exudate was eluted as described previously (Kuboyama et al. 1997) and separated by SDS PAGE with 10% polyacrylamide gel and electrotransferred to PVDF membrane. For immunological detection, the membrane was incubated with a 1 : 500 dilution of the anti-SE32 antiserum and then with alkaline phosphatase-conjugated secondary antibodies against mouse IgG (Vector Laboratories, Burlingam, CA, USA) as described in the manufacturer’s instruction manual. For lectin blotting, the membrane was incubated with a peroxidase-conjugated lectin, and glycoproteins on the membrane were detected by enzymatic development of color as described by Kijimoto-Ochiai et al. (1985). Peroxidase-conjugated *Aleuria aurantia* lectin (AAL) and Con A were obtained from Honen Corporation Inc., Tokyo, Japan.

*Immunohistochemistry*

Tissue and cellular localization of SE32 and SE35 was investigated by immunohistochemistry. The stigma and apical part of the style were fixed in 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight at 4°C. For paraffin sectioning, tissues were dehydrated through a graded ethanol series, and then a n-butanol series (Sass 1958), and finally embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO). Microtome sections (10 μm thick) were placed on charged slide glasses (Fisher Scientific, Pittsburgh, PA). The sections were deparaffinized in xylene, and rehydrated through a graded ethanol series. For resin sectioning, tissues were dehydrated through an ethanol series and then embedded in 7100 resin (Kulzer, Wehrheim, Germany) according to the manufacturer’s instruction manual. The tissues were sectioned at 0.7 μm in thickness using an ultramicrotome. After being blocked, the deparaffinized and rehydrated sections or the resin sections were incubated with a 1 : 50 dilution of the anti-SE32 antiserum for an hour. After being washed, the sections were incubated with a 1 : 100 dilution of colloidal gold-coupled goat anti-mouse IgG secondary antibody (Vector Laboratories, Inc., Burlingame, CA). Colloidal gold on the sections was visualized by Silver Enhancing Kit for LM (British BioCell International, Cardiff, UK) according to the manufacturer’s instruction manual. After signal development, the sections were dehydrated through a graded ethanol series and then mounted in Eukitt (O. Kindler, Germany).

*Results*

*N-terminal amino acid sequencing of SE32 and SE35*

The N-terminal amino acid sequence of SE32 was analyzed as described in materials and methods and eight amino acid residues were determined as “AETDFKKAXA”. This sequence was coincident with the amino acid sequence of stigma-specific allergen-like protein of tobacco, PPAL (Feron et al. 1998). This suggests that SE32 is PPAL. The corresponding region was located from amino acid 40 to 48 of PPAL precursor protein that was deduced from the nucleotide sequence. Thus, first 39 amino acids should be cut off from precursor protein of PPAL to produce SE32. The N-terminal amino acid sequence of SE35 was also analyzed and 7 amino acid residues were determined as “ALFTLAN”. This amino acid sequence is not included in the PPAL precursor protein and we found no proteins with an identical sequence in the protein databases (SwissProt, Genes, PIR, PRF and PDBSTR).
Protein gel blot analysis using anti-SE32 antiserum and lectins

Antiserum raised against SE32 was used to investigate the temporal and spatial distribution of SE32. First, the specificity of the anti-SE32 antiserum was examined. The proteins of the stigma exudate were separated by SDS-PAGE, electrotransferred to PVDF membrane, incubated with the anti-SE32 antiserum and were detected with alkaliphosphatase-conjugated secondary antibodies. Not only a 32 kDa protein band but also a 35 kDa protein band was detected, and the other stigma-exudate proteins did not react with the antiserum (Fig. 1 lane 1 and 2). The stigma exudate proteins were also examined with lectin blotting. Although most of the stigma-exudate proteins reacted with AAL, the 32 kDa protein did not react with either Con A or AAL in the stigma exudate (Fig. 1, lane 3 and 4). Thus, SE32 does not seem to be glycosylated. A faint band was observed at 35 kDa when the membrane was incubated with AAL or Con A (Fig. 1, lane 3 and 4), but it was not clear whether SE35 was glycosylated or not.

The distribution and accumulation in the stigma and style of SE32 and SE35 in tobacco

The distribution of SE32 and SE35 in floral and vegetative tissues was investigated by immunoblot analysis (Fig. 2A). Both SE32 and SE35 were detected in the stigma exudate and the stigma (Fig. 2A lane 1 and lane 2), but only SE35 was detected in the style (Fig. 2A lane 3 and 4). This result showed that SE32 was specific to the stigma and not secreted from the style. Usually, SE32 was more abundant than SE35 in the stigma exudate (Fig. 1 lane 1). The results of immunoblot analysis also showed that the signal of SE32 was stronger than that of SE35 (Fig. 2A lane 1, 2B lane 8). However, in the stigma extract, the signal of SE35 was conversely stronger than that of SE32 (Fig. 2A lane 2, 2B lane 5, 6 and 7). These signal ratios indicate that more SE35 exists inside of the tissue than in the stigma exudate. Neither SE32 nor SE35 was detected in the ovary (Fig. 2A lane 5), anther (Fig. 2A lane 6), leaf (Fig. 2A lane 7) or root (Fig. 2A lane 8). The accumulation patterns of SE32 and SE35 in the stigma at various developmental stages were studied (Fig. 2B). SE32 was detected at stage 4 and increased during flower maturation, but SE32 was not detected at either stage 1, 2 or 3 (Fig. 2B lane 1 to lane 5). Stage 4 was coincident with the stage that the stigma starts secreting exudate. On the other hand, SE35 was already detected at stage 1 (Fig. 2B lane 1) and the content in the stigma did not increase

![Image of Protein gel blot analysis using anti-SE32 antiserum and lectins](image1)

![Image of The distribution and accumulation in the stigma and style of SE32 and SE35 in tobacco](image2)

Proteins were detected with anti-SE32 antiserum after SDS-PAGE and blotting. A: Tissue specificity of SE32. Lane 1, stigma exudate; lane 2, tip of stigma; lane 3, apical part of style; lane 4, middle part of style; lane 5, ovary; lane 6, anther; lane 7, leaf; lane 8, root. 16 µg protein was loaded in each lane, except lane 1. B: SE32 or SE35 at different developmental stages of the flower. Flower development was divided into five stages according to the difference between the height of the tips of the corolla and that of the bract. Stage 1, the tip of the corolla is approximately the same height as that of the bract; stage 2, the difference is less than 1 cm; stage 3, the difference is about 3 cm; stage 4, corolla is fully developed and the tip of the corolla is pink; stage 5, anthesis. Lane 1-5, each number indicates the stage of flower development. Lane 6, unpollinated flower one day after anthesis; lane 7, pollinated flower one day after anthesis; lane 8, proteins from stigma exudate. In each lane, except lane 8, 16 µg protein was loaded.
during flower development (Fig. 2B lane 1 to lane 5). Thus, SE32 and SE35 differently accumulated in flower development. There was no difference in the relative amount of SE32 to SE35 between unpollinated and pollinated flower (Fig. 2B lane 6 and lane 7), thus pollination would not affect the accumulation of these proteins.

We investigated various species of the genus *Nicotiana* for the presence or absence of SE32- and SE35-related proteins. Proteins reacting with SE32 antiserum were present in the stigma extract of all *Nicotiana* species so far examined (Fig. 3). A protein with a molecular mass from 34 kDa to 36 kDa protein was detected as a major band in each species tested (Fig. 3 from lane 3 to lane 8) and it was considered to be the counterpart of SE35. However, there were no other proteins that reacted with anti-SE32 antiserum in *N. repanda* (Fig. 3 lane 3), *N. rustica* (Fig. 3 lane 4), *N. trigonophylla* (Fig. 3 lane 5) and *N. sylvestris* (Fig. 3 lane 6). On the other hand, *N. glauca* and *N. longiflora* had extra protein bands that were smaller than the major one (Fig. 3 lane 7 and 8).

SE32 and SE35 were accumulated in the intercellular space of transmitting tissue

The tissue localization of antigen of anti-SE32 antiserum was examined. The cross section of the upper part of the style (just under the stigma) showed a reaction with the anti-SE32 antiserum and the signal was specifically localized in the transmitting tissue (Fig. 4A). Subcellular signal localization was investigated with the resin section. The signal was detected exclusively in the intercellular space (Fig. 4C).

**Discussion**

The N-terminal amino acid sequence of SE32 showed that SE32 is identical with PPAL (Feron et al. 1998). Feron et al. (1998) deduced the amino acid sequence of PPAL from the nucleotide sequence of cDNA and studied its expression pattern using an antibody against recombinant PPAL. Their anti-PPAL antibody specifically reacted with PPAL and there were no other proteins that cross-reacted with the antibody. On the other hand, anti-SE32 antiserum used in this study cross-reacted with the SE35. The antigen used for immunizing SE32 was protein eluted from the 2D PAGE gel. The difference of the method for antigen preparation might have caused the difference in specificity. The anti-SE32 antiserum reactivity to SE35 shows that SE32 and SE35 share some epitope and have structural similarity. SE35 has the same pI as SE32 and these proteins show the same brown color in silver stained 2D PAGE (Kuboyama et al. 1997). Thus, SE35 might belong to the same protein family as SE32. However, SE35 had an accumulation pattern different from SE32 and had a unique N-terminal amino acid sequence. Thus, SE32 and SE35 should be encoded by different genes and SE32 should not be produced from post translational processing of SE35.

All *Nicotiana* species used in this study had a protein that cross-reacted with anti-SE32 antiserum. Thus, SE32- or SE35-like proteins might have certain function and be advantageous to the plants under natural selection. *N. glauca* and *N. longiflora* had multiple bands and the smaller protein bands were considered to be SE32 counterparts. These two species are rich in stigma exudate. Probably, this is why the SE32 counterparts could be detected in these two species. The molecular masses of the SE32 counterparts of *N. glauca* and *N. longiflora* were different from SE32, and this indicates some polymorphism in SE32 among *Nicotiana* species.

![Fig. 3](image-url) Immunoblot showing the presence of SE32-like protein in various *Nicotiana* species. Lane 1, stigma exudate of *N. tabacum*; lane 2, *N. tabacum*; lane 3, *N. repanda*; lane 4, *N. rustica*; lane 5, *N. trigonophylla*; lane 6, *N. sylvestris*; lane 7, *N. glauca*; lane 8, *N. longiflora*. From lane 2 to lane 8, proteins extracted from a stigma were loaded on each lane.

![Fig. 4](image-url) Immunohistochemical detection of antigens of anti-SE32 antiserum in the style just under the stigma. Each section was incubated with anti-SE32 antiserum (A and C) or normal serum (B and D), and then incubated with immunogold-labeled secondary antibody as described in Materials and Methods. A and B, paraffin-embedded cross sections; C and D, resin-embedded cross sections. tt, transmitting tissue; co, cortex. Bars = 500 μm in A and B. Bars = 30 μm in C and D.
Expansin has been known to affect cell-wall stress relaxation (Cosgrove 1998); and β-expansin has been reported mainly in monocots, especially in grass pollen. It has been predicted to be secreted at the tip of the pollen tube and diffuse into the intercellular spaces of the stigma to aid in the invasion of the pollen tube by softening the cell walls of maternal tissues (Cosgrove 1998). Thus, SE32 might also soften the cell walls of stigma and promote the pollen-tube penetration into stigma (Feron et al. 1998). The fact that β-expansin is one of the major proteins of the stigma exudate may explain why transmitting tissue is soft and why cells of the transmitting tissue have a thin and elongated shape (Bell and Hicks 1976). The antigenicity of SE35 to anti-SE32 antibody implies that SE35 also belongs to the expansin family. Thus, SE35 might also soften the transmitting tissue and might play a role in elongation of transmitting-tissue cells. However, SE35 starts to accumulate much earlier than SE32 and is detectable in the young flower bud (Fig. 2B). The distribution of SE35 is also different from that of SE32 (Fig. 2A). Thus, the function of each protein might be differentiated according to their accumulation pattern, although SE32 and SE35 have a common distribution in the mature stigma.

Acknowledgements

We wish to thank Professor Hisashi Hirano of Kihara Institute of Biological Research, Yokohama City University for amino acid sequencing of SE32.

Literature Cited


