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# Immunological Evidence of Thaumatin-Like Proteins during Tobacco Floral Differentiation[1]

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## ABSTRACT

**Tobacco proteins that share homology with thaumatin, a sweet protein of *Thaumatococcus daniellii* Benth., are produced in various physiological situations such as pathogenesis-related stress or water deficit stress. Using purified polyclonal anti-thaumatin antibodies, we have detected other thaumatin-like proteins in tobacco (*Nicotiana tabacum* var Samsun) that have been related with floral differentiation. Thaumatin-like proteins with apparent molecular masses of 42.6, 31.6, and 26.3 kilodaltons were found in immature and mature flower organs *in vivo*, and others of 46.7, 41.7, and 27.5 kilodaltons were exclusively detected in thin cell layer explants forming flowers. *In situ* immunolocalization revealed their synthesis in newly differentiated floral meristems, in tracheids, and in parenchyma cells.**

The sweet-tasting protein thaumatin is a protein of 22 kD, synthesized in the cytoplasma of fruit cells of *Thaumatococcus daniellii* Benth., a tropical monocotyledon plant (4, 22). The conformational structure of thaumatin is stabilized by the presence of eight disulfide bonds that have been conserved among several plant proteins during evolution (2, 21). In *Nicotiana tabacum*, osmotin (16) and some pathogenesis-related proteins (1, 24) share amino acid sequence similarity with thaumatin. The primary structures of osmotin and pathogenesis-related proteins are 54 and 65% homologous to thaumatin, respectively, and the positions of all of the cysteine residues are conserved. Nevertheless, these proteins have not been recognized as sweet-tasting proteins.

These Thl[2] proteins and thaumatin differ from each other in their molecular weight, their tissue specificity, and their cell localization. The 26-kD osmotin has been detected in vacuoles of root cells in response to osmotic stress (8) and in cell suspension (5). The 24-kD pathogenesis-related protein has been detected in the extracellular space of tobacco mosaic virus-infected leaf tissue (23, 24). Thl proteins seem to have adapted to various physiological functions in the same plant species. Because thaumatin is absent from roots or leaves of *Thaumatococcus*, the physiological function of thaumatin is probably different from those of osmotin and pathogenesis-related proteins.

The synthesis of thaumatin and its accumulation in floral derived organs such as fruit suggests that thaumatin may have a physiological role in the flowering of *Thaumatococcus* other than its sweet-tasting properties. The question raised concerns the possible existence of other Thl proteins in plants that might be related to developmental process such as floral differentiation.

In this study, we searched for Thl proteins in *Nicotiana tabacum* that could be recognized by their cross-reactivity to polyclonal antibodies raised against purified thaumatin. Using rabbit anti-thaumatin antibodies, we detected several Thl proteins by immunological methods. The relationships of these proteins with flower development were analyzed in the entire plant by the comparison of plant organs at different development stages. The analysis was extended by the use of a simplified experimental system of tissue culture in which flower, vegetative bud, and root organogenesis can be selectively induced *de novo* (6, 7, 13, 18). The expression of Thl proteins during floral organogenesis was analyzed by immunohistochemistry.

## MATERIALS AND METHODS

### Anti-Thaumatin Antibodies

Commercial thaumatin from Sigma (11, 22) was used as an antigen to obtain anti-thaumatin antibodies. A rabbit was immunized intradermally with 1 mg of thaumatin emulsified in complete Freund's adjuvant. After 1 month, a booster injection of 0.1 mg thaumatin was given. Blood was collected weekly starting 1 week after the first booster injection and then every 4 weeks. The positivity of the sera was tested by an enzyme immunoassay using thaumatin as adsorbed antigen, donkey biotinylated anti-rabbit antibodies (Immuno Research Laboratories), and streptavidin-peroxidase conjuguate (Sigma) as secondary reagents. Hydrogen peroxide and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Boehringer Mannheim) were used as substrates (12).

Anti-thaumatin antibodies were purified by affinity chromatography on a CNBr-activated Sepharose adsorbent (Pharmacia) on which commercial thaumatin was linked (10 mg/

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[2] Abbreviations: Thl, thaumatin-like; TCL, thin-cell layer; IBA, indole butyric acid; KIN, kinetin; PBST, PBS (50 mM sodium phosphate, 150 mM NaCl [pH 7.3]) and 0.05% (v/v) Tween 20; PBST-milk, PBST containing 3% skim milk powder.

g adsorbent) using standard procedures. The γ-globulin fraction obtained by precipitation at 35% $(NH_4)_2SO_4$ was dialyzed in PBS (10 mM phosphate, pH 7.4) and adsorbed on the affinity gel. Specific antibodies were eluted with 0.2 M glycine buffer (pH 2.8) and dialyzed extensively against PBS.

Silver-staining SDS-PAGE (15) of the commercial thaumatin used as antigen revealed the presence of not only the 22-kD thaumatin band but also two minor bands of 20 and 41 kD. These bands have been described as being some naturally occurring forms of thaumatin (11). Their immunological relationship with thaumatin was checked to confirm the monospecificity of the antibodies toward thaumatin epitopes. For this purpose, the three proteins were separated on 10% SDS-PAGE from 1 mg of commercial thaumatin and transferred to a nitrocellulose filter. After staining with 0.1% Ponceau red, the three bands were cut and used as affinity matrices to purify subfractions of antibodies (15). The immunological specificity of each affinity-purified subfraction of antibodies was analyzed on separate blots of 0.5 μg of commercial thaumatin.

The monospecificity of antibodies toward thaumatin epitopes was reciprocally characterized by negative proof. Antibodies were preincubated in the presence of the blotted 22-kD thaumatin purified by SDS-PAGE from 1 mg of commercial thaumatin. Any binding capacity changes were visualized on blots of 0.5 μg of commercial thaumatin. Analogous experiments were also performed using tobacco protein extracts.

### Plant and TCL Culture

Tobacco (*Nicotiana tabacum* L. cv Samsun) plants were grown on vermiculite using a 16-h photoperiod of natural light complemented by artificial light of 100 to 150 μmol $m^{-2}$ $s^{-1}$ at 24°C with an RH of 50%. Vegetative, early floral (25–30 internodes and approximately 20 immature floral buds), and floral stage plants were used for analysis.

TCL explants of 1 × 10 mm were removed from sterilized floral branches of a terminal green fruit stage plant. They were cultured on 0.8% agar-solidified medium as described previously (18). IBA, KIN, and sugars were added in different concentrations to Murashige-Skoog salts to induce separately the differentiation of flowers (1 μM IBA, 1 μM KIN, 30% glucose), vegetative buds (1 μM IBA, 10 μM KIN, 30% glucose), and roots (10 μM IBA, 0.1 μM KIN, 10% sucrose) from the TCL explants. Each organogenic program was studied using TCL explants of 30-d culture. Solid instead of liquid medium was chosen in this study because the average number of organs formed per TCL explant was greater (20).

### Protein Extraction and SDS-PAGE

Total cellular extracts of proteins were prepared from TCL explants and plant organs. Sterilized plant material was ground in liquid nitrogen and homogenized (2 mL/g fresh weight) into 4.6% (w/v) SDS, 0.25 M Tris-HCl (pH 6.8), 10% (v/v) β-mercaptoethanol, and 20% glycerol (9). Equal amounts of total proteins from each sample (50 μg) and thaumatin used for internal immunological control were separated on a polyacrylamide gel slab containing 0.1% SDS, 10% acrylamide, and 0.4 M Tris-HCl (pH 8.8). Electrode buffer was 25 mM Tris-HCl, 200 mM glycine, and 0.1% SDS. A high molecular weight calibration kit (Pharmacia) was used to ascertain protein molecular weight.

### Immunoblotting

Gels were incubated in a transfer buffer containing 10 mM Tris-HCl (pH 8.2), 50 mM NaCl, 2 mM EDTA, and 0.1 mM DTT for 30 min. Proteins were blotted by bidirectional diffusion on two BA-83 nitrocellulose filters (Schleicher and Schuell) overnight at 18°C. One of the two filters was used for the control. Filters were blocked for 1 h in PBST-milk. Purified polyclonal anti-thaumatin antibodies (0.24 μg/mL) were preincubated for 2 h at 37°C in PBST-milk before being exposed to the filters. After overnight incubation in the presence of antibodies, filters were washed four times for 15 min each in PBST-milk and incubated for 1 h in biotinylated anti-rabbit antibodies from donkey (Amersham) diluted 1:500 in PBST-milk. After washing as described above, the filters were incubated for 2 h in PBST containing 0.5 μCi/mL of [$^{35}$S] streptavidin (Amersham). Filters were then washed three times in PBST and vacuum dried before autoradiographic exposure (X-OMAT AR diagnostic film from Kodak).

### *In Situ* Immunolocalization

After 30 d of culture, TCL explants forming flowers were fixed overnight at 4°C in 50 mM PBS (pH 7.3) containing 1% (v/v) paraformaldehyde and 0.7% (v/v) glutaraldehyde. They were washed four times in PBS, dehydrated in ethanol, and embedded in paraplast (Monoject Scientific Inc.). Serial sections of 10 μm were mounted on 1% (w/v) gelatin-covered slides, deparaffinized in toluene, and rehydrated in a decreasing series of ethanol. After washes in PBS, slides were processed as described for the immunoblotting method. Dry sections were then used for autoradiographic or nuclear emulsion exposure (LM1-Amersham). Slides were viewed through Olympus SZH or Reichert microscopes.

### Control Conditions

Immunological inhibition of anti-thaumatin antibodies by thaumatin during preincubation was systematically performed as a control for Western blot and immunohistochemistry studies. The immunological specificity of signals detected after immunolabeling with anti-thaumatin antibodies alone was estimated according to their change of intensity in the control. The control condition was obtained by using immunolabeling with anti-thaumatin in the presence of 0.1 mg/mL of thaumatin.

## RESULTS

### Characterization of the Monospecificity of the Anti-Thaumatin Antibodies toward Thaumatin Epitopes

Anti-thaumatin antibodies could be a useful tool to detect some cross-reactive proteins in tobacco insofar as the monospecificity of the antibodies is rigorously assessed. Polyclonal anti-thaumatin antibodies were obtained after immunization

of rabbit with commercial thaumatin. Silver-staining SDS-PAGE of the thaumatin preparation used showed the unique 22-kD thaumatin band when 10 μg were loaded (Fig. 1A), although higher amounts of proteins revealed the presence of two additional bands of 41 and 20 kD (Fig. 1, B–D). The occurrence of these 41- and 20-kD bands has been previously described as some natural forms of thaumatin (11).

The immunological relationship of these proteins with thaumatin was further checked by immunoblotting of the commercial thaumatin preparation after SDS-PAGE (Fig. 2). The obtained results indicated that purified anti-thaumatin antibodies recognized the 22-kD thaumatin and the 41- and 20-kD bands. Their recognition was inhibited by the presence of thaumatin. Nitrocellulose affinity matrices containing the 41-, 22-, and 20-kD bands were obtained from commercial thaumatin and were used to purify the anti-41-, anti-22-, and anti-20-kD antibodies, respectively, from total antibodies. These affinity-purified subfractions of antibodies were tested on separate blots of commercial thaumatin (Fig. 2). The subfractions against 41- and 20-kD antibodies recognized the 22-kD thaumatin and the 41-kD band in the same manner as the subfraction against the 22-kD thaumatin. The fact that the 20-kD band was not detectable may be due to competition between fractions of the commercial thaumatin. These results clearly demonstrated the immunological relationship between thaumatin and the 41- and 20-kD proteins and corroborated the monospecificity of the antibodies toward thaumatin epitopes.

Although the 41-kD protein may contain other specific epitopes in addition to thaumatin epitopes that could raise anti-41-kD antibodies not recognizing thaumatin, the complete adsorption of the antibody preparation on a blotted 22-kD thaumatin strip of nitrocellulose indicated that the 41-kD protein should contain only thaumatin epitopes (Fig. 3A).

To ascertain that the immunoreactive signals detected in plant extracts were due to the presence of thaumatin epitopes, blots of proteins extracted from immature stamen were incubated with antibodies in the presence of individual serial nitrocellulose strips of a blot of commercial thaumatin (Fig. 3B). Antibody binding (Fig. 3C, lane j) was completely inhibited by the presence of thaumatin in solution (lane k). The

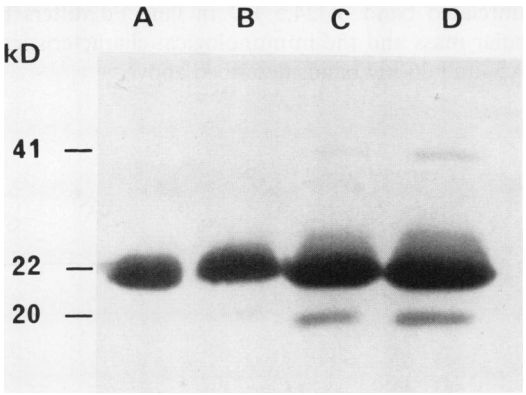


**Figure 1.** Characterization of the commercial thaumatin. Silver-staining SDS-PAGE of 10, 20, 40, and 60 μg of thaumatin (A, B, C, and D, respectively).

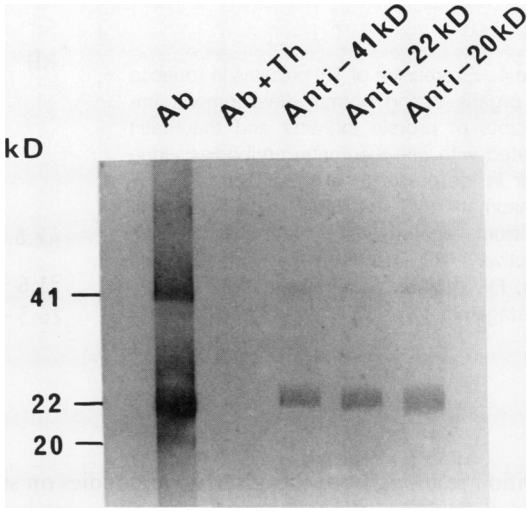


**Figure 2.** Evidence of the monospecificity of the anti-thaumatin antibodies toward thaumatin epitopes. Immunoblots of thaumatin were incubated with total antibodies (Ab), total antibodies in the presence of thaumatin (Ab+Th), or subfractions anti-20, anti-22, and anti-41 kD separately.

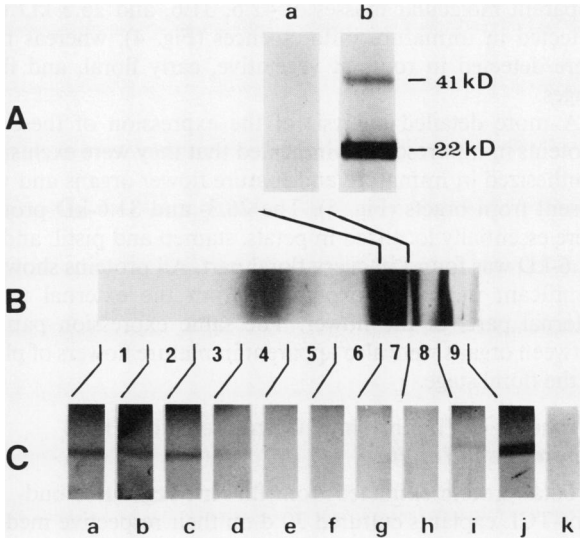


**Figure 3.** Common epitopes between thaumatin and stamen tobacco proteins. A, Immunoblots of thaumatin after incubation with anti-thaumatin antibodies alone (lane b) or in the presence of the blotted 22-kD thaumatin band (lane a) corresponding to nitrocellulose strip 7 in B. B, Silver staining of a longitudinal strip sliced from a preparative SDS-PAGE of 1 mg of the commercial thaumatin preparation. C, After the preparative gel referred to in B was blotted, the blot was cut into pieces (corresponding to the fractions shown at the bottom of B). Strips 4, 7, and 8 correspond to the 41-, 22-, and 20-kD bands, respectively. Strip 9 corresponds to the migration front. Immunoblots of stamen extracts incubated with anti-thaumatin antibodies alone (lane j), in the presence of 0.1 mg/mL of thaumatin (lane k), and in the presence of strips 1 to 9 (lanes a–i, respectively).

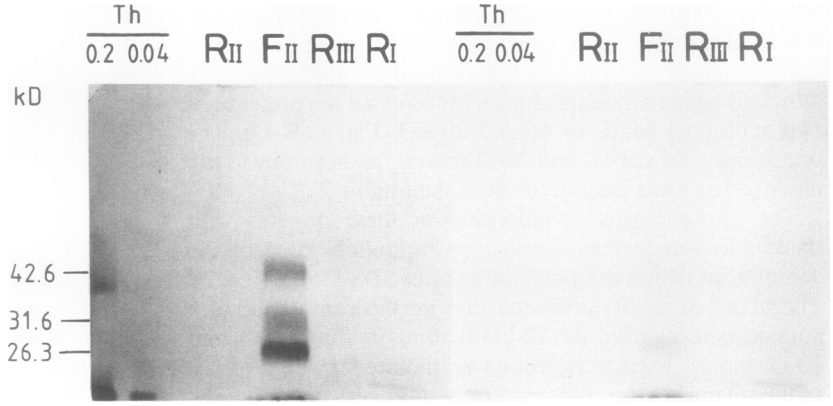


**Figure 4.** Expression of Thl proteins in tobacco plant organs during normal development. Immunoblots of protein extracts and thaumatin incubated with anti-thaumatin antibodies alone (Ab) or in the presence of thaumatin (Ab+Th). Th, thaumatin (0.2 and 0.04 μg); RI and RIII, roots from vegetative and floral stage plants, respectively; RII, root from early floral stage plants; FII, immature inflorescences from early floral stage plants.

inhibition resulting from the adsorbed antibodies on strips 4, 7, and 8 (lanes d–h) indicated that tobacco protein shares common epitopes with thaumatin and the 41- and 20-kD proteins.

### Occurrence of Thl Proteins during Tobacco Flowering *in Vivo*

Proteins extracts from inflorescences and roots of the early floral stage plant and roots at vegetative and floral stage plants were studied. Several immunologically related bands with apparent molecular masses of 42.6, 31.6, and 26.3 kD were detected in immature inflorescences (Fig. 4), whereas none were detected in roots at vegetative, early floral, and floral stages.

A more detailed analysis of the expression of these Thl proteins in inflorescences indicated that they were exclusively synthesized in immature and mature flower organs and were absent from bracts (Fig. 5). The 26.3- and 31.6-kD proteins were essentially localized in petals, stamen and pistil, and the 42.6-kD was found in every floral part. All proteins showed a significant increasing expression from the external to the internal parts of the flower. The same expression patterns between organs were also apparent in mature flowers of plants at the floral stage.

### Synthesis of Thl Protein Is Correlated with Floral Differentiation *in Vitro*

Total protein extracted from flower-, vegetative bud-, and root-TCL explants cultured 30 d on their respective medium were analyzed by western blot. The neoformation of floral meristems only occurred at the proximal pole of flower TCL explants, which is close to the base of the floral branch, and the distal pole of the same explant remained macroscopically unchanged during culture. Comparative analysis of the expression of Thl proteins between poles was also carried out.

Several immunorelated proteins were synthesized in explants depending on their organogenic fate (Fig. 6). The flower TCL explants contained three Thl proteins with apparent molecular masses of 46.7, 41.7, and 27.5 kD, close to those detected in flowers *in vivo*. The 27.5-kD protein was only detected in the proximal pole, whereas the 46.7- and the 41.7-kD proteins occurred throughout the explant. Only traces of Thl proteins were found in vegetative bud TCLs and a unique immunoreactive band of 26 kD occurred in explants forming roots. This 26-kD protein was detected in trace amounts in the proximal pole of flower TCL explants.

The analysis of protein extracts from TCL explants revealed the presence of unspecific bands that were still apparent when the incubations with antibodies were performed in the presence of thaumatin. Contrary to the thaumatin band (lanes Th), used as internal control and to the immunoreactive bands described above, the recognition of the nonspecific bands was completely unaffected by the blocking effect of thaumatin. The corresponding proteins were not related to Thl proteins. The unrelated band of 24.5 kD in lane Fd differs by the molecular mass and the immunological characteristics from the 27.5- and 26-kD bands described above.

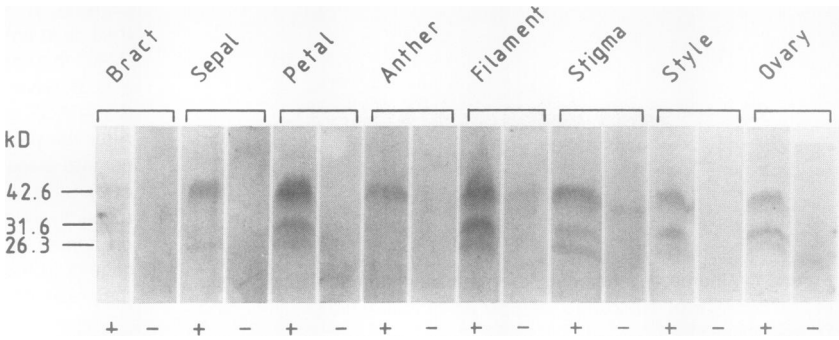


**Figure 5.** Distribution of Thl proteins in immature flower organs of tobacco plant at early floral stage. Immunoblots of total protein extracts incubated with anti-thaumatin antibodies alone (+) or in the presence of thaumatin (−).

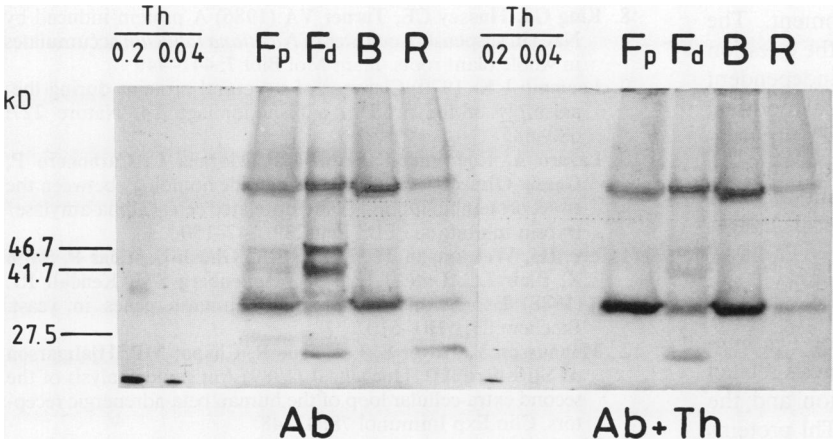


**Figure 6.** Expression of Thl proteins in 30-d-old TCL explants forming flowers, vegetative buds, and roots. Immunoblots of protein extracts and thaumatin incubated with anti-thaumatin antibodies alone (Ab) or in the presence of thaumatin (Ab+Th). Th, thaumatin (0.2 and 0.04 μg); Fp, proximal pole of flower TCL explants; Fd, distal pole of flower TCL explants; B, vegetative bud TCL explants; R, root TCL explants.

### *In Situ* Localization of Thl Protein Expressed during Floral Differentiation

Longitudinal sections of flower TCL explants were used for immunohistochemical localization of the Thl proteins. The observations account for the presence of Thl proteins in newly formed tissues. They were confined to the dividing cells of floral meristem corresponding to the basal sepal primordia (Fig. 7A) and to the tracheary elements of vascular nodules (Fig. 7B). Thl proteins were also detected throughout the parenchyma cell layer (Fig. 7C).

Cytoplasmic expression of the immunoreactive proteins was observed in tracheids and parenchyma cells. Such cell localization has also been reported for thaumatin in *Thaumatococcus* fruit cells. Thl proteins were absent from the vacuole and the intercellular space, which are the two main cellular compartments for osmotin and most of the pathogenesis-related proteins, respectively.

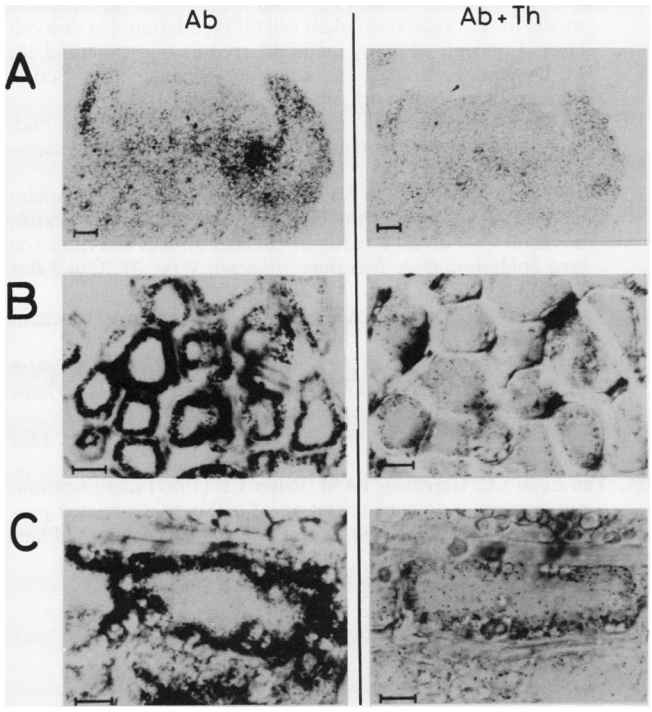


**Figure 7.** *In situ* localization of Thl protein in 30-d flower TCL explants. Longitudinal sections of explant incubated with anti-thaumatin antibodies alone (Ab) or in the presence of thaumatin (Ab+Th). Autoradiographic emulsions of floral meristem (A), tracheary elements of vascular nodule (B), and parenchyma cells (C). Bar, 30 μm.

## DISCUSSION

Using monospecific anti-thaumatin antibodies we have detected several Thl proteins in specific plant organs and in TCL explants undergoing specific organogenic processes. Because the conformational structure of these proteins was not conserved under the denaturing conditions used for their extraction (9), their recognition by the anti-thaumatin antibodies must depend essentially on the conservation of the primary structure. The Thl proteins of 42.6, 31.6, and 26.3 kD in inflorescence *in vivo* and of 46.7, 41.7, and 27.5 kD in flower TCL explants distinguished on the basis of their molecular weight do not seem to be related to the 24-kD pathogenesis-related protein and the 26-kD osmotin (1, 16) and to the 29-kD precursor of osmotin (17) previously characterized in tobacco plants. They may represent as yet undescribed tobacco Thl proteins.

The immunological analyses of plant organs at different development stages indicate that Thl protein occurrence can be correlated with floral differentiation. The results obtained by the use of a tissue culture method in which flower development is independent of both external physicochemical factors and physiological relationships between plant organs corroborated this correlation with floral differentiation.

Floral and vegetative organogenesis display identical histological features, such as neocambium and tracheid differentiation (19), whereas the immunoreactivity of these tissues was only expressed in explants undergoing floral organogenesis. The relationship between the expression of Thl proteins and floral differentiation is also confirmed by their occurrence in floral meristem, where their asymmetric distribution accords with the phyllotactic emergence of floral primordia (3).

The presence of Thl proteins in sepal primordium of floral meristem indicated that their synthesis occurs early in the course of flower organ formation. Their appearance in immature and mature flower organs suggests that they may be

constitutively expressed during flower development. The changes in the expression of these proteins raise the question of whether they correspond to some genetically independent isoforms of Thl proteins or to some posttranslational modified products occurring during floral differentiation. Posttranslational modifications of the higher molecular weight Thl proteins might have occurred, producing Thl proteins of lower molecular weight, concomitantly with the histological changes appearing in the course of flower development.

The molecular weights, the tissue specificity, and the developmental appearance of these proteins indicate that they are physiologically more related to the Thl proteins of *Thaumatococcus* than to the osmotin and the pathogenesis-related proteins. The evolutionary structural conservation and the various related physiological expressions of the Thl proteins (16) denote their possible important roles in plant physiology. Although amino acid sequence comparisons suggest that thaumatin could have a similar function to that of the bifunctional trypsin/$\alpha$-amylase inhibitor (10, 14), the biological function of these proteins have yet to be determined. The construction of a cDNA library using an expression vector (in progress) will allow the identification of the reported Thl proteins and the study of their physiological role during floral development.

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