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The Tomato 66.3-kD Polyphenoloxidase Gene: Molecular Identification and Developmental Expression

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A gene coding for a polypeptide abundant in tomato floral meristems was isolated and shown to represent a tomato 66.3-kD polyphenoloxidase. Analysis of cDNA clones and a corresponding intronless genomic clone indicated that the plastid-bound 587-residue-long polypeptide, designated P2, contains two conserved copper-binding domains, similar to those found in fungal and mammalian tyrosinases. P2 transcripts and polypeptides are accumulated in the arrested floral primordia of the *anantha* mutant inflorescences and are equally abundant in primordia of wild-type flowers; the gene continues to be expressed at high levels in developing floral organs. In young expanding leaves, P2 protein is concentrated in palisade cells and in epidermal trichomes. Expression patterns of P2 in plant meristems permit molecular distinction between floral and vegetative primordia, and, in a companion study, comparison with dUTPase suggests that the two genes mark two alternative complementary developmental programs in the floral and vegetative meristems of the tomato plants.

INTRODUCTION

Shoot and root apical meristems are set apart during the embryonic stage, persist throughout the life span, and continuously and repeatedly generate all primary tissues and lateral meristems of the plant body (Cutter, 1980; Walbot, 1985; Goldberg, 1988; Steeves and Sussex, 1989; Sussex, 1989; Poethig, 1990).

Unlike vegetative meristems, floral meristems are not embryonic in origin but evolve as a result of transformation of the vegetative meristem: transformation is initiated by transmissible inductive signals that evoke a transition stage, followed by floral differentiation (for recent review, see Bernier, 1988). Advanced stages of floral organogenesis are currently amenable to genetic and molecular analysis due to the impressive collection of homeotic genes and their recent cloning in Antirrhinum, Arabidopsis, and tomato (Coen et al., 1990; Sommer et al., 1990; Yanofsky et al., 1990; Ma et al., 1991; Pnueli et al., 1991). However, the genetic nature of the newly induced floral program is not understood. One suggestion is that apical cells fated to form an inflorescence acquire, upon induction and evocation, a cell-autonomous floral program that both characterizes the floral state throughout development and is a prerequisite for organ formation and terminal differentiation (Lifschitz, 1988).

To identify developmental regulatory components of such a hypothetical floral program, a search has been initiated for gene markers that are upregulated in floral meristems and operate continuously in all developing floral organs. The experimental approach we have taken involves the comparison of protein repertoires from meristematic tissues, growing leaves, and developing flowers. These comparisons have been conducted to ensure that a given polypeptide is not specific to only one stage of floral development nor to one particular floral organ. The source of meristematic material was floral buds from inflorescences of the *anantha* mutant. The *anantha* recessive mutation was described by Helm (1951) and Paddock and Alexander (1952). Inflorescences of *anantha* differentiate normally, but floral buds are arrested at an early preorganogenesis stage. The arrested primordia then duplicate repeatedly, generating a loose, cauliflower-like structure composed of thousands of floral primordia. Polyclonal antibodies were prepared against polypeptides that are more abundant in *anantha* meristems than in leaves or flowers, and the corresponding genes were isolated from a phage expression library.

The P2 protein and the corresponding cDNA and genomic sequences isolated by the above procedure were shown to represent the gene that most likely encodes the tomato 66.3-kD polyphenoloxidase (PPO). PPO catalyses the *ortho*-hydroxylation of monophenols (like tyrosine), as well as the oxidation of *ortho*-diphenols to *ortho*-diquinones. We describe the isolation and identification of the cDNA and genomic clone, present detailed mapping of the expression domains of P2 in floral and vegetative meristems, and examine the developmental significance of the expression patterns.

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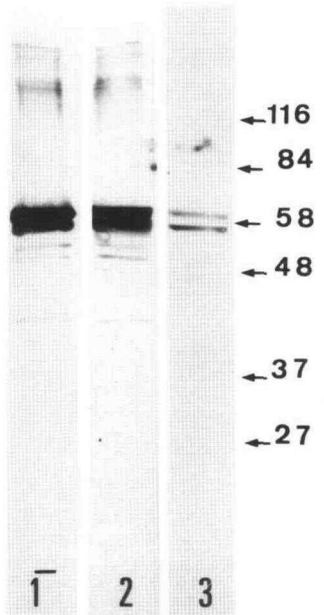


Figure 1. Identification of the P2 protein and Its Immunodetection in Extracts of Pelleted Proteins.

Immunoblot analysis of P2 proteins. Blots of total pelleted proteins from *anantha* floral meristems (lane 1), mature flowers (lane 2), and young leaves (lane 3) were probed with anti-P2 antibody. Peroxidase-conjugated second antibody was employed. The two polypeptides also reacted specifically with affinity-purified antiserum for the P2 portion of the lacZ-P2 protein.

RESULTS

Analysis of protein profiles containing ~1500 bands from *anantha* meristems, expanding leaves, and normal mature flowers was conducted to identify flower-specific and meristem-related genes (Samach et al., 1991; Pri-Hadash et al., 1992). In this article, we report the isolation of a 58- to 60-kD protein (hereafter P2) found to be abundant among proteins in the 100,000*g* pellet from *anantha* floral meristems and normal flowers but hardly detected in mature leaves. The P2 polypeptide was extracted from 8% SDS-PAGE (Laemmli, 1970) gels and rabbit anti-P2 antibody was prepared as described in Methods. The antibody identifies two adjacent polypeptides of the expected size in the protein fractions of *anantha* meristems and normal flowers, as shown in Figure 1, lanes 1 and 2. To a lesser extent, the antibody also recognizes polypeptides of similar size in the protein fraction of young leaves (1- to 2-cm wide) (Figure 1, lane 3), but not in mature leaves (<10 cm). cDNA expression libraries, constructed in λgt11 from mRNA of *anantha* meristems and normal flowers, were probed with anti-P2 antibody. Three positive clones were found among 10^5 clones of the *anantha* library and one positive clone among 6×10^4 of the floral library.

Organization of the P2 Gene

All four cDNA clones identified by the antibody were found to be homologous to one another. We chose to continue with the longest clone, cP2/A1. The 772-bp EcoRI insert recloned into the plasmid vector pTZ18 is composed of an open reading frame (ORF) of 196 amino acids, a stop codon, 181-bp-long nontranslated 3′ end, and a stretch of poly(A). The ORF is in frame with that of the LacZ gene of λgt11, and its 5′ EcoRI site is part of the legitimate sequence of the P2 gene. The cP2/A1 EcoRI insert was used to isolate the genomic clone, λgP2/B. Upon sequencing, it became apparent that the base sequence of the cDNA cP2/A1 clone and the corresponding coding region of the genomic clone, gP2/B, are not identical but rather 93% homologous. The few base substitutions do not alter the size of the ORF. Consequently, we screened the cDNA library again, this time with the ^{32}P-labeled cP2/A1 probe, rather than with P2 antibody. None of the newly isolated cDNA clones was of the complete size expected of a gene coding for a 60-kD protein. The longest cDNA insert, cP2/A7, is 1350 bp and contains an internal EcoRI site, which, upon digestion, yields a fragment identical in size and sequence with cP2/A1. Among the four other cDNA clones that we sequenced, two were identical to cP2/A1 and two had nucleotide sequences identical to that of the genomic clone gP2/B. There are, therefore, at least two similar P2 genes, designated P2/A and P2/B, both of which are transcribed.

The physical maps of the λgP2/B genomic clone, which represent the P2/B gene, and the cDNA clones cP2/A1 and cP2/A7, corresponding to gene P2/A, are shown in Figure 2A. The complete nucleotide and deduced amino acid sequence of gene P2/B, including portions of the 5′ and 3′ nontranslated ends, is given in Figure 2B. It is compared with the nucleotide and deduced amino acid sequence of the cP2/A7 clone, which represents most of the P2/A gene (see legend to Figure 2B). Evidently, the extreme similarity of the coding sequences of P2/A and P2/B extends to their 3′ nontranslated regions as well.

The genomic clone representing the gene P2/B is devoid of intervening sequences. Conceptual translation permits the assignment of an uninterrupted ORF of 587 amino acids with the M_r of 66.310 kD and predicts a slightly acidic isoelectric point of 6.1.

In SDS-PAGE, P2 proteins migrate as a 58- to 60-kD rather than the expected 66.3-kD polypeptide. The 90-residue-long N-terminal portion of P2 is, however, characteristic of plastid transit peptides (Heijne et al., 1988; Keegstra et al., 1989), which is in agreement with the cytological observations (see below). The presumptive transit peptide is divided into three domains (marked by opposite arrows in Figure 2B). Serine and threonine residues comprise the majority (60%) of the

first 40 N-terminal amino acids, which are interrupted only once by a charged residue. A central domain with above average charged residues (positions 40 to 68) is followed by an extremely hydrophobic C-terminal sequence (68 to 88).

P2 Protein Contains Two Conserved Copper-Binding Domains

No overall significant similarity with known proteins was found, but a search through the protein data base (Swiss prot 14) revealed the presence of copper-binding domains similar to those of the binucleate copper proteins hemocyanins and tyrosinases. The location of the histidine ligands and the primary and secondary structure of the copper A- and copper B-binding domains were determined for hemocyanin of the arthropod *Panulirus interruptus* from the three-dimensional structure of the protein (Gaykema et al., 1984). Copper B-binding domains are similar in hemocyanins and tyrosinases, despite the difference in their functions, whereas copper A-binding domains are unique for each class of protein (Gaykema et al., 1984; Lerch et al., 1986; Müller et al., 1988). The comparison of regions A and B of known tyrosinases with the corresponding sequences of the P2 genes is shown in Figure 3. In region A (Figure 3A), we have extended the comparison to an adjacent C-terminal sequence of ~15 residues. In *Neurospora crassa*, this region is separated from the main domain by an insertion of 20 amino acids. These homologies strongly suggest that P2 genes represent the Solanaceae 66.3-kD PPO, a synonym for tyrosinase.

Temporal and Spatial Expression of the P2 Gene

To verify that the isolated cP2/A1 clone represents a gene that is upregulated in *anantha* meristems and mature flowers, the abundance of the homologous RNA was monitored in various parts of the plant. The RNA gel blot analysis in Figure 4 indicates that although cP2/A1 transcripts are found in all parts of the plant, the gene is particularly upregulated in *anantha* meristems and is expressed at high levels in mature flowers as well. Unlike the results obtained with the antibody, only one size species of RNA is recognized by the cP2/A1 cDNA probe. The two polypeptides identified by the P2 antibodies may reflect differential processing, post-translational modifications, or different translation products of P2/A and P2/B (see legend to Figure 2).

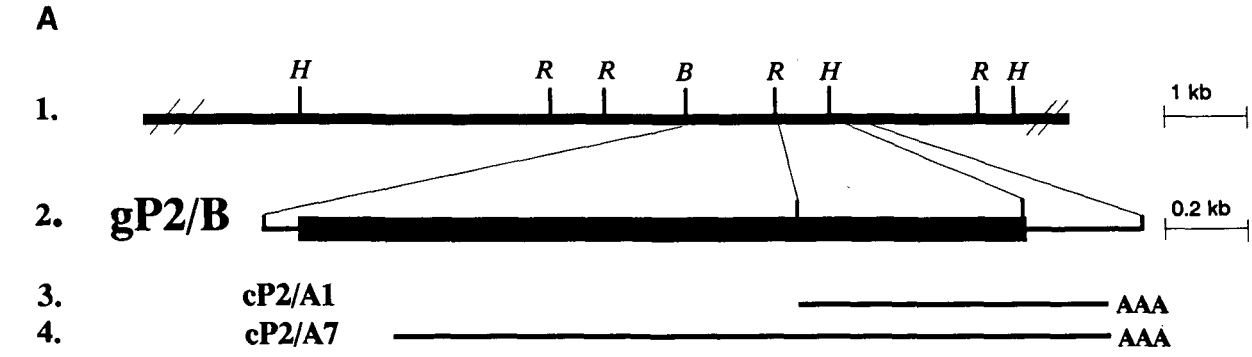
We have followed the spatial and temporal expression of the P2 gene using the immunogold detection procedure. These studies were corroborated by in situ hybridization experiments with complementary RNA. To ensure that the antibody employed in the immunodetection experiments actually represents the legitimate product of the P2/A gene, a new antibody for the fusion protein expressed by the λcP2/A1 clone was prepared. This was essential because the purity of the initial gel-isolated P2 polypeptide was not verified, the sequence of the protein was not available, and the original antibody recognized two polypeptides in protein blots. Furthermore, screening of expression libraries with polyclonal antibodies often results in the isolation of unrelated genes. Following affinity elimination of the antibodies for the β-galactosidase epitopes, the cP2/A1 antiserum was shown to react with the same two polypeptides as the original antiserum (see Figure 1), and the two antibodies decorated the same cells in all plant tissues. Nevertheless, all cytological data were collected using the antibody for the P2/A1 fusion protein only.

The localization of the P2 antigen in tissues of three different plant meristems is shown in Figures 5A to 5C. In accordance with the results obtained for the corresponding mRNA, the P2 protein is most abundant in *anantha* floral primordia. The tissue-specific pattern is unique; P2 is accumulated in the ground and pith parenchymal cells but not in the apical cells of this meristem or in the provascular tissue (Figure 5A).

In the vegetative shoot and root meristems, however, the most conspicuous signals are found in the future epidermal cells, whereas low signals characterize the other meristematic layers and the ground cells (Figures 5B and 5C). The low proportion of dermal cells in shoots and roots is reflected in the results shown in Figure 4. Pith cells of the shoot apex are labeled but not as intensely as the floral pith cells.

The spatial distribution of P2 antigen in successive stages of floral development is documented in Figures 6A to 6G. The buds shown in Figure 6A are slightly advanced in comparison with those arrested by the *anantha* mutation. Sepal primordia have already emerged and exhibit strong signals, but, notwithstanding, the overall pattern is identical to that of *anantha*. Elevated levels of P2 antigen are observed only in parenchyma cells of the newly differentiated ground and pith tissues but not in provascular tissues. A rather interesting pattern of low signals is monitored in the layered zone of the floral apex. P2 is expressed in the LI cell layer and in the scrambled LIII layers, which are the progenitors of epidermal and the other somatic tissues, respectively (see Stewart, 1978). The gene is repressed, however, in LII cells—the progenitors of the archesporium (Figures 6A1 and 6A2). In archesporial derivatives of the anther, P2 is not expressed in pollen mother cells (PMC) and endothecium, but the antigen is found in plastids of the tapetal cells (Figure 6C).

Figure 6B features a longitudinal oblique section through the normal floral bud after emergence of all organ primordia. Intensive labeling is observed in sepals, especially in the adaxial side, in all mesophyll cells of the petals, in stamens (with the exception of sporogenic tissue), in ovaries, and in pith cells of the receptacle and stalk. Following completion of differentiation, the P2 antigen level in floral organs drops and is restricted to specific tissues and cell types (Figure 6C). In mature sepals, P2 is found mostly in the adaxial side, and prior to anthesis most of the protein in the petals is localized



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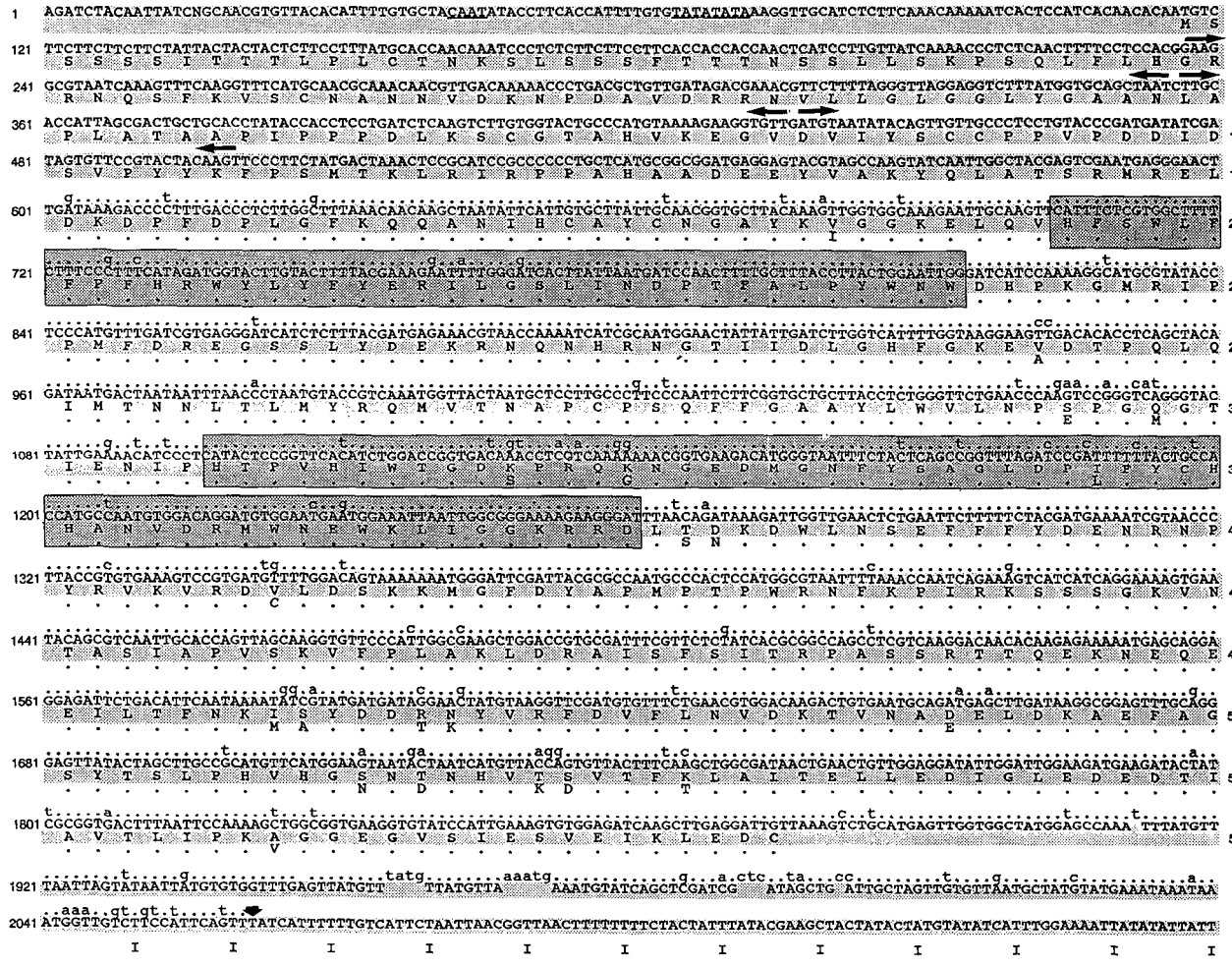

Figure 2. Physical Map and Sequence Analysis of the Genomic and cDNA Clones Coding for P2 Proteins.

(A) Schematic representation of the physical maps of the cDNA clones and the genomic clone for the P2 gene. 1, the λ clone from the genomic library; 2, the genomic segment containing the gP2/B gene, the sequence of which is shown in **(B)** (the translated region is marked with a bold line); 3, cP2/A1 cDNA clone, isolated from the *anantha* cDNA library using P2 antibody as a probe; 4, cP2/A7 cDNA clone, isolated from the floral cDNA library with cP2/A1 as a probe. B, BglII; H, HindIII; R, EcoRI.

(B) Nucleotide and amino acid sequence of the genomic clone gP2/B and the cDNA clone cP2/A7. P2/A and P2/B refer to different but nearly

A

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P2/B 197   HFSWLFFPFHRWYLYFYERILGSLIN--------------------DPTFALPYWNW 233
S.g   54   HRSPSFLPWHRRYLLEFERALQSV----------------------DASVALPYWDW  88
N.c   96   HSSILFITWHRPYLALYEQALYASVQAVAQFKFPVEGGLRAKYVAAAKDFRAPYFDW 152
M.m  184   HEAPGFLPWHRLFLLLWEQEIRELTG--------------------DENFTVPYWDW 221
           ....!....!....!....!....!....!....!....!....!....!....!..
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B

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           *   *                       *
P2/B 328   HTPVHIWTGDKPRQKNGEGMGNFY-SAGLDPIFYCHHANVDRMWNEWKLIGGKRRD 382
P2/A       HTPVHIWTGDSPRQGNGEDMGNFY-SAGLDPLFYCHHANVDRMWNEWKLIGGKRRD
S.g  190   HNRVH-------VWVGGR-MATGM-SP-NDPVFWLHHAYVDKLWAEWQ-----RRH 230
N.c  277   HNEIH-----DRTGGNGH-MSSLEVSA-FDPLFWLHHVNVDRLWSIWQDLN----- 320
M.m  345   HNALH-------IFMNGT-MSQVQGSA-NDPIFLLHHAFVDSIFEQW-L----RRH 386
E.c  320   HNWGH-----------GVMDDTSTSL-RDPIFYRYHRWMDNIFQEY-L----HRL 375
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Figure 3. Homology between Copper-Binding Domains of P2 and Tyrosinases of Different Species.

(A) Comparison of conserved region A of tyrosinases and related P2 sequence. Comparison of residues 1 to 20 follows Lerch et al. (1986) and Müller et al. (1988). We suggest the extension of this homology to additional downstream residues (16 to 18) of P2 and tyrosinases of *S. glancescens* (S.g) and *M. musculus* (M.m). In *N. crassa* (N.c), the second homologous region is separated from the major A domain by insertion of ~20 amino acids. Shading indicates identity with P2. Note, however, that in several positions (4, 7, 9, 21, and 52) a compatible residue replaces an identical one.

(B) Conservation of amino acid sequences surrounding copper B ligands. Comparison of the binucleate copper proteins follows the assignment of histidine ligands in hemocyanin of the arthropod *P. interruptus* (Gaykema et al., 1984). Similarity with the copper-binding domain of tyrosinases from different species was analyzed by Gaykema et al. (1984), Lerch et al. (1986), and Müller et al. (1988). Here we compare the relevant sequences of the two P2 genes with tyrosinases from *S. glaucescens* (S.g) (Huber et al., 1985), *N. crassa* (N.c) (Lerch et al., 1982; Huber et al., 1985), and *M. musculus* (M.m) (Müller et al., 1988), and with hemocyanin from *E. californicum* (E.c) (Scharlau et al., 1983). The two tomato genes differ among themselves in three positions (11, 15, and 19) in a region that is also variable in other tyrosinases. Shaded letters refer to identity in a given position with P2. A compatible residue replaces an identical one in several positions (32, 38, 42, 43, and 44). Asterisks mark the copper ligands.

in the lateral (growing) ends. In anthers, following differentiation of PMC, P2 is found mostly around the vascular bundles of the filament, in the outer epidermal layers, in the tapetum, and in the parenchymal cells of the connective. At this stage, no signal was observed in PMC, endothecium, or stomium. In the ovary, P2 is most conspicuous in the integuments of ovules (Figure 6D) and in the style, where the antigen is found in the ground tissue but not in the conducting tissue (Figure 6E). The expression in the style is contrasted with that of dUTPase (Figure 6F) to demonstrate the complementary manner in which the two genes are expressed (see Discussion and Pri-Hadash et al., 1992). For control, the localization of the light-harvesting protein of photosystem II in a cross-section of a mature flower (Figure 6G) is presented.

In situ hybridization experiments with an antisense RNA probe derived from the cP2/A1 cDNA clone established the one-to-one relationship between antigen accumulation and gene expression. In *anantha* floral meristems (Figure 7A), in normal floral buds (Figure 7B), in the ovary (Figure 7C), or in the young anther (Figure 7D), the distribution of P2 mRNA

Figure 2. (continued).

identical P2 genes. Only the sequence of gP2/B represents an entire P2 gene. The complete sequence of P2/A is not available. Nucleotide and amino acid sequences of gP2/B are lightly shaded. One-letter codes are used for amino acids. Comparison of cP2/A7 with the genomic clone of P2/B starts at position 603. Changes in base sequence between the genes are indicated by lower case letters above the shaded genomic sequence, whereas substituted amino acids are indicated by the code letters below the genomic sequence. Possible TATA and CAAT boxes are underlined. Arrowhead at position 2061 marks the beginning of the poly(A) tail in cP2/A7 and in cP2/A1 clones. Horizontal arrows delineate three subdomains of the presumptive transit peptide. Gaps in the 3′ nontranslated end of the genomic clone, gP2/B, were introduced to achieve maximum homology with the cP2/A7 clone. Note how invariant the 3′ ends of the two genes are. The heavily shaded boxes refer to the protein domains that are conserved among all tyrosinases that contain the presumptive copper A and B binding sites. They are compared with other tyrosinases in Figures 3A and 3B. The P2/B sequence also contains five potential N-glycosylation sites, a protein with a moderately hydrophobic nature, an overall predicted PI of 6.1, and a more acidic PI (6.0) for the presumptive processed polypeptide.

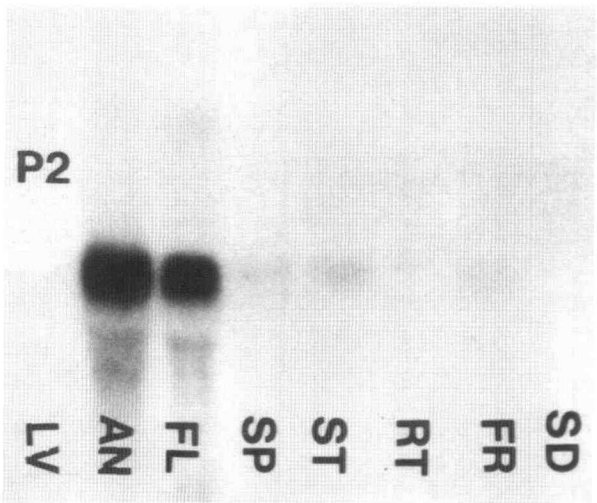

Figure 4. Representation of P2 mRNA in Floral and Vegetative Organs of the Tomato Plant.

Total RNA was isolated from 5 to 10-cm-long leaves (LV), *anantha* floral meristems (AN), normal sepal-less mature flowers 3 to 4 days before anthesis (FL), sepals of mature flowers (SP), shoots of 20-cm-high plants (ST), roots 2- to 10-cm long (RT), fruits 0.5 cm in diameter (FR), and seeds (SD). RNA, 15 μg per lane, was fractionated, transferred to nitrocellulose membrane, and hybridized with a ^{32}P-labeled gel-purified fragment of the cP2/A1 cDNA clone. The level of P2 mRNA in *anantha* floral meristems was approximately 100-fold higher than in mature leaves, as suggested by the results of longer exposures. Higher levels of P2 mRNA were found in very young leaves (1 cm wide).

agrees well with that of the P2 antigen, lending support to the assumption that the detailed picture revealed in Figures 5 and 6 faithfully reflects the expression of the P2 gene and not only the accumulation of the protein. In Figure 7E, the hybridization pattern of dUTPase is shown as an additional illustration of the alternative expression domains of the two genes.

The presence of a plastid transit peptide and the results of immunogold labeling suggest that P2 is found in cellular organelles, which is also supported by the association of at least one type of PPO with plastids (Vaughn et al., 1988). Because the 66.3-kD PPO activity in other plants was analyzed mainly in leaves and trichomes (Steffens et al., 1990) and because it was clear that P2 is expressed also in leaf primordia (Figure 5B), we probed leaves of various ages with P2 antibody. In leaf buttresses, P2 is expressed first in all dermal cells but is gradually accumulated in cells fated to form the palisade layer. In the intermediate stage, shown in Figure 8A, adaxial and abaxial epidermal layers are labeled and increasingly strong signals are observed in the now differentiated palisade cells. At this stage, the leaflet is ~100 cells wide. At approximately the time of vascular differentiation (leaflet width, ~5 mm), P2 antigen disappears from the epidermal tissues and almost all labeling is concentrated in the palisade cells and glandular trichomes (Figure 8B). It is difficult, using this technique, to detect P2 in tomato leaves wider than 2 to 3 cm.

To investigate further the relationship between P2 (PPO) and plastids, preliminary studies of the localization of P2 in ultrathin sections of *anantha* floral meristems were conducted. In the section shown in Figure 9A, anti-P2 antibody decorates a protein body in amyloplast-like plastids. P2 is also accumulated in less condensed protein bodies of proplastids (Figure 9B) and is sometimes detected in proplastids that lack protein bodies altogether. Accumulation of P2 in protein bodies explains the initial abundance of P2 in the 100,000*g* pellet from *anantha* meristems. In light of the crucial role played by the 66.3-kD PPO in defense mechanisms (Steffens et al., 1990), its target plastids in epidermal and mesophyll cells of the leaf and the relation between plastid biogenesis and gene activity (Taylor, 1989) deserve a separate, detailed study.

DISCUSSION

Molecular Analysis of the P2 Genes

P2 Genes Code for a 66.3-kD PPO

Sequence comparisons establish that P2 genes contain two potential copper-binding domains, designated A and B, characterizing all known tyrosinases (Figure 3). The copper B domain is specific to all binucleate copper proteins, but the less characterized A domain is specific only to tyrosinases (Gaykema et al., 1984; Huber et al., 1985; Lerch et al., 1986; Müller et al., 1988). The homology of the B domain of P2 to the published domains is as extensive as that of the hemocyanins and tyrosinases among themselves. No appreciable homology is found between P2 and other tyrosinases outside the A and B domains; this is also the case when proven tyrosinases from fungal and mammalian sources are compared.

Two different PPO enzymes (monooxygenases) are now recognized in Solanaceae. The first is a 45-kD PPO, ubiquitous in all plant tissues, localized exclusively in plastids, and associated with thylakoids. No specific substrate has been assigned to it and no physiological role agreed upon; it is unique in that no evidence for a precursor protein has been found (for review, see Vaughn et al., 1988). The tomato PPO 59-kD protein, with its 66.3-kD precursor, is only remotely related —if at all—to the ubiquitous 45-kD enzyme described by Vaughn et al. (1988). P2 cloned sequences recognize only one species (~2 kD) of mRNA; of 16 clones isolated so far from cDNA libraries of flowers or *anantha* meristems, none differs from P2/A or P2/B. Furthermore, no 45-kD protein is recognized by the antibody employed in this study. The antibody was raised against a denatured C-terminal portion of

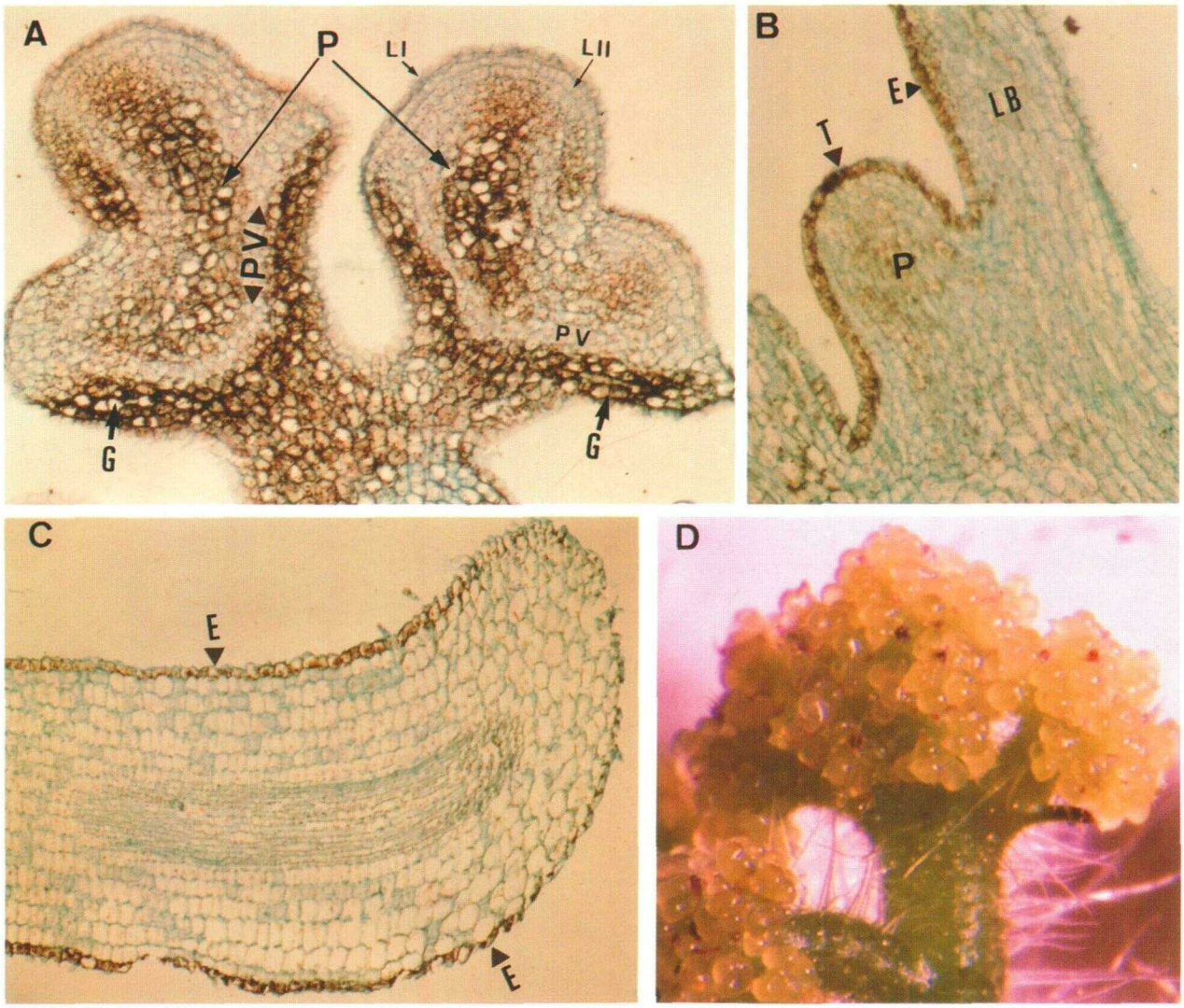

Figure 5. Immunogold Localization of the P2 Antigen in Meristematic Tissues of Floral and Vegetative Apices.

(A) *Anantha* floral meristem. A bifurcated floral primordia is shown.
(B) Longitudinal section of a vegetative shoot apex.
(C) Oblique section of a root apex.
(D) A branch of *anantha* inflorescence.
E, epidermal tissue; G, ground tissue; LI and LII, layered apical meristematic cells; LB, leaf buttress; P, pith cells; PV, provascular strands; T, tunica.

the P2 protein that forms a part of the lacZ-P2 fusion construct. It is impossible to categorically exclude interaction between P2 antibody and the 45-kD monooxygenase, but the patterns of upregulation shown in Figures 5, 6, and 8 most probably do not reflect the ubiquitous distribution of the 45-kD PPO.

A second PPO primarily associated with the so-called type A glandular trichomes and epidermal cells of the wild potato, *Solanum bethuletii*, has recently been described (Kowalski et al., 1990; Steffens et al., 1990). It is represented by a 67-kD precursor and a 59-kD mature polypeptide. These figures agree with the size of the P2 precursor and presumptive mature proteins. The potato 59-kD PPO is found in many Solanacea species, including tomato, and is claimed to be the only phenoloxidase in type A glandular trichomes and epidermal cells. Likewise, P2 is found in trichomes and in young epidermal cells of tomato (Figures 8A and 8B), and the tomato antibody recognizes an abundant antigen in the epidermal cells of potato leaves as well (Figure 8C). Its transient expression in the palisade cells of young tomato and

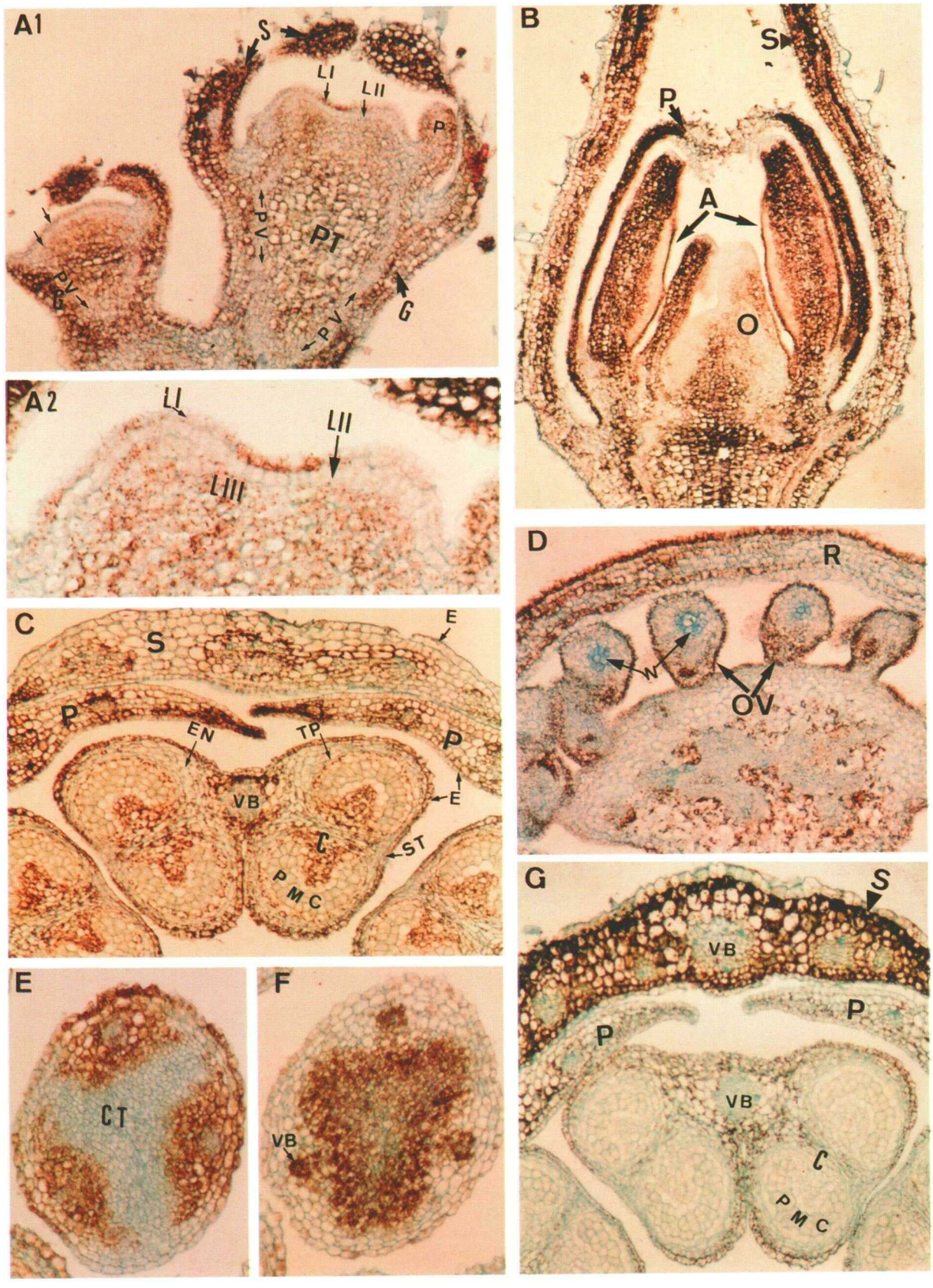

Figure 6. Spatial and Temporal Localization of P2 Antigen during Floral Development.

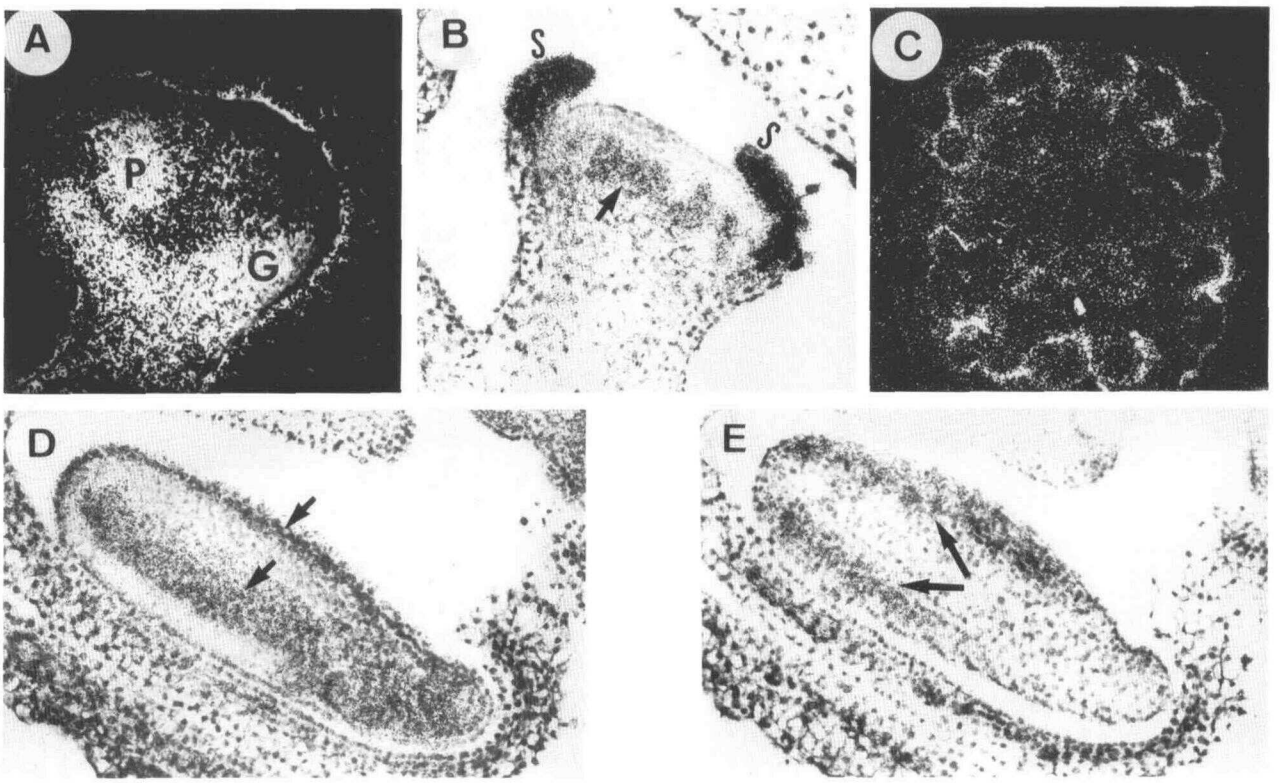

Figure 7. In situ Localization of P2 Gene Transcripts in Floral Meristems and in Normal Floral Organs.

(A) *Anantha* floral meristems. Dark-field photography. Compare with Figure 5A.
(B) Normal floral buds. Bright-field microscopy. Compare with Figure 6A.
(C) Ovary of 7-mm-long flower. Dark-field photography. Compare with Figure 6D.
(D) Localization of P2 transcripts in longitudinal sections of developing immature anther. Bright-field photography.
(E) Localization of dUTPase transcripts in longitudinal sections of developing immature anther. Compare with **(D)**. Bright-field photography.
Arrows in **(B)**, **(D)**, and **(E)** point to localization of silver grains.
G, ground cells; P, pith cells; S, sepal primordium. Exposure after 12 days.

Figure 6. (continued).

(A1) Longitudinal section through normal floral apices during emergence of sepal primordia.
(A2) Enlargement of a primordial floral apex. Assignment of LI to LIII after Stewart (1978 and references therein).
(B) Longitudinal section of young floral bud (1.2 mm long) after primordia of all floral organs have emerged but before differentiation of sporogenic cells is complete.
(C) Transverse sections of 7-mm-long Tiny Tim flower after differentiation of PMC. Sepals, petals, and anthers are shown. Only one out of five anthers with its two pollen sacs and the adjacent petals and sepals is shown.
(D) Part of a longitudinal section through the ovary of 7-mm-long Tiny Tim flower.
(E) Localization of P2 antigens in the style. Transverse section of 7-mm-long flower.
(F) Localization of dUTPase antigen in the style. Transverse section of the same style shown in **(E)**. Note the complementary patterns of P2 and dUTPase.
(G) Transverse section of 7-mm-long flower probed with anti-LH antibody (light-harvesting protein of photosystem II). Compare with **(C)**.
A, anthers; C, connective; CT, conducting tissue; E, epidermis; EN, endothecium; IN, integumental epidermis; N, nucellus; O, ovary; OV, ovules; P, petals; PT, pith cells; PV, provascular strands; R, pericarp; S, sepals; ST, stomium; TP, tapetum; VB, vascular bundles.

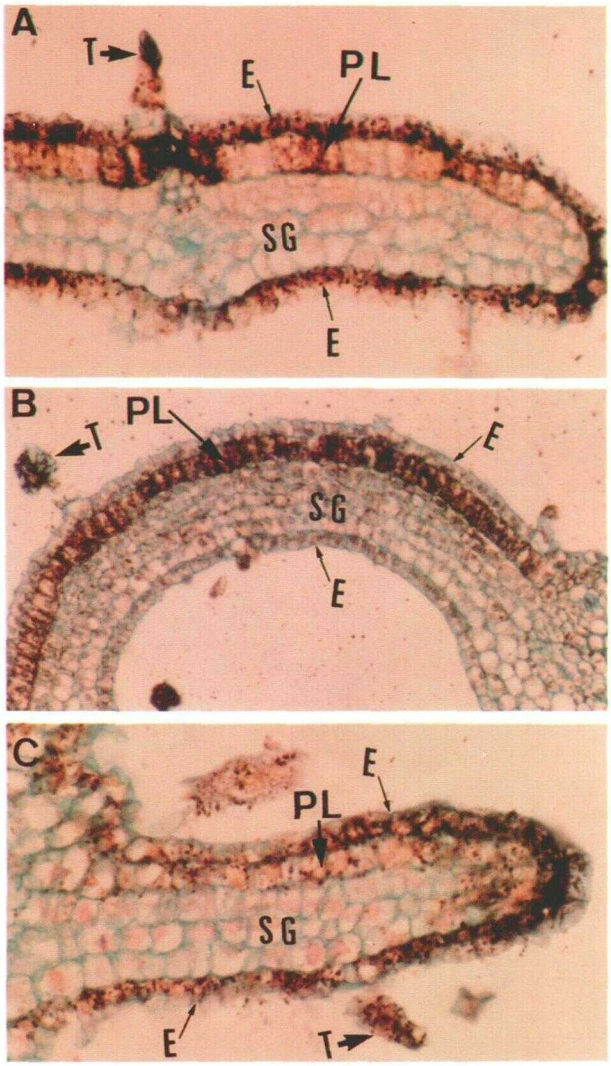

Figure 8. Differential Localization of P2 Antigen in Tissues of Young Tomato and Potato Leaves.

(A) A leaf primordium, 100 cells wide.
(B) Differentiated young tomato leaf.
(C) Primordium of potato leaf.
E, epidermal cells; PL, palisade cells; SG, spongy parenchyma cells; T, trichomes.

potato leaves (Figures 8B and 8C) would have gone unnoticed by Steffens et al. (1990) because these authors analyzed mature leaves. We do not know how similar the expression patterns are in floral primordia of tomato and potato, and it is possible that developmental expression of homologous genes in different species of Solanacea may be different. Excitatory postsynaptic potential synthase, for example, is upregulated in petals of petunia only (Gasser et al., 1988), and threonine deaminase is overexpressed 500-fold in floral organs of tomato but not in petunia or tobacco (Samach et al., 1991).

We conclude, therefore, that the P2 genes most probably code for a 66.3-kD tomato PPO, the plant monooxygenase synonymous with tyrosinase or phenoloxidase (Butt and Lamb, 1981).

Genomic Organization of the P2 Gene

Molecular evidence implies that at least two similar genes are transcribed in *anantha* meristems or in flowers of the Tiny Tim

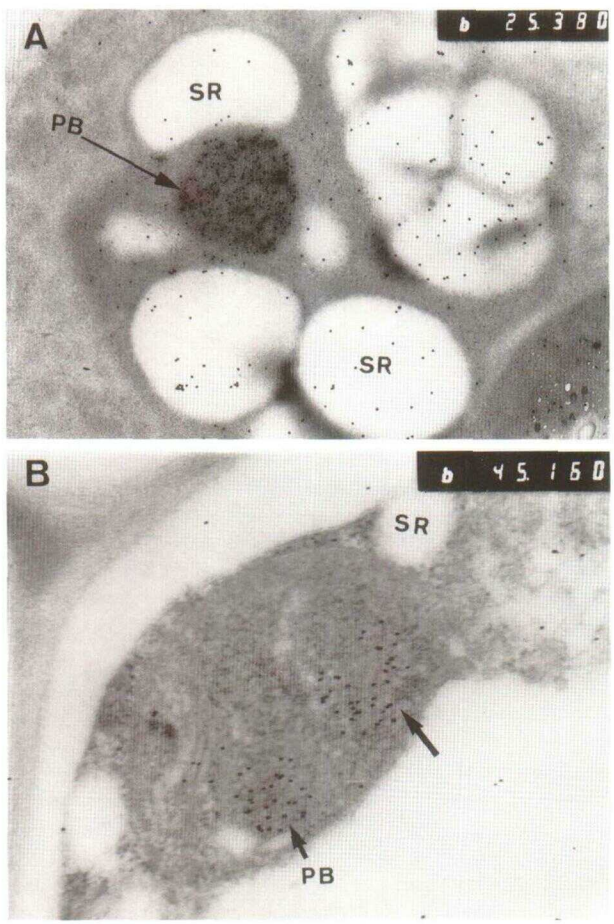

Figure 9. Immunogold Localization of P2 in TEM Sections of *Anantha* Ground Meristem Cells.

(A) Mature amyloplasts. Protein bodies labeled with anti-P2 antibody are most conspicuous in amyloplast of *anantha* meristem ground cells. Embedding was done in Epon.
(B) Accumulation of P2 antigen in proplastids (or etioplasts) with immature protein bodies. P2 antigen is often detected in the stroma of proplastids that are devoid of organized protein bodies as well. Embedding was done in LR White.
PB, protein body; SR, starch granules.

cultivar. DNA gel blot analysis, using cP2/B1 or gP2/A1 probes, provided no evidence for the presence of more than two genes. Restriction fragment length polymorphism (RFLP) mapping experiments, using P2/A and P2/B probes, unequivocally assigned both to one locus on chromosome VIII (D. Zamir, M. Abu-Abeid, and E. Lifschitz, manuscript in preparation) between RFLP genomic markers TG330 and TG127, five Morgan units from the latter and in close proximity to the Aps-2 gene. The coding sequences of the two genes in regions available for comparison are 93% similar. The 3′ nontranslated ends are also 85% similar and could not be used to distinguish the genomic sequences or mRNA species. Furthermore, the antibodies against the full-size P2 polypeptides and against the lac-Z fused portion of P2/A1 recognize the same polypeptides; probes of the two gene clones hybridize to transcripts of the same size (~2 kb). The sequence similarity and proximity of chromosomal loci suggest that they represent two extremely polymorphic alleles or, more likely, a small gene family that is the result of a recent gene duplication. In the developmental analysis, we refer to P2 as a single gene.

Another feature is the lack of introns in gene P2/B. We note with no further implications that, like the P2 genes, the zein storage genes of corn are devoid of introns (Hu et al., 1982; Pedersen et al., 1982), are clustered in adjacent chromosomal loci (Heidecker and Messing, 1986), and are targeted to protein bodies as well (Burr and Burr, 1976).

Developmental Expression of the Tomato PPO Gene

Comparisons of expression domains of genes active in several organs or during successive stages are likely to expose developmental realms with common properties (Lifschitz, 1988).

Detailed analysis of antigen distribution in vegetative and floral meristems reveals an intricate pattern of gene regulation. In the shoot apex, P2 genes are upregulated in the peripheral tunica layer and in its derived dermal cells. Similarly, P2 is upregulated in the dermal tissue of the young roots (Figures 5B and 5C). This expression pattern evidently represents the common rather than diverse properties of the two meristems (for discussion, see Steeves and Sussex, 1989). In leaf primordia, high levels of P2 are found initially in protodermal cells (Figure 5B) derived from the peripheral tunica (or LI) layer. Subsequently, however, P2 genes are downregulated in the mature epidermal cells (although not in trichomes) of growing leaves and are activated in the differentiated palisade cells (Figure 8). The preferential expression of P2 (PPO) in palisade cells (Figure 8) and that of threonine deaminase in the spongy parenchyma cells of young tomato leaves (Samach et al., 1991) are good examples of differential regulation of nonphotosynthetic genes in bifacial leaves.

P2 displays a different distinct pattern of expression in floral meristems and during flower development, which is particularly evident in *anantha* meristems. The meristematic cells of the apical domes are the immediate progenitors of the provascular strands and of the parenchymal derivatives of the pith and ground tissues. P2 is expressed at low levels everywhere but is upregulated in the parenchymal derivatives of the apical cells and in the parenchymal cells of all emerging floral organs, though not in the provascular strands. Temporal and spatial patterns are then unique for each floral organ (Figure 6).

We suggest, therefore, that P2 activity in vegetative and floral organs of tomato is under the control of two different developmental systems and that the floral mode of regulation initiated in the early meristems and later evolved in all floral organs reflects developmental programs shared by all of them prior to final differentiation.

In the accompanying article, we describe the isolation and developmental expression of a meristem-related gene coding for the tomato dUTPase (Pri-Hadash et al., 1992). P2 and the dUTPase genes are expressed in a complementary manner in vegetative and floral meristematic domains, as well as in almost every organ in which both genes are expressed (Figures 6E to 6F and Figures 7D and 7E).

The complementary expression patterns in meristems and developing organs support the hypothesis that the activity of the two genes in tomato exposes two complementary, alternative developmental programs.

METHODS

Plant Material

Lycopersicon esculentum cv Tiny Tim (LA154) served as a major source of normal plant organs (Stevens and Rick, 1986). For RNA and proteins, flowers were harvested 2 to 4 days before anthesis. Young leaves were collected at ~10 to 15% of their final size. Arrested floral meristems of *anantha* mutant plants (Helm, 1951; Paddock and Alexander, 1952) were collected from inflorescences of ~5 cm wide. Tomato lines were kindly provided by C. Rick (University of California at Davis, Davis, CA).

Nucleic Acid Techniques and Tomato Libraries

cDNA expression libraries were constructed in λgt11 (Gubler and Hoffman, 1983; Huynh et al., 1984) from mRNA prepared from normal flowers or *anantha* floral meristems. A tomato genomic library was constructed from DNA partially digested by Sau3AI and cloned into the BamHI site of λEMBL4, as in Maniatis et al. (1982). Isolation of RNA and DNA was as referred to in Samach et al. (1991).

Isolation of P2 and Preparation of Antibodies

Tissues were homogenized in 25 mM NaCl/100 mM Tris-HCl, pH 7.4/1 mM phenylmethylsulfonyl fluoride (4 mL/g) five times for 20 sec in a Waring blender. Fractionation and analysis of the soluble proteins were described (Samach et al., 1991), and the filtered homogenate was centrifuged at 100,000*g* for 20 min. For P2, the 100,000*g* pellet

was dissolved in 2% SDS/2% βME/50 mM Tris-HCl, pH 7.4, boiled for 3 min, and subjected to one-dimensional SDS-PAGE (8%; Laemmli, 1970). The designated P2 polypeptide was electroeluted twice from gel slices in 0.3% SDS/0.1% βME/50 mM Tris-HCl, pH 7.4, and used in complete Freund adjuvant to immunize rabbits (200 μg per immunization). Subsequent immunization was carried out after 4 weeks, using P2 (100 μg) in an incomplete adjuvant.

Cytological Techniques

Antigen localization for the light microscope was performed using gold-labeled (5 nm) goat anti-rabbit antibody (Amersham International) as second antibody. Tissues were fixed in ethanol/acetic acid, 3:1 (v/v) for 24 to 48 hr at 4°C. Primary antibody at a dilution of 1:100 was applied to 8 μm of paraffin-embedded tissue. Staining was performed according to manufacturer's protocol, and slides were counterstained with alcian green and safranin (Yoel, 1983).

Electron Microscopy

Anantha floral meristems were fixed overnight in 1.5% glutaraldehyde in 0.05 M cacodylate buffer, treated with 1% OsO_4 dehydrated through a graded ethanol series, and embedded in Epon or LR White. Ultrathin sections (100-nm thick) were mounted on nickel grids for immunogold electron microscopy. Gold conjugated protein A (10 nm; Sigma) was used as a second antibody. Sections were stained with uranyl acetate.

In Situ Hybridization

In situ hybridization was carried out according to Cox and Goldberg (1988), with ^{35}S-labeled sense and antisense as probes. Tissues were fixed in FAA (3.7 formaldehyde: 50% ethanol and 5% acetic acid) embedded in paraffin, and 8-μm sections were hybridized overnight with 8×10^5 cpm of fragmented probe. Exposure time was 12 days.

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