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Specific Peroxidase Isoenzymes Are Correlated with Organogenesis[1]

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ABSTRACT

We have examined isoperoxidase patterns obtained from buffer-, salt-, and enzyme-extractable fractions and correlated them with histological changes in tobacco (*Nicotiana tabacum* L., cv Wisc. 38) 'epidermal' explants induced to produce either callus, vegetative buds, or floral buds. By utilizing a combination of extraction and electrophoretic procedures different from any hitherto used for this kind of investigation, we were able to resolve 47 isoperoxidases distributed between the three types of fractions. The majority of these isoperoxidases were common to all explants regardless of their developmental fate. Correspondingly, a number of histological changes were observed in all explants (*e.g.* the initiation of cell division by day 2, lignin deposition by day 4, and the formation of clustered tracheary elements by day 8). We have made correlations between 25 isoperoxidases and specific developmental events based on the time when certain isoperoxidases were detected relative to observed histological changes: 3 were correlated with desuppressed/sustained cell division, 3 to 6 with lignification/tracheary element maturation, 7 with callus formation, 1 with localized suppression of growth, 3 with determinate axial organization, 4 with leaf development, and 1 with stamen development. These results suggest that a continued investigation using this system could lead to a better understanding of the role of specific isoperoxidases in different developmental processes.

Organ initiation and development in plant explants involves the promotion of meristematic activity, its maintenance in some regions, and its concomitant suppression in regions of 'maturation.' The enzymes that may be crucial in the above are not sufficiently understood and warrant more thorough investigation. One enzyme of particular interest in this regard is peroxidase (EC 1.11.1.7). In separate studies conducted over nearly four decades, peroxidase activity has been correlated both with the initiation of meristematic activity (11, 30) as well as with the suppression of growth (18, 25), an extreme manifestation being terminal differentiation (*e.g.* tracheary elements). A partial explanation for the apparently conflicting roles of peroxidase in organogenesis lies in the occurrence of this enzyme in multimolecular forms, *i.e.* isoenzymes. Individual isoperoxidases may differ in their substrate specificity, pH optima, and distribution within cellular compartments, etc. (10, 12, 15). There is considerable evidence that different isoperoxidases are correlated with the activities of different plant growth regulators which primarily act to either promote or suppress growth, including terminal differentiation (3, 8, 17 *inter alia*). Similarly, there is a strong indication that different isoperoxidases may be involved in separate processes related to organogenesis (7, 14, 16, 22 *inter alia*). Nevertheless, no specific isoperoxidase has been identified, isolated, and characterized with respect to its possible role in organ initiation, development, or maturation/differentiation.

An excellent experimental system for correlating enzymic changes with cellular processes involved in the initiation, development, and maturation of plant organs *in vitro* has been described by Tran Thanh Van (27). We have adapted this system with very little modification for our investigation. The procedures used to extract and separate isoperoxidases by earlier workers varied considerably. None seemed completely satisfactory for our purposes. We therefore designed our own protocol. This protocol in conjunction with the experimental control of organogenesis afforded by Tran Thanh Van's system and a simultaneous histological examination of the tissues has enabled us to find a number of consistent correlations between specific isoperoxidases and specific developmental events.

As a result of our observation, that differences in the procedures we used to extract and to separate isoperoxidases could profoundly affect our ability to detect isoperoxidases related to specific morphogenetic events, we report fully the separate steps in extraction and the results of different electrophoretic conditions.

MATERIALS AND METHODS

Tissue Culture. A modification of the procedure reported by Tran Thanh Van (27, 28) was used. Thin layer explants (10 × 3 mm), consisting of the epidermis and underlying cortex, were taken from the basal portion (between the stem and the first flower) of the flowering branches of vigorously growing plants of *Nicotiana tabacum* L., cv Wisc. 38. Only inflorescences with a full-size, green terminal fruit were selected. Using sterile technique throughout, surface-sterilized tissue was excised and inoculated into 60 × 15 mm plastic Petri dishes containing an appropriate culture medium and the dishes sealed with 'Parafilm M.' The basal medium was that of Murashige and Skoog (24) macro- and microelements, supplemented with 0.55 mM myo-inositol, 0.33 mM thiamine·HCl, and 1% (w/v) bacto-agar. To induce organogenesis or callus formation, the basal medium was supplemented as follows: for VB[2] induction, 2 μM IAA, 20 μM BA, and 88 mM sucrose; for FB, 1 μM IAA, 1 μM Kin, 83 mM glucose, and 83 mM sucrose; for CF, 10 μM IBA, 0.1 μM Kin, and 73 mM sucrose. The pH of all media (including phytohormones) was adjusted to 5.1 with 0.05 N NaOH before autoclaving.

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[2] Abbreviations: VB, vegetative bud; FB, floral bud; CF, callus forming; Kin, 6-furfurylaminopurine; IBA, indole butyric acid; PER, peroxidase; TE, tracheary element; isoPER, isoperoxidase.

Culture Conditions. Cultures were incubated in a room thermostatically controlled to maintain a temperature of 25 ± 2°C. Cultures on different media were subject to the following light regimes: for VB, 16 h photoperiod, 6 W m^{-2}; for FB, continuous light, 15 W m^{-2}; for CF, total darkness. The light source was a mixture of fluorescent and incandescent with equal wattage in the visible range. The total incubation period was 20 d. Experiments were repeated three to five times.

Tissue Sampling. To determine the minimum number of d explants had to be maintained under inductive conditions before a particular pattern of organogenesis was 'fixed,' samples were transferred from experimental medium to phytohormone-free medium at 24 h intervals during the 20 d incubation period.

To perform histological and electrophoretic analyses, explants were sampled at the start and every 2 d thereafter.

Histology. Explants were fixed *in vacuo* at 4°C in either 3% gluteraldehyde or a mixture of 1% gluteraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2 to 7.4. They were rinsed with the same buffer, ice-cold, until all traces of aldehyde were gone and stored at 4°C until dehydration. Rapid dehydration using acidified 2,2-dimethoxypropane (20) was followed by an acetone, tertiary butyl alcohol series, and paraffin embedding. Tissues were sectioned at 15 μm on a rotary microtome and stained with 0.05% w/v aqueous toluidine blue.

Line drawings were based on microscopic observation and photomicrographs.

Extraction Procedures. All of the following were carried out at 4°C unless otherwise indicated. The lyophilized tissue was ground in a prechilled mortar and pestle in 20 times its weight (1 volume), in a buffer containing 50 mM Tris·HCl, 0.5 M sucrose, 10 mM $MgCl_2$, and 6 mM mercaptoethanol, at pH 7.2. The slurry was centrifuged 10 min at 13,500*g*; the resultant supernatant fluid was the soluble fraction. The pellet was washed twice in 2 to 4 volumes of 1% Triton X-100, 6 times in 2 to 4 volumes distilled deionized H_2O, each time followed by a 10 min centrifugation. The pellet was washed twice in 0.5 volume 1 M NaCl to obtain the ionically bound fraction (the supernatant fluid). The pellet was again washed in 2 to 4 volumes of solution: twice in 0.5 M $NaHCO_3$, 6 times in 1 M NaCl, and 3 times in H_2O. Then the pellet was incubated overnight at 25°C in 0.1 M sodium acetate buffer (pH 5.5), containing 0.5% (w/v) cellulase and 2.5% (w/v) pectinase (both ICN Pharmaceuticals). This was repeated. The combined supernatant fluids were the covalently bound fraction. All three extraction fractions were dialyzed in 2 L of 0.025 M borate buffer (pH 8.0). Dialysis was especially necessary for good resolution of the ionically bound fraction. The three fractions, concentrated via lyophilization, were rehydrated just before using for electrophoresis.

Electrophoresis. Thin layer horizontal slab polyacrylamide gel electrophoresis was performed using an LKB 2117 Multiphor apparatus. The slot-former was hand crafted to allow the samples to be placed in the middle of the gel (25 cm × 11.5 cm × 2 mm), perpendicular to the long axis. Water in the cooling plate was at 10°C. Acidic and basic gels, polymerized with ammonium persulfate, both consisted of 7.5% acrylamide. Acidic gels were made to a final concentration of 20 mM acetate ion (pH 4.5), while basic gels contained the same concentration of borate ion (pH 8.0). The buffer tanks contained the same buffer as that of the gel. Gels were prerun for 30 min to remove impurities. The sample and bromophenol blue, as an indicator, were placed in the sample slots and then subjected to a 10 min 'concentrating' phase, at 140 V, 10 mamp, before the ~5 h run at 500 V, 40 mamp. Six samples could be run at a time; horseradish peroxidase (Sigma, P-8000) was used as a marker.

Staining for PER. The gels were immersed in a freshly prepared solution consisting of *o*-dianisidine in 95% ethanol (1 g/L), diluted 1:1 (v/v) with acetate buffer (pH 4.5) (0.88 M sodium acetate plus 0.62 N acetic acid). After 30 min the above solution was drained off and replaced with a 0.03% H_2O_2 solution until the PER bands were visible. These were recorded immediately and then again after 30 min. Duplicate gels were stained using guaiacol. A 1% guaiacol solution in 28.5% ethanol was substituted for the *o*-dianisidine-ethanol solution in the above procedure. The banding pattern was recorded immediately, as this stain faded rapidly. Although guaiacol revealed fewer bands (43% fewer than *o*-dianisidine) it was used to ensure that PER isoenzymes with differing substrate specificities would be detected. The sequential application of substrate and then H_2O_2 allowed detection of more PER bands than did the simultaneous application of substrate and H_2O_2.

RESULTS

By transferring explants from inductive media to phytohormone-free media at 24 h intervals, it was determined that normal induction of both vegetative and floral buds required 6 d on inductive media. Outward manifestation of bud development was not visible until d 12, regardless of whether the explants were maintained on inductive media during the entire 20 d incubation period or were transferred to phytohormone-free medium after d 6.

A number of internal changes that occurred during the course of the experimental incubation period were observed in histological preparations. These are shown in Figure 1. Our observations are in agreement with those of Dien and Tran Thanh Van (6, 29) though they sampled their explants at different intervals.

Electrophoretic Analysis of Isoperoxidases. Even though aliquots of the same extracts were used, and the staining procedures were exactly the same, markedly different isoPER patterns were obtained with the two buffer systems we tried. With borate buffer (pH 8.0) only two-thirds as many isoPER bands were revealed compared to those detected with acetate (pH 4.5) buffer. An example of this can be seen by comparing Figure 3 with panel D of Figure 2. More important for our objectives, few (if any) of the isoPER detected in the basic, borate-buffered gels could be correlated with specific events in VB or FB formation or sustained callus growth.

Twenty-five of the 47 isoPER detected in acetate-buffered gels appear to be correlated with histologically identified developmental events (Fig. 4). Nine of these were detected in all tissues regardless of inductive conditions. These are: C12, C15, A2, A3, and A6 of the soluble fraction; C12, C15, and A5 of the ionically bound fraction; and C12 of the covalently bound fraction. The fact that these isoPER appeared at the same times and stained with approximately the same intensities in tissues incubated under the three different inductive regimes suggests that they may be correlated with the processes that were occurring simultaneously in zone 3 of all tissues (bottom, Fig. 4).

Correlated with callus proliferation were seven isoPER: C1 of the soluble fraction; C14, C2, C1, and A7 of the ionically bound fraction; and A5 and A6 of the covalently bound fraction. The last three could only be detected in bud forming tissues up until cell divisions could be observed in zone 3 after which they disappeared; in callus tissues they persisted throughout the incubation period. The band C1 (ionically bound) was also found for a period in VB tissues, when there was some limited callus formation there.

The isoPER correlated with bud initiation, (*i.e.* initiation of organized meristems) whether VB or FB, were: A4 of the soluble fraction C7 of the ionically bound fraction, and C4 of the covalently bound fraction. Vegetative bud-forming tissues had, in addition, four unique isoPER that were not found in FB tissues: C6 and C17 of the ionically bound fraction, and C1 and C9 of the covalently bound fraction. Only one isoPER appeared to be peculiar to FB forming tissues, C2 of the soluble fraction. It is noteworthy, however, that FB tissue was the only type in which C16, of the soluble fraction, was not detected.

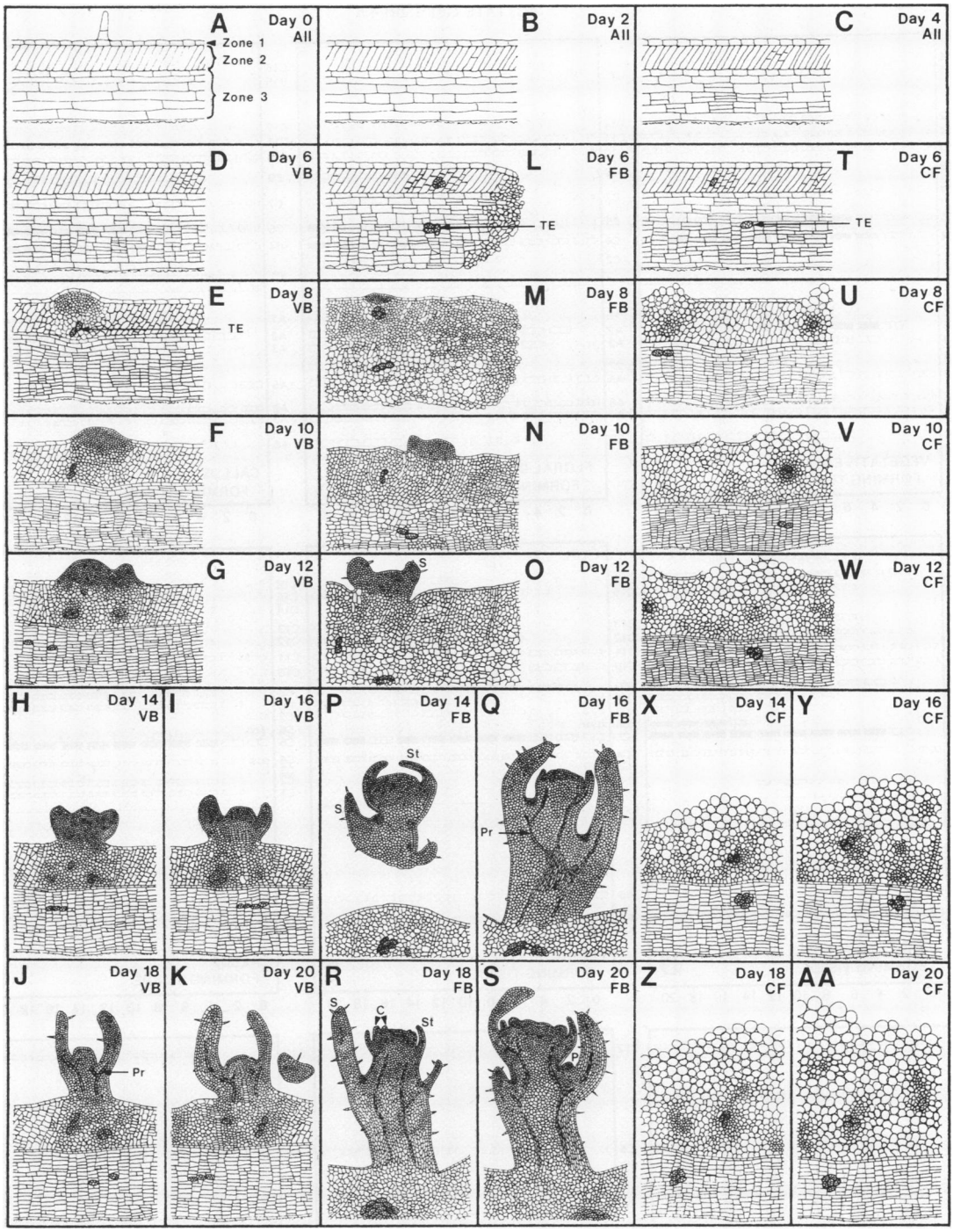

FIG. 1. Line drawings of longitudinal sections of developing tobacco epidermal explants. The sections are identified according to organogenetic regime (A–C, all regimes; D–K, VB formation; L–S, FB formation; T–AA, CF), and by number of days in culture (upper right hand corner). Magnifications are: A–D, ×54; E–G, ×42; H–K, ×36; L, ×54; M–O, ×36; P–S, ×30; T, ×54; U–V, ×32; W–Y, ×26; Z–AA, ×22. Specific features identified are: TE, tracheary elements; Pr, procambium; S, sepal; St, stamen; C, carpel; P, petal. Lignification of the lower (wounded) surface was apparent via differential staining by d 4 in all explants.

A summary of the correlations between isoPER and developmental events and/or processes is given in Table I.

DISCUSSION

There is an extensive body of literature that suggests that involvement of certain isoperoxidases may be important in plant development and differentiation. Yet it has been very difficult to identify specific isoPER and define their role in any specific developmental process. Previous attempts to use Tran Thanh Van's system to find such correlations (9, 26) were unsuccessful. We think the reasons for this may be 2-fold. First, the original studies were performed with electrophoretic buffers that were basic (~pH 8.4). We too were unable to detect any qualitative differences in isoPER correlated with developmental events when we used a basic, borate buffer. We suspect that basic buffers might interfere with the staining reaction. Most peroxidases have a pH optimum around 4.5 (19). When we used an electrophoretic buffer system that approximated the pH optimum of most peroxidases, the number of isoPER we were able to detect

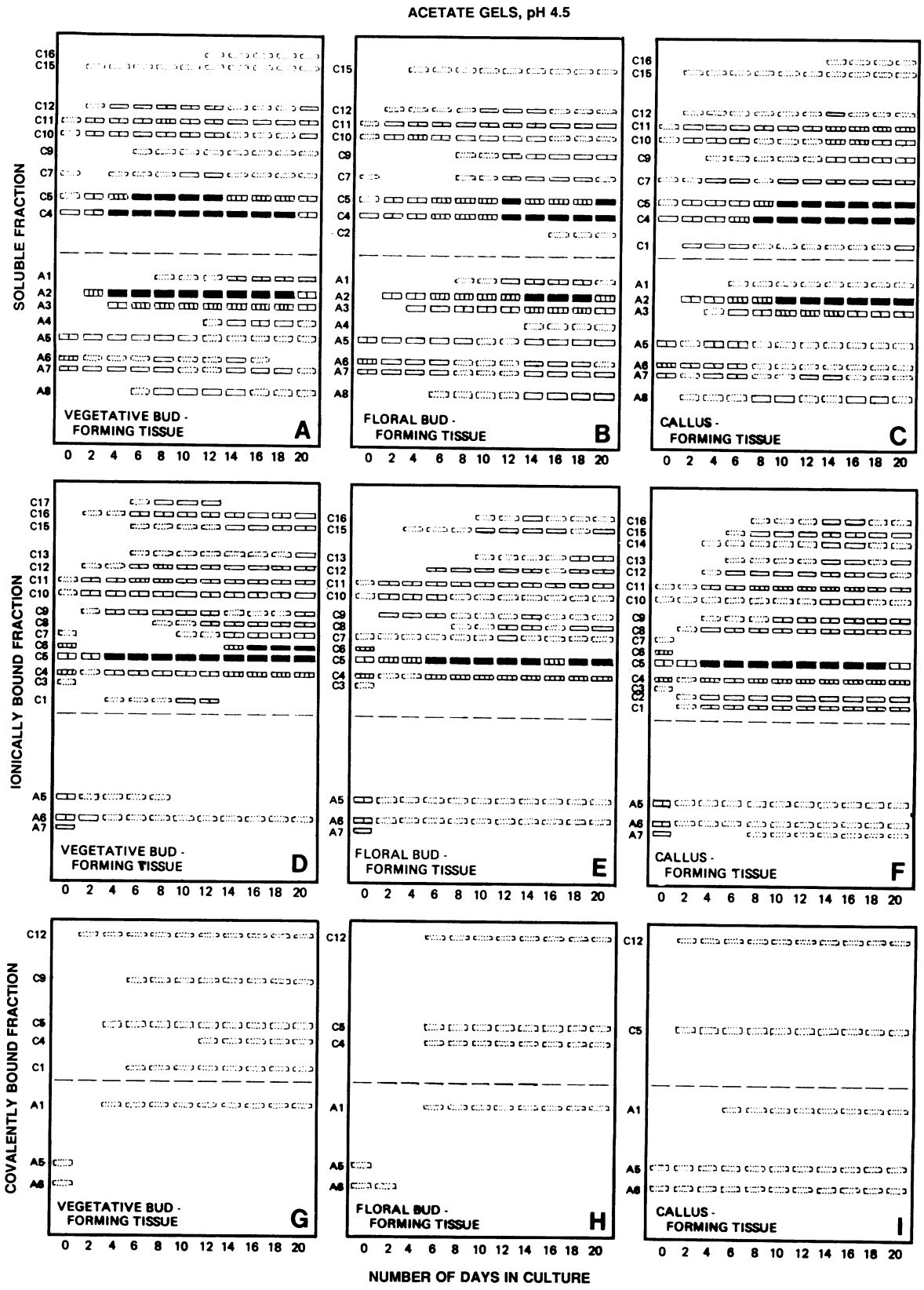

FIG. 2. Isoperoxidase bands as seen in acid-buffered (acetate, pH 4.5) polyacrylamide electrophoresis gels. Three extraction fractions for each organogenetic regime were examined. Specific isoperoxidases that were unique to one regime, or that correlated with events during organogenesis, are shown in Figure 4 and listed in Table I. Intensity of staining is shown in order, from most intense to faint: ■ ▥ □ ▭ ▭.

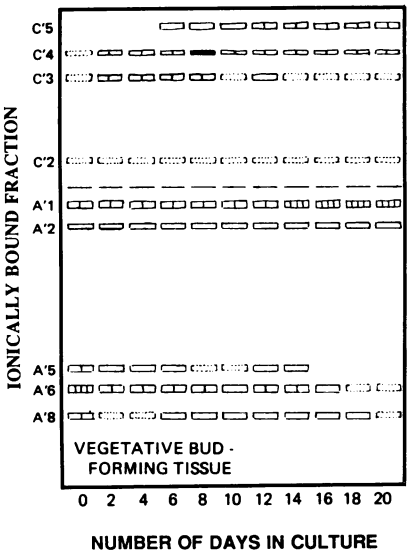

FIG. 3. An example of the isoperoxidase banding pattern as seen in base-buffered (borate, pH 8.0) polyacrylamide electrophoresis gels. Although the samples applied to the borate gels were aliquots of exactly the same extracts as those applied to the acetate-buffered gels (see D of Fig. 2) the banding pattern is extremely different. This is also in spite of the staining protocols being exactly the same. The number of isoperoxidases seen here is about half that seen in panel D of Figure 2; this is typical of all three extraction fractions in all three organogenetic regimes. Staining intensity is as in Figure 2.

increased by about 50%. More important, we were able to detect qualitative differences in isoPER that could be correlated with different developmental events. We think the second reason for this study yielding more isoPER was our use of a far more extensive extraction procedure. Indeed, two-thirds of the isoPER of interest were in the bound (nonbuffer-soluble) fractions. (See Berger *et al.* [5] for comments on extraction procedures.)

We perceived seven more or less broadly circumscribed categories of developmental change with which isoPER could be correlated. These serve as column headings in Table I. The assignment of an isoPER to any category is largely based on the data summarized in Figure 4. Some discussion of the rationale used in making the correlations as well as how our findings relate to those of others follow.

Correlation of isoPER with desuppressed and/or sustained cell division activity in explants cannot be unambiguously separated from that of lignification and TE differentiation. Evidence of both desuppressed cell division and lignification appeared between d 2 and 4 and both processes continued for the remainder of the experimental incubation period. Therefore, our correlation of any isoPER with either cell division or TE differentiation at this point must remain arbitrary.

It is because of their very early and persistent occurrence in tissues on all three inductive regimes that we think the three isoPER indicated in Table I may be correlated with desuppressed and sustained cell division in the explants. It may prove significant that all three behaved as anions in acetate buffered gels. As early as 1947, Van Fleet (30) described peroxidase as being present in all organized meristems and observed that sites of organ initiation and other presumptive sites of metabolic activity could be predicted from localized increases in peroxidase activity. More recently, Mäder (21) found that meristemoid formation in tobacco callus was accompanied by a sharp rise in activity in several isoPER. Gordon (12) found only one isoPER associated with wound healing in tomato stems. But since bound fractions were not analyzed and wounded tissues were not subjected to exogenous growth regulators, differences in the results presented here and those of Gordon may be expected.

With respect to lignification and TE differentiation, we are uncertain as to whether the cationic isoPER C12 and C15 that have similar electrophoretic mobility and are present in both soluble and bound fractions are the same isozymes in different states of solubility-insolubility. These two appear in the bound fraction between d 2 and 4 when the first differentiated TE were detected. We think, therefore, their presence may be better correlated with TE differentiation. The soluble isoPER A3 also appears at a time which suggests it is better correlated with TE differentiation than with cell division. Therefore, depending on whether the isoPER C12 and C15 of the three extraction fractions represent five different isoenzymes or two in different stages of becoming associated with cell walls, there are either 6 or 3 isoPER correlated with lignification-TE differentiation. That peroxidase is involved in lignification of cell walls has been well demonstrated by Harkin and Obst (13), among others. The probability that three rather than six isoPER are correlated with TE differentiation is suggested by the findings of Fukuda and Komamine (7). They observed only two isoPER correlated with TE differentiation of isolated *Zinnia* mesophyll cells. One peaked earlier just before lignification, the second at the time of lignin synthesis.

It should be clear that the correlations being made with respect to callus formation are with processes other than mitosis which clearly was not restricted to callus forming tissues in these experiments. Some of the more obvious features by which dividing callus cells differ from desuppressed cortical parenchyma and bud meristem cells are: cell enlargement, central vacuole formation, and lack of constraints with respect to the direction of cell enlargement and cell division. The seven isoPER we think correlated with callus formation are presumably correlated with processes not specific for mitosis or other features common to the three experimental regimes. Because the auxin levels in callus-inducing medium were 5 to 10 times higher than in bud-inducing, one or more isoPER detected in these tissues may be in direct response to high auxin levels. The production of specific isoPER in response to high auxin levels has been reported (8, 18). A comparative analysis of calli derived from different parts of tobacco plants enabled Bassiri and Carlson (4) to identify three isoPER in calli not present in the parent tissues.

There are eight isoPER correlated with some aspect of VB or FB initiation and/or development. Because three of these are found in both vegetative and bud-forming explants, we correlated the isoenzymes with general processes involved in the organization of shoot apical meristems rather than with those specific for vegetative or reproductive shoot meristems. What characterizes floral and vegetative bud-formation cannot be cell division or cell enlargement because these processes begin well before the buds are formed (Fig. 4) and continue to occur in the callus-forming explants. Quite the contrary, cell division and enlargement are for the most part constrained/suppressed in the buds, especially in the cells that make up the condensed axes. Ridge and Osborne (25) found a close correlation between the buildup of peroxidases and suppression of axial growth in pea plants.

A more striking example of suppression is found in the case of the soluble isoPER C2. This isoPER appears in VB and CF tissues on d 12 and 14, respectively. At the same time we find that TE differentiation and growth in general cease in zone 3 of these explants. The FB forming tissues continue to produce TE in zone 3, and never reveal band C2. Lee (17) found similar results in tobacco callus (looking only at the IAA oxidizing isoenzymes, however). When callus growth was suppressed by high levels of 2,4-D the activity of two isoenzymes was greatly increased. A somewhat similar phenomenon was found in tomato (23). A genetically induced repression of leaf lamina growth was correlated with increased peroxidase activity. Unfortunately, the isoPER composition was not examined.

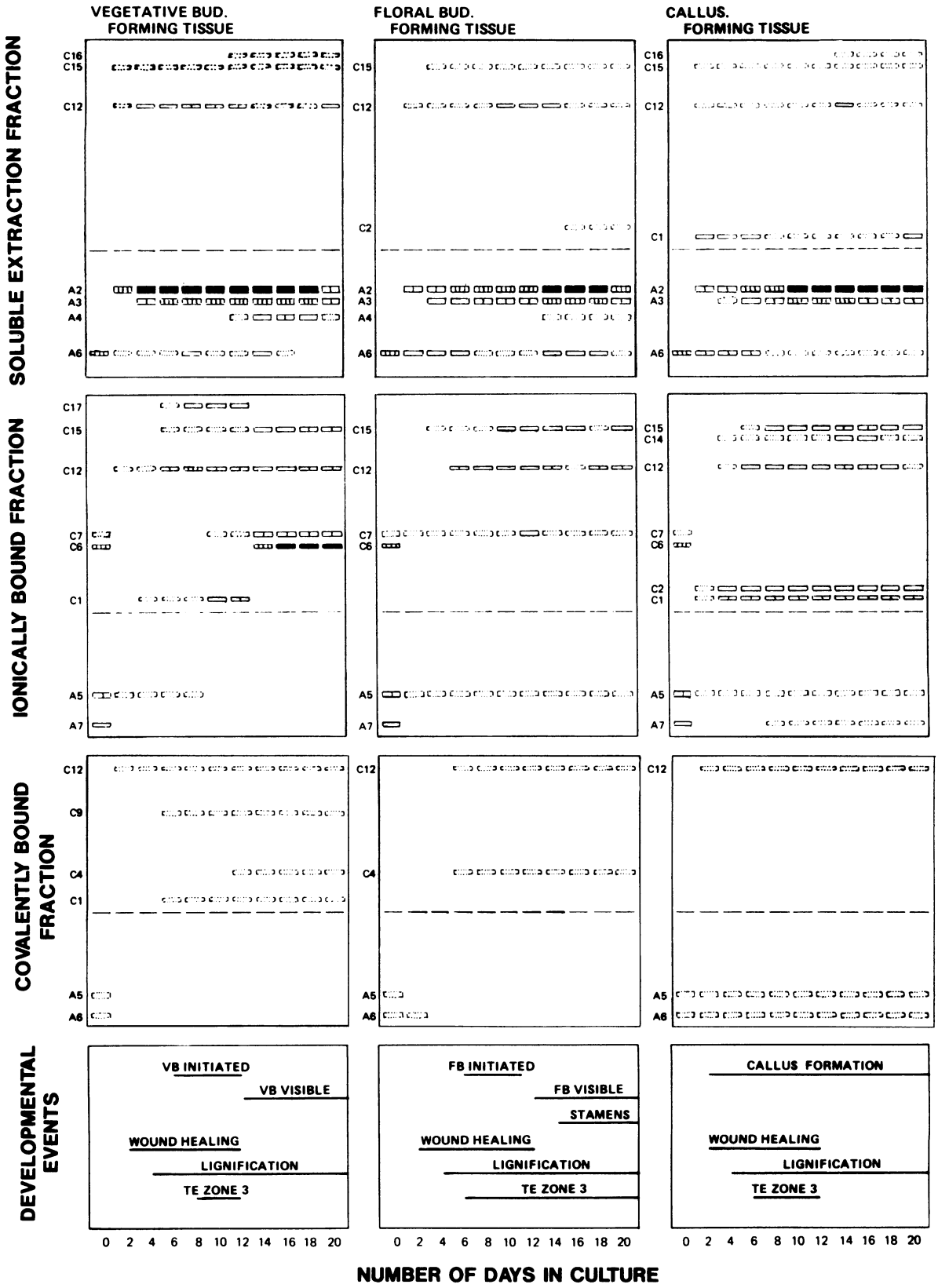

FIG. 4. Isoperoxidase bands of interest, as seen in acid-buffered gels, that occur in only one organogenetic regime or whose occurrence correlates with a specific event during organogenesis or explant development. The top three rows of figures show select isoperoxidases, the lowest row of figures indicates the time frame of events occurring in the developing epidermal explant that are of interest.

Table I. *Correlations between Isoperoxidase Bands and Developmental Processes Related to Organogenesis that were Experimentally Induced in Tobacco Epidermal Explants*

The prefixes A and C refer to the anionic and cationic behaviors of the isoPER in acetate buffer (pH 4.5), during electrophoresis using a horizontal slab polyacrylamide gel unit. Data are based on Figure 4.

	Wound Healing/ Desuppression	Lignin Synthesis TE Maturation	Callus Development	Localized Growth/ TE Suppression	Axial Organization/ Suppression	Vegetative Organ Development	Floral Organ Development
Soluble isoPER	A2 A6	A3 C12 C15	 C1 	 C16	A4 	 	C2
Ionically bound isoPER	A5	 C12 C15	A7 C1 C2 C14	 	 C7 	 C6 C17	
Covalently bound isoPER		C12	A5 A6 	 	 C4	 C1 C9	

Four other isoPER are correlated specifically with VB formation, presumably leaf development. Mäder *et al.* (22) similarly found that in going from shoots to callus and back to (regenerated) shoots, there were three isoPER that typified shoot tissues of tobacco.

The eighth isoPER associated with bud formation was found only in FB beginning at d 16 and showing a close correlation with stamen development (Fig. 4). Kahlem (14), using immunohistochemical procedures reported a 'flower specific' isoPER localized in the microspore and tapetal tissues of stamens in *Mercurialis annua*. A total of seven isoPER were reported correlated with specific stages of FB development by Koul and Bhargava (16).

The correlations we have been able to make based on the combination of methods used must perforce be tentative. Nevertheless, we consider attaining the kind of data presented here to be extremely useful in our search (1, 2) to identify factors important in the correlative control of suppression/desuppression in relation to organogenesis. This study enabled us to resolve 47 isoperoxidases and to choose from them a relatively small number for which a possible role with respect to suppression/desuppression of growth warrants further investigation.

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