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Spatial and Temporal Gene Expression Patterns Occur during Corm Development

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We investigated gene expression patterns that occur during taro corm development. Two-dimensional gel electrophoresis identified several different prevalent proteins that accumulate during corm development. Microsequencing studies indicated that some of these proteins are related to taste-modifying proteins, such as curculin and miraculin, and proteins found in other storage organs, such as sporamin and the Kunitz trypsin inhibitor. A curculin-encoding cDNA clone, designated as TC1, was identified that corresponds to a highly prevalent 1-kb corm mRNA. The TC1 mRNA accumulates during corm development, is more prevalent in corm apical than basal regions, and is either absent, or present at low concentrations, in other vegetative organs such as the leaf and root. In situ hybridization experiments showed that the TC1 mRNA is highly concentrated in corm storage parenchyma cells and is absent, or present in reduced concentrations, in other corm cells and tissues. Our results show that corm development is associated with the differentiation of specialized cells and tissues, and that these differentiation events are coupled with the temporal and spatial expression of corm-specific genes.

INTRODUCTION

Vegetative propagation is an important reproductive strategy in flowering plants that involves the generation and dispersal of genetically identical individuals, or clones. Generally, vegetative propagules are derived from axillary buds on aerial stems or from specialized underground storage stems, such as tubers, rhizomes, and corms (Richards, 1986).

Corms are perennial underground storage stems that have meristematic zones which can differentiate into roots, stems, and leaves (Raven et al., 1986). The taro plant has a well-differentiated corm and grows abundantly in tropical regions where it is used as a food source (Pate and Dixon, 1982). Corms accumulate storage proteins and carbohydrates that are involved in the maintenance of the developing sporophyte, as well as in supporting the morphogenetic events that are required for vegetative propagation. The deposition of corm storage products is under developmental control (Strauss et al., 1980; Wills et al., 1983); however, the mechanisms and genes that control storage product accumulation are not known.

In this study, we investigated the gene expression patterns that occur during taro corm development. Our results show that differentiation events occur early in corm development to establish a precise pattern of specialized cells and tissues within this underground stem. The differentiation of storage parenchymal cells is associated with the accumulation of prevalent proteins and mRNAs, some of which are related to taste-modifying and sweetener proteins present in other tropical plants. One mRNA, designated as TC1, encodes a corm globulin protein related to curculin. The TC1 mRNA is corm specific, accumulates preferentially in apical regions early in development, and is localized specifically within storage parenchymal cells. Our findings indicate that corm development is associated with the temporal and spatial expression of corm-specific genes.

RESULTS

Growth and Development of Taro Corms

Taro is the most prominent of the edible aroid crops. It has an herbaceous appearance with a few large leaves and one or more specialized underground stems, or corms (Wang, 1983). A typical taro plant is shown in Figure 1A. It contains a leaf blade (LB), leaf petioles (LP), roots (R), and corms at three different developmental stages (C2, C3, and C5). We divided corm development into five stages, designated as C1 to C5, on the basis of size, growth, external morphology, presence of young corms, and tissue patterns. Corms at stages C1 to C5 are shown in Figures 1B to 1E, and bright-field photographs of C1 to C4 corm sections are shown in Figure 2. Developmental markers characteristic of each stage of corm development are summarized in Table 1.

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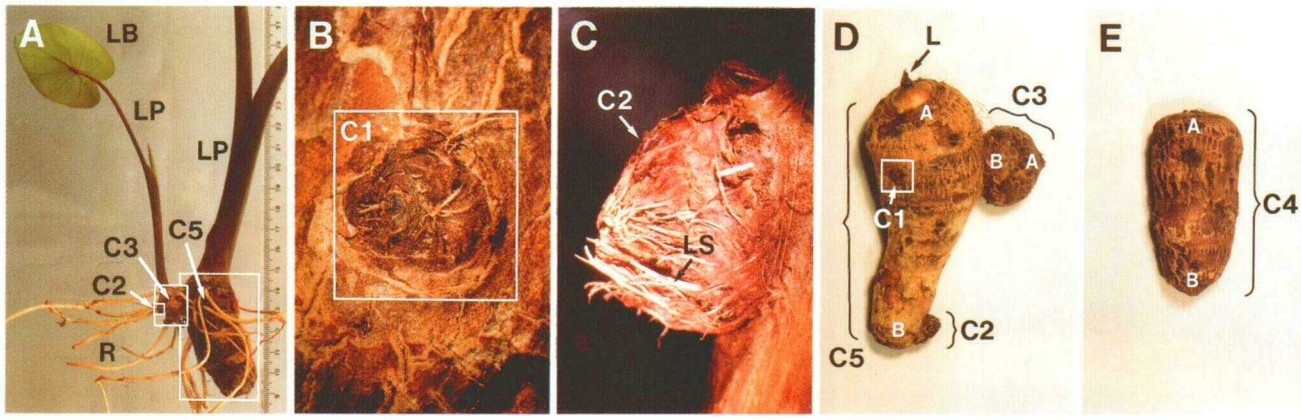

Figure 1. Taro Corm Development.

(A) A taro plant containing corms at developmental stages C2, C3, and C5. R, LP, and LB refer to root, leaf petiole, and leaf blade, respectively.
(B) A corm at developmental stage C1.
(C) A corm at developmental stage C2. LS refers to leaf scales.
(D) Corms at developmental stages C1, C2, C3, and C5. L designates the site of leaf emergence. A and B refer to apical and basal regions, respectively.
(E) Corm at developmental stage C4. A and B refer to apical and basal regions, respectively.

C1 corms differentiated on the surface of stage C3 to C5 corms and were protected by scale-like leaves (Figure 1B). Corms at this stage were usually less than 2 months old and were small, having a diameter of less than 10 mm (Figures 1B and 1D). Corms at stages C2 and C3 were also attached to older, stage C5 corms (Figures 1A and 1D). By contrast, stage C4 corms were not attached to the C5 corms and grew independently (Figure 1E). C5 corms represented the terminal stage of corm development and were derived from growth and expansion of stage C4 corms (Figures 1A and 1D). Leaves and roots were observed only in the apical regions of corms at stages C3 to C5 (Figures 1A and 1D). In addition, differentiating stage C1 buds were observed only on corms at stages C3 and later (Figures 1B and 1D). Together, these data indicate that corms undergo specific programs of growth and development, and that events controlling the establishment and differentiation of corm meristematic zones are regulated temporally and spatially.

Differentiation of Corm Cells and Tissues

We studied the cells and tissues present within corms at stages C1 to C4 to obtain cell markers for corm development and to determine the relationships between clonal propagation activity and corm anatomical structure. Figure 2 shows bright-field photographs of transverse sections from corm apical regions (Figures 2A, 2B, 2C, and 2E) and basal regions (Figures 2D and 2F) at different developmental stages. The cells and tissues observed in these sections are summarized in Table 1.

Figures 2A and 2C show a differentiating C1 corm that is embedded within an older stage C3 corm. C1 corms were surrounded by an epidermis (Ep) and collenchyma cell layers (Co), and had prominent ground parenchyma cells (GP), storage parenchyma cells (SP), and pith parenchyma cells (P) that were organized in a typical stem pattern. Storage parenchyma was distinguished from other parenchymal cell types by the presence of starch grains. The vascular cells (VC) in the C1 corms were organized as a circle around the inner storage and pith parenchyma and were indicators of stem primary growth (Fahn, 1974). By contrast, Figures 2B to 2F show that at stages C2 (Figure 2B), C3 (Figures 2C and 2D), and C4 (Figures 2E and 2F) this pattern was modified. Collenchyma cells were no longer observed and the vascular cells were not organized in a prominent cylinder, but were scattered throughout the ground and storage parenchyma tissues. New cells and tissues, such as periderm (Pd) and mucilage ducts (MD), were present as markers indicative of stem secondary growth. In addition, all parenchymal cells appeared morphologically similar and storage parenchyma cells became the dominant corm cell type (Figures 2B to 2E). At stage C3 and later, roots (R) differentiated from meristematic cells adjacent to vascular tissue located in the storage parenchyma (Figure 2E). Root organogenesis was observed primarily in the apical regions of C3 and C4 corms (Figure 2E) as compared with the basal regions (Figures 2D and 2F). Similarly, corms differentiated from meristematic foci more frequently in the apical region at these stages (Figures 2A and 2C). Together, these observations indicate that cell, tissue, and organ differentiation events occur in a precise temporal and spatial pattern during corm development.

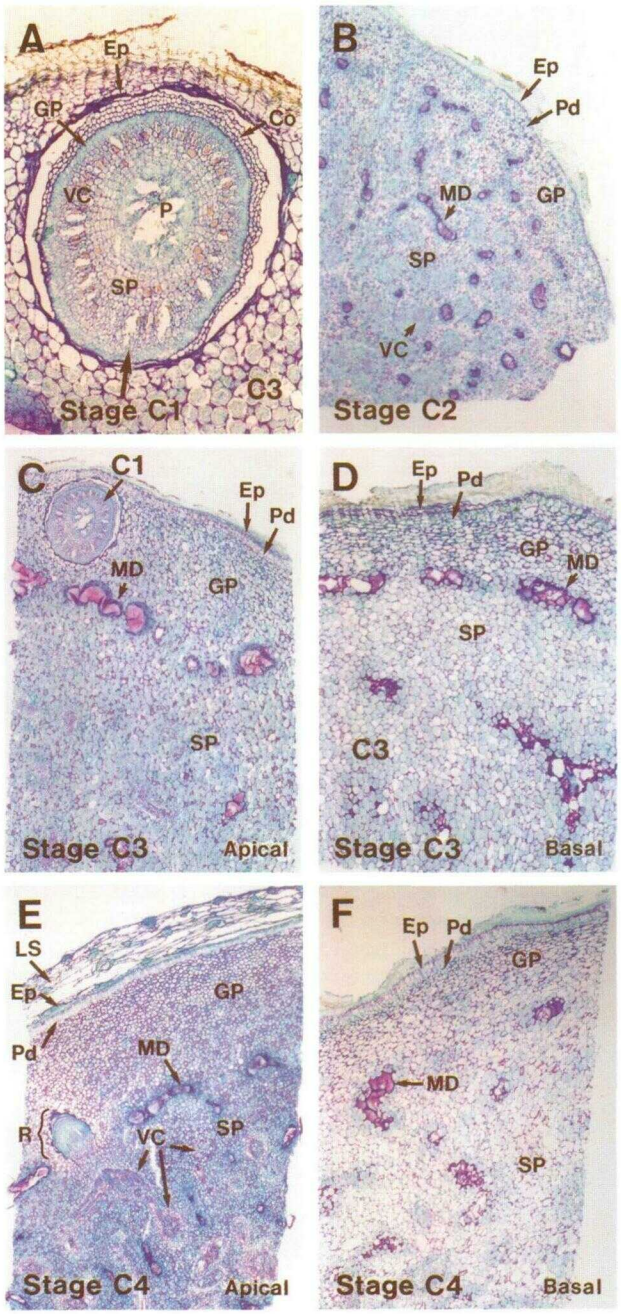

Figure 2. *Bright-Field Photographs of Corm Sections at Different Developmental Stages.*

Corms at different developmental stages were fixed, embedded in paraffin, and sliced into 10-μm transverse sections, as described in Methods. The fixed sections were stained with toluidine blue and photographed with bright-field illumination. MD, SP, GP, VC, R, P, Ep, Pd, Co, and LS refer to mucilage duct, storage parenchyma, ground parenchyma, vascular cells, root, pith parenchyma, epidermis, periderm, collenchyma, and leaf scales, respectively.

(A) *Transverse section of a corm at the C3 developmental stage.* Section was taken from the corm apical region (see Figure 1D). Arrow points to a C1 stage corm that has differentiated within the C3 corm shown in this photograph. Magnification, 40×.

(B) Transverse section of a corm at the C2 developmental stage. Section was taken from the apical region of the corm (see Figure 1D). Magnification, 40×.

(C) Transverse section of a corm apical region at the C3 developmental stage (Figure 1D). The emerging C1 corm shown at high magnification in **(A)** can be seen within this section. Magnification, 10×.

(D) Transverse section of a corm basal region at the C3 developmental stage (Figure 1D). Magnification, 10×.

(E) Transverse section of corm at the C4 developmental stage. Section was taken from the apical portion of the corm. An emerging root tip can be visualized within the C4 corm. Magnification, 10×.

(F) *Transverse section of a corm at the C4 developmental stage from the basal region.* Magnification, 10×.

Abundant Proteins Accumulate during Corm Development

We extracted proteins from corms at stages C1 to C5 (Table 1) and then analyzed these proteins by two-dimensional gel electrophoresis (see Methods). Figure 3 shows the two-dimensional gel electrophoresis patterns of silver-stained proteins at different periods of corm development. At each stage, a small number (<20) of highly prevalent proteins was observed. In addition, ~100 less prevalent protein spots were present. We divided the prevalent proteins into four different groups on the basis of their size, isoelectric point, and solubility in dilute salt solutions (Carneiro et al., 1990). Two of the groups contained globulin proteins, designated as G1 (14 kD; pI, 5.5 to 7.6) and G2 (22 kD; pI, 5.7 to 6.3). The other two groups contained albumin proteins and were designated as A1 (12 to 14 kD; pI, 4.5 to 5.3) and A2 (55 to 66 kD; pI 5.5 to 6.0). These groups contained between two (G2) and eight (A1) distinct protein spots each (Figure 3).

Figure 3 shows that the two-dimensional protein patterns changed during corm development and that changes occurred independently among the four protein groups. All four groups were present at the C1 stage. The G1 and G2 protein groups increased in prevalence from stages C1 to C3 and then declined in concentration at stage C4. Similarly, the A2 protein group accumulated between stages C1 and C2 and then declined later in corm development. By contrast, the A1 protein group declined continuously from stages C1 to C4. All protein groups were present in stage C5 corms; however, this was probably due to the presence of corms at different developmental stages that were attached to the C5 corms (Figures 1A and 1D). Each protein group also had a higher prevalence in the apical region of corms at stages C4 and C5 as compared to the basal region (data not shown). Together, these data show that the expression of genes encoding the prevalent globulin and albumin proteins is regulated with respect to both time and region during corm development, and that the accumulation patterns for the G1, G2, A1, and A2 proteins are not coordinated with each other.

Table 1. Major Events during Taro Corm Development

Stage	Diameter[a]	Weight[b]	Growth[c]	Tissues Present[d]	Morphological and Anatomical Markers
C1	<10	ND	<2	Ep,Co,GP,SP,VC,P	Observed as buds in stages C3 through C5 parental corms. Stem primary growth characterized by the presence of collenchyma tissue and distinct cell types in cortical and core parenchyma tissues. Cylindrical arrangement of vascular cells and tissues. Roots and leaves absent.
C2	10–20	1	2–3	Ep,Pd,GP,SP,VB,MD,P	Attached to parental corms at stage C5. Stem secondary growth characterized by the presence of periderm, mucilage ducts, and vascular bundles. Leaves and roots absent.
C3	20–30	10–30	3–6	Ep,Pd,GP,SP,MD,VB,P	Attached to corms at stage C5. Storage parenchyma heavily vascularized. Roots emerging from the storage parenchyma primarily from the corm apical region. C1 corms differentiating from cortical parenchyma. Leaves present.
C4	30–40	30–50	6–9	Ep,Pd,GP,SP,MD,VB,P	Corm grows independently. No significantly different anatomical markers compared to C3. C1 corms visible at the surface of the corm.
C5	>40	>50	>9	Not Analyzed	Corm has several cormel stages, such as C2 and C3, growing at the surface. Leaves and roots present.

[a] Range of size of >10 individual corms expressed in millimeters. Corms at each stage shown in Figure 1.
[b] Range of weights of >10 individuals expressed in grams. C1 weight was not determined (ND).
[c] Expressed in months.
[d] Ep, epidermis; Co, collenchyma; GP, ground parenchyma; SP, storage parenchyma; VC, vascular cells; P, pith; Pd, periderm; VB, vascular bundle; MD, mucilage duct. Information was taken from the histological sections shown in Figures 2 and 7.

Corm Globulins Are Related to Taste-Modifying and Storage-Organ Proteins

We sequenced the N-terminal amino acids of the corm G1 and G2 globulin proteins (Figure 3) to compare their sequence relationships and to determine whether they were related to other known proteins (see Methods). Figure 4A presents the N-terminal amino acid sequences of the G1a, G1b, G1c, G1d, G2a, and G2b proteins (Figure 3). No amino acid homologies were observed between the N termini of proteins in the G1 and G2 globulin groups (Figure 4A). By contrast, significant amino acid homologies were observed between the N termini

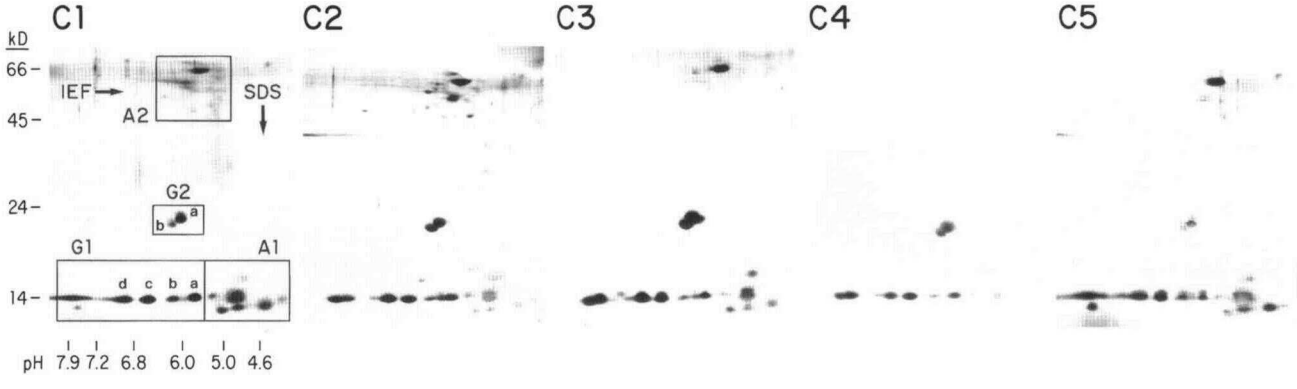

Figure 3. Two-Dimensional Gel Electrophoresis Patterns of Prevalent Corm Proteins.

Corm proteins from the designated stages were fractionated by two-dimensional gel electrophoresis and stained with silver, as described in Methods. Approximately 60 μg of protein was utilized for each stage. Isoelectric focusing (IEF) and SDS gel electrophoresis directions are indicated by arrows. Molecular weight markers are shown in kilodaltons (kD); C1 to C5 designate corm developmental stages (Table 1). G1, and G2, A1 and A2 designate globulin (G) and albumin (A) protein groups, respectively. a, b, c, and d refer to proteins within each group that were sequenced.

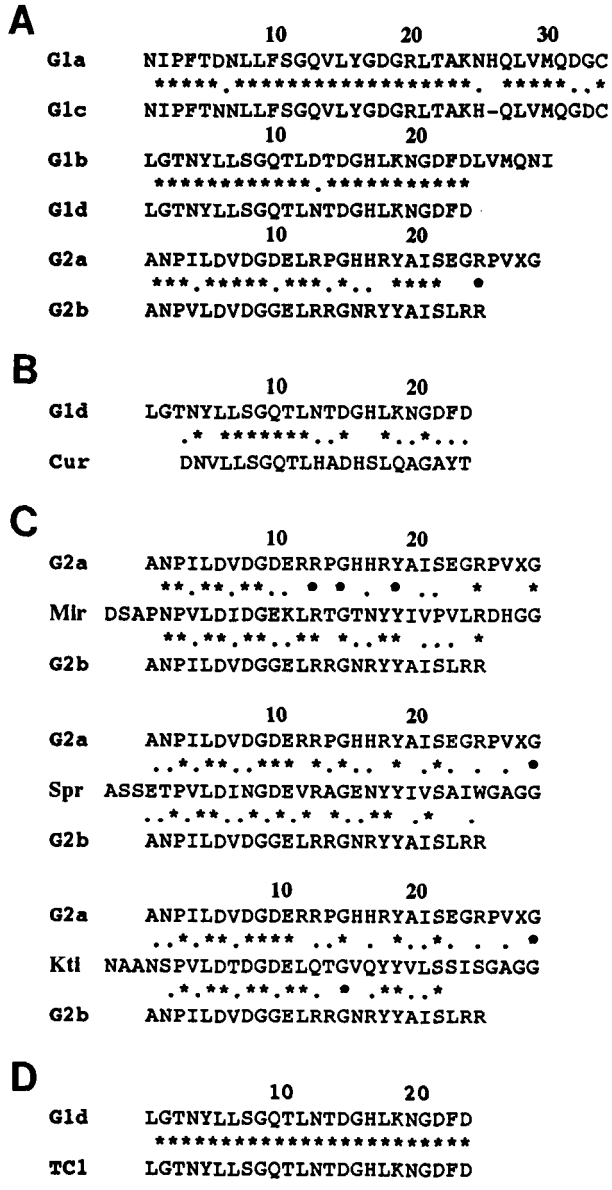

Figure 4. Amino Acid Sequence Homologies between G1 and G2 Globulin Proteins and Other Proteins

N-terminal amino acid sequences were obtained for the G1a, G1b, G1c, G1d, G2a, and G2b proteins shown in Figure 3, as outlined in Methods. The N-terminal amino acid sequences of curculin (Cur), sporamin (Spr), miraculin (Mir), and Kunitz trypsin inhibitor (Kti) were taken from Yamashita et al. (1990), Hattori et al. (1985), Theerasilp et al. (1989), and Jofuku and Goldberg (1989), respectively. TC1 represents the computer-translated amino acid sequence of the TC1 cDNA clone, which is homologous to the N-terminal amino acid sequence of G1d. Stars show amino acids that are identical to each other, dots show amino acids that are similar, and the dash indicates a gap in the amino acid sequence.

(A) Comparison of the N-terminal amino acids of G1 and G2 globulin proteins.

of proteins within a given group (Figure 4A). For example, the G1a and G1c N-terminal amino acid sequences are 88% identical to each other, whereas the G1b and G1d sequences are 96% identical. The G2a and G2b N-terminal sequences are 68% identical to each other. By contrast, the G1b/G1d and G1a/G1c subgroups had only 25% identity in their N-terminal sequences. Together, these data indicate that the G1 and G2 globulins comprise small families of unrelated proteins and that the G1 protein family contains two subfamily groups that are more closely related to each other.

We compared the corm G1 and G2 globulin amino acid sequences to those of other proteins found in the GenBank. Figure 4B shows that the G1 globulin group is related to the sweet-tasting protein curculin, found in fruits of the tropical plant *Curculigo latifolia* (Yamashita et al., 1990), and that the G1d N-terminal amino acids are 50% identical and 86% similar to those of curculin. By contrast, Figure 4C shows that the G2 globulin protein group is related to three different known proteins: the taste-modifying protein miraculin, found in fruits of the miracle-berry plant, *Richardella dulcifica* (Theerasilp et al., 1989); the sporamin storage protein present in tuberous roots of the sweet potato, *Ipomea batata* (Hattori et al., 1985); and the Kunitz trypsin inhibitor present in soybean seeds (Jofuku and Goldberg, 1989). For example, the G2a N-terminal amino acid sequence is 38% identical and 79% similar to the N-terminal amino acid sequences of sporamin and Kunitz trypsin inhibitor, and 38% identical and 69% similar to the miraculin N-terminal protein sequence. Together, these data show that the taro corm G1 and G2 globulin proteins are related to taste-modifying and sweetener proteins found in other tropical plants, as well as prevalent proteins present in other storage organs such as tubers and cotyledons.

Corm-Specific cDNA Clones Were Identified in a Stage C3 Corm Library

Figure 5A shows that poly(A) mRNA isolated from stage C3 corms contains three highly prevalent mRNAs, 0.6, 0.8, and 1 kb in size. These mRNA classes were visualized more clearly within a ^{32}P-cDNA population synthesized from stage C3 corm poly(A) mRNA (Figure 5A). We constructed a cDNA library of stage C3 corm mRNA and identified cDNA clones corresponding to these prevalent mRNA classes. One cDNA clone, designated as TC1, was found to be a representative of the 1-kb mRNA class. As shown in Figure 4D, sequence

(B) Comparison between G1 globulin and curculin N-terminal amino acids.

(C) Comparison between G2 globulins, miraculin, sporamin, and Kunitz trypsin inhibitor N-terminal amino acids.

(D) Comparison between the computer-translated TC1 amino acid sequence and the G1d globulin N-terminal amino acid sequence.

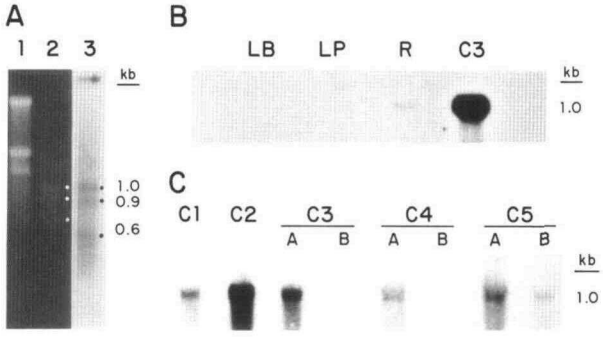

Figure 5. TC1 Gene Expression during Taro Corm Development.

Total and poly(A) mRNAs were isolated from leaf blades (LB), leaf petioles (LP), roots (R), and corms at designated developmental stages (C). The RNAs were fractionated by denaturing gel electrophoresis, transferred to nitrocellulose, and hybridized with a labeled TC1 cDNA plasmid probe, as described in Methods.

(A) Presence of prevalent sequences in corm mRNA and cDNA. rRNA (3 μg) and poly(A) mRNA (3 μg) from stage 3 corms were fractionated by denaturing gel electrophoresis and then stained with ethidium bromide to visualize RNA bands. ^{32}P-cDNA was synthesized from stage 3 corm poly(A) mRNA and then fractionated by electrophoresis on a nondenaturing agarose gel. Lanes 1, 2, and 3 contain rRNA, poly(A) mRNA, and ^{32}P-cDNA, respectively. Dots highlight the prevalent mRNA and cDNA classes. Sizes are given in kilobases (kb).

(B) Hybridization of the TC1 cDNA plasmid probe with leaf and root poly(A) mRNAs (5 μg). The C3 lane contains an equivalent amount of poly(A) mRNA from stage C3 corm. Autoradiogram was exposed for 24 hr.

(C) Hybridization of the TC1 cDNA plasmid probe with corm total RNAs (10 μg) at different developmental stages (C). A and B refer to RNAs isolated from the apical and basal corm regions, respectively (Figure 1). Autoradiogram was exposed for 96 hr.

analysis of the TC1 cDNA clone indicated that it encoded the curculin-related, G1d globulin protein. We hybridized a corm DNA gel blot with a TC1 cDNA plasmid probe to determine the representation of TC1 sequences in the taro genome. As seen in Figure 6, the TC1 probe reacted with single-copy intensity to three or four HindIII and EcoRI DNA fragments. These results, and the two-dimensional protein gels presented in Figure 3, indicate that the corm G1 globulin group is encoded by a small gene family.

TC1 Globulin G1d mRNA Is Corm Specific and Highly Regulated during Taro Development

We hybridized the TC1 cDNA plasmid with gel blots containing stage C3 corm, root, leaf blade, and leaf petiole mRNAs. Figure 5B shows that a strong 1-kb hybridization signal was obtained with corm poly(A) mRNA and a 100-fold weaker signal was observed with root poly(A) mRNA. By contrast, no detectable hybridization signals were observed with either leaf petiole or leaf blade poly(A) mRNAs (Figure 5B).

We hybridized the TC1 cDNA plasmid with corm total RNAs from different developmental stages, as well as with total RNAs from corm apical and basal regions (Figure 1). Figure 5C shows that the TC1 globulin G1d mRNA accumulated from stage C1 to C2 and then decreased slightly in prevalence at later stages of corm development. In addition, the TC1 globulin G1d mRNA was highly prevalent in corm apical regions at stages C3, C4, and C5 and was either absent or present at a much reduced level in the basal regions of corms at the same developmental stages (Figure 5C). Together, these data show that the TC1 globulin G1d mRNA is corm specific and that the accumulation of this mRNA is regulated with respect to both region and time during corm development.

TC1 Globulin G1d mRNA Is Present in Specific Corm Cell Types

We hybridized a labeled TC1 anti-mRNA probe with corm sections in situ to localize TC1 globulin G1d mRNA sequences within corm cell types at different developmental stages. Figures 7A to 7C show bright-field photographs of stage C1 and C3 corm transverse sections used for in situ hybridization. Figures 7D and 7E show that the TC1 anti-mRNA probe produced an intense hybridization signal over the storage parenchyma of stage C1 corms. No hybridization grains above background levels were observed within the pith, vascular tissue, ground parenchyma, or surrounding collenchyma cell layers. Figures 7D and 7F show that the TC1 anti-mRNA probe also produced an intense hybridization signal within storage parenchyma cells of stage C3 corms and to a lesser extent within ground parenchyma cells. No detectable hybridization signal was observed within mucilage ducts, vascular bundles,

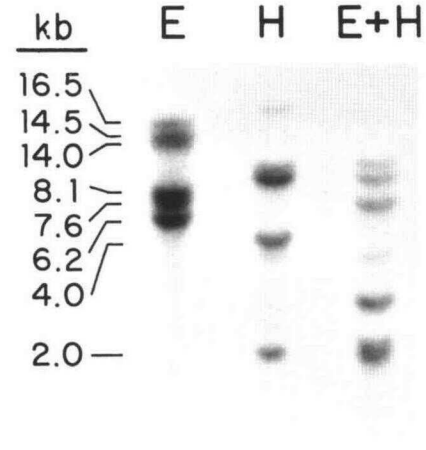

Figure 6. Representation of TC1 DNA Sequences in the Taro Genome.

Taro DNA was digested with either EcoRI (E), HindIII (H), or both enzymes (E + H), fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with a labeled TC1 cDNA plasmid probe, as described in Methods. Size calibrations are given in kilobases (kb).

periderm, or epidermis (Figure 7D). In addition, the TC1 anti-mRNA probe did not hybridize detectably with a differentiating root emerging from the stage C3 corm apical region (Figure 7F).

Figures 7G to 7I show results obtained with a TC1 mRNA control probe used to monitor background hybridization. No detectable hybridization grains were observed over any corm tissues (Figures 7G and 7H) or over an emerging root (Figure 7I). However, white patches were observed with this probe under dark-field illumination over storage parenchyma tissue (Figures 7G and 7I). Figures 7J and 7K show similar sections that were hybridized with the TC1 anti-mRNA probe and photographed by bright-field illumination. Dark hybridization grains (HG), representing RNA/RNA hybrids, were visualized specifically within storage parenchyma cells (Figure 7K). By contrast, Figure 7L shows a bright-field photograph of an analogous section that was hybridized with the TC1 mRNA control probe. Starch grains were visualized within the storage parenchyma cells (boxed area), but dark grains representing RNA/RNA hybrids were not present. This indicates that the white patches observed with the TC1 mRNA control probe under dark-field illumination were due to dark-field scattering effects by the starch grain–filled storage parenchyma cells. Together, these results show that the TC1 globulin G1d mRNA is localized preferentially within storage parenchyma cells of stage C1 and C3 corms and to a lesser extent within the ground parenchyma at the C3 stage of development.

DISCUSSION

Vegetative propagation is a widespread reproductive strategy among vascular and nonvascular plants (Silander, 1985). Although flowering plants may utilize different organs for somatic propagation, the establishment and dispersal of genetic clones in higher plants are carried out frequently by specialized underground stems, such as tubers, rhizomes, and corms (Richards, 1986). A corm is a short, swollen, modified stem that develops in a vertical position at or below ground level (Bell, 1991). Corms generate scale and foliage leaves, roots, and new corms from localized meristematic zones. These specialized stems are found among both monocots and dicots. In this investigation, we studied the morphological and gene expression patterns that occur during corm development.

Corm Clonal Propagation Is a Late Developmental Event

We divided corm development into five different stages (Figure 1 and Table 1). The C1 stage corm is typical of an early developing corm and is localized inside an older, parental corm. C1 corms are characterized by the presence of primary tissues and the absence of organogenesis and new corm propagules (Figures 1B and 2A). The C2 stage corms are present on the surface of C5 stage corms (Figures 1A, 1C, and 1D). Although no leaf or root primordia are present at the C2 stage (Figure 2B), cells and tissues that are markers of stem secondary growth are present (Figure 2B and Table 1). Later stages of development, represented by stages C3 through C5, are characterized by increasing independence from the parental corm (Table 1, Figures 1D and 1E). The deposition of storage products (starch and proteins) in parenchyma cells, the appearance of young corms within the cortical parenchyma, and the generation of roots and leaves contribute to the establishment of a fully independent, self-propagating corm (Figures 1D and 2E). These results suggest that corm development is characterized by two phases, an early phase, in which the cellular pattern of the corm is established, including lateral meristems which give rise to new corms later in development, and a late phase, which is characterized by the developing corm as an independent propagative center.

Clonal Propagation Occurs within Corm Cortical Parenchyma Tissue

Corm clonal propagation results from localized meristematic activities (Figures 1A and 2C). C1 stage corms are found exclusively among the cortical parenchyma cells of parental corms at stages C3 or later (Figures 2A and 2C). No developing corms were detected within the storage parenchyma or other tissue layers of the parental corm (Figures 2A and 2C). These observations indicate that the establishment of corm primordia is both spatially and temporally controlled during corm development. In addition, root and leaf development are uncoupled spatially from corm specification. Leaf differentiation is restricted to the upper apical region of the parental corm (Figure 1D), and root primordia are adjacent to vascular bundles embedded within the core, or storage parenchyma tissue (Figure 2E). These data indicate that (1) independent meristematic activities are responsible for establishing roots, leaves, and new cormels from the parental corm; and (2) a mature, differentiated corm is a complex structure, in which meristematic centers directly related to vegetative propagation are developmentally distinct from foci controlling the establishment of organs involved in the maintenance of the parental corm (e.g., roots and leaves).

Corm Globulin Proteins Are Related to Taste-Modifying and Sweetener Proteins Found in Other Tropical Plants

We identified four prevalent protein groups that are regulated during corm development (Figure 3). These protein groups are concentrated preferentially within corm apical regions (Figures 1 and 2), suggesting that they are localized primarily within the storage parenchyma (Figure 2). Sequencing studies

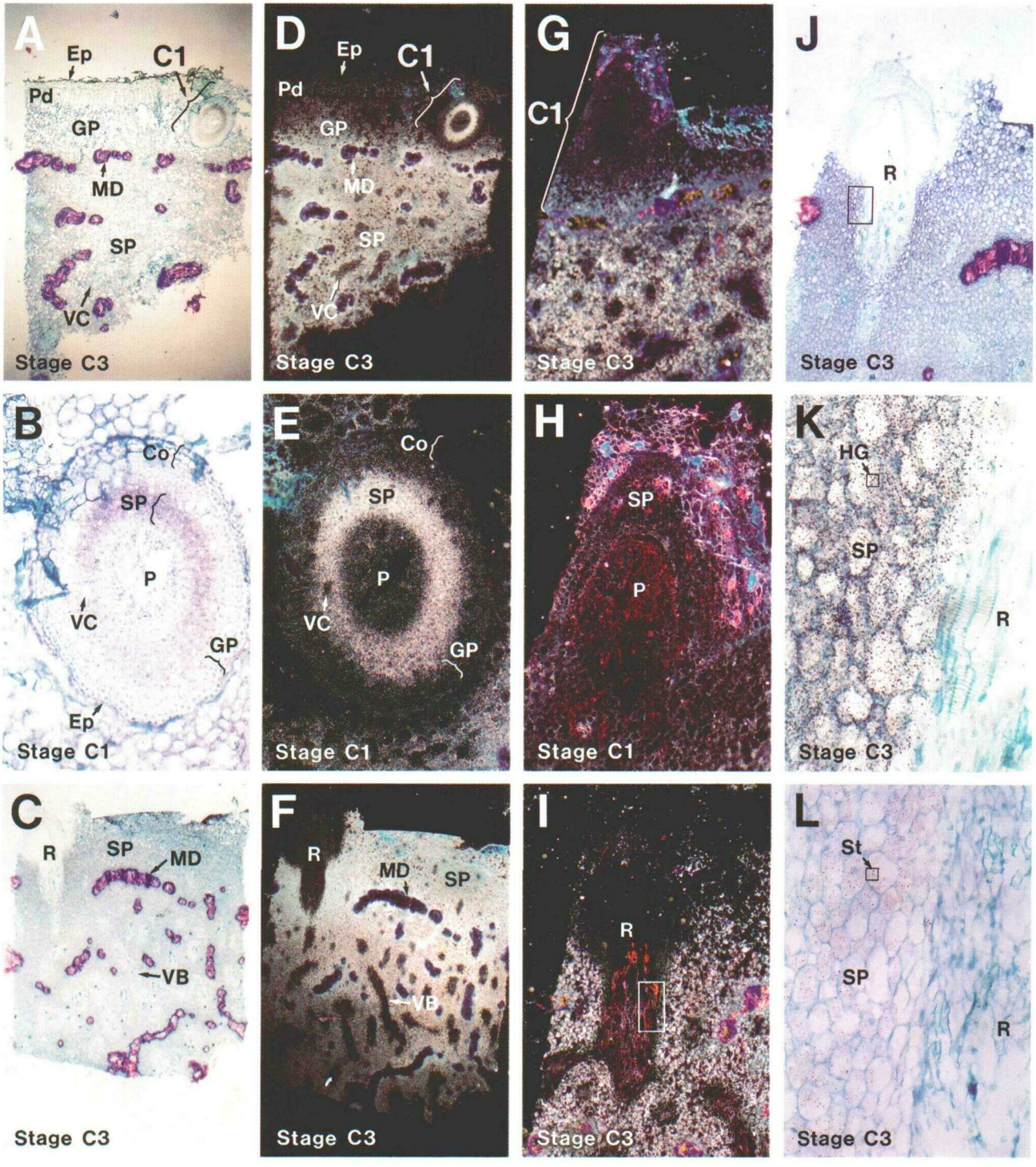

Figure 7. Localization of TC1 mRNA within Developing Corms.

Corms at the designated developmental stages were fixed, embedded in paraffin, sliced into 10-μm sections, and hybridized with TC1 single-stranded ^{35}S-RNA probes, as outlined in Methods. Corm developmental stages are described in Table 1 and shown in Figures 1 and 2. Ep, Pd, GP, MD, SP, Co, P, R, HG, VB, VC, and St refer to epidermis, periderm, ground parenchyma, mucilage duct, storage parenchyma, collenchyma, pith, root, hybridization grains, vascular bundle, vascular cells, and starch grains, respectively.

indicated that the G1 globulin proteins are related to the curculin sweetener and taste-modifying protein (Figure 4B; Yamashita et al., 1990). By contrast, the G2 globulins are related to the sporamin storage protein, the Kunitz trypsin inhibitor, and the miraculin taste-modifying protein (Figure 4C; Hattori et al., 1985; Jofuku and Goldberg, 1989; Theerasilp et al., 1989). The latter three proteins are all related to each other (Figure 4C).

What biological roles do the globulin proteins perform within the taro plant? Because the G1 and G2 globulins are related to proteins that either have a sweet taste (curculin) or that modify sour to sweet tastes (curculin and miraculin), it is possible that these proteins attract animal predators that disperse corms over large distances. It is also possible that the G2 globulins play a defensive role within the corm because of their relatedness to the Kunitz trypsin inhibitor, although we have no evidence that the G2 globulins possess trypsin inhibitor activity. Alternatively, the G1 and G2 globulin proteins might be storage proteins, analogous to the sporamin protein of tuberous roots and used for the growth and development of new corms and associated organ systems. Regardless of their exact role, the studies presented here indicate that the G1 and G2 globulin proteins are encoded by small gene families (Figures 3 and 6) that are highly regulated during the taro life cycle (Figures 3, 5, and 7).

Temporal, Regional, and Cell-Specific Gene Expression Patterns Occur during Corm Development

We identified cDNA clones representing prevalent corm mRNAs (Figure 5A) to study the gene expression patterns that occur during corm development. One cDNA clone, TC1, encodes the curculin-related G1d globulin (Figures 3 and 4D) and represents the prevalent 1-kb corm mRNA class (Figure 5A). The TC1 globulin G1d mRNA accumulates during early corm development and is concentrated preferentially within corm apical regions (Figure 5C), similar to that of the G1d globulin protein (Figure 3). In addition, the TC1 globulin G1d mRNA is not detectable within leaves and is present at a very reduced prevalence within roots (Figure 5B).

The in situ hybridization studies indicated that the TC1 globulin G1d mRNA is preferentially concentrated in corm storage parenchyma cells and is not detectable in most other corm cell types (Figure 7). The absence of a TC1 mRNA signal within roots emerging from stage C4 corms indicates that this mRNA probably has a reduced prevalence in most root cell types, rather than being highly concentrated in a few cells within the root (Figures 5B, 7F, and 7K). Together, the results obtained with the TC1 globulin G1d mRNA and those obtained by two-dimensional gel electrophoresis of prevalent corm proteins indicate that corm-specific gene expression programs occur during the taro life cycle and that these programs are regulated with respect to time, region, and cell type within the corm (Figures 3, 5, and 7). Clearly, the mechanisms responsible for regulating gene expression during corm development remain to be determined.

METHODS

Plant Material

Taro plants (*Colocasia esculenta*) were grown in the greenhouse and leaves, roots, and corms were harvested at the relevant developmental stages (Table 1).

Isolation and Analysis of Proteins

Proteins were extracted from corms at stages C1 to C5 (Table 1 and Figure 1) and then fractionated by two-dimensional gel electrophoresis, as described previously (de Castro et al., 1987). Isoelectric focusing was carried out using the procedures specified by Pharmacia. Silver staining of the two-dimensional gels utilized the Stratagene stain kit and followed the manufacturer's procedures.

Figure 7. (continued).

(A) to **(C)** Bright-field photographs of corm sections that were utilized for in situ hybridization with the TC1 anti-mRNA probe. The magnification factors were 10, 100, and 10× for the sections shown in **(A)**, **(B)**, and **(C)**, respectively.

(D) to **(F)** Dark-field photographs of the sections shown in **(A)** to **(C)**. White grains represent localization of RNA/RNA hybrids. **(D)** and **(E)** corm sections were exposed for 3 days, and **(F)** was exposed for 5 days. Magnification factors were 10, 100, and 10× for **(D)**, **(E)** and **(F)**, respectively. Arrow in **(D)** points to the C1 corm shown at higher magnification in **(E)**.

(G) to **(I)** In situ hybridization of corm sections with a TC1 mRNA control probe. Photographs taken by dark-field microscopy. Sections **(G)**, **(H)**, and **(I)** were exposed for 3, 3, and 5 days, respectively. The magnification factors for **(G)**, **(H)**, and **(I)** were 40, 100, and 40×, respectively.

(J) and **(K)** Bright-field photographs of C3 corm sections that were hybridized in situ with a TC1 anti-mRNA probe. **(K)** Section is an enlargement of the boxed region in **(J)**. The dark grains (HG) show the localization of RNA/RNA hybrids. Sections were exposed for 5 days. The magnification factors were 40 and 100× for **(J)** and **(K)**, respectively.

(L) Hybridization of a C3 corm section contiguous to those shown in **(J)** and **(I)** with a TC1 mRNA control probe. Photograph is an enlargement of the boxed area in **(I)** taken by bright-field illumination. St arrow points to starch grains (box) within parenchyma cells. Exposure and magnification factors were 5 days and 100×, respectively.

Proteins used for sequencing were fractionated by two-dimensional gel electrophoresis and then transferred overnight to Bio-Rad PVDF membranes, as outlined by Bio-Rad. Protein microsequencing was performed by an Applied Biosystem 4758 gas-phase protein sequencer at the UCLA Protein Microsequencing Facility using 500 pmoles per sample. Homology comparisons were carried out using the GenBank data base (Pearson and Lipman, 1988).

Isolation and Analysis of RNA

Polysomal mRNAs were isolated according to the procedures of Kamalay and Goldberg (1980). Poly(A) mRNAs were selected by oligo(dT)-cellulose chromatography (Davis et al., 1986). Total RNAs were prepared as described by Cox and Goldberg (1988). RNAs were size fractionated by denaturing gel electrophoresis following the glyoxal method of McMaster and Carmichael (1977), or the formaldehyde method of Berger and Kimmell (1987). RNA gel blot analysis was carried out according to the procedures of Davis et al. (1986) for the nitrocellulose and glyoxal gel analysis, or the procedures outlined by Schleicher & Schuell for the Nytran and formaldehyde gel analysis.

Construction and Screening of a Corm cDNA Library

A cDNA library was constructed for stage C3 corm polysomal poly(A) mRNA utilizing the the Amersham cDNA cloning system and manufacturer's procedures. Double-stranded cDNA was tailed with poly(dG) and annealed with poly(dC)-tailed, PstI-digested pBR329 plasmid DNA as described by Berger and Kimmell (1987). Transformation of *Escherichia coli* HB101 cells with the recombinant cDNA plasmids followed the protocol outlined by Bethesda Research Laboratories Life Technologies. Tet^R Amp^S recombinant cDNA clones were digested with PstI and analyzed by agarose gel electrophoresis. Plasmids containing cDNA inserts greater than 0.3 kb were chosen for further study. Ten cDNA clones were digested, labeled by nick translation using the Amersham kit following the manufacturer's procedures, and hybridized with C3 corm mRNA gel blots to identify clones corresponding to the prevalent mRNAs and cDNAs observed in Figure 6A. One clone, designated as TC1, represented the prevalent 1-kb corm mRNA class and was chosen for further analysis.

Corm Anatomical Sections and In Situ Hybridization

In situ hybridization experiments were performed as described by Cox and Goldberg (1988) and by Perez-Grau and Goldberg (1989). Corm histological sections were made according to the procedures of Cox and Goldberg (1988). Single-stranded ^{35}S-RNA probes were synthesized using the Promega pGEM transcription system.

DNA Isolation and Gel Blot Analysis

Taro DNA was isolated as described by Jofuku and Goldberg (1988). DNA gel blot analysis was carried out according to the method of Koltunow et al. (1990).

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