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A Novel Cell-Ablation Strategy for Studying Plant Development

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A novel cell-ablation strategy for studying plant development

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SUMMARY

The processes controlling the differentiation of plant cells are not well understood. Two alternative, but not mutually exclusive, mechanisms probably play a major role in plant-cell differentiation. One mechanism utilizes a position-independent, cell-autonomous differentiation process. The other, employs a position-dependent, cell–cell interaction process that requires signals from neighbouring cells. Cell ablation studies can be used to distinguish between these two models of plant-cell differentiation. In this article we outline a new cell-ablation strategy that utilizes promoters with distinct, but overlapping, cell specificities that are fused with cytotoxic and anticytotoxic structural genes. We present preliminary observations on how this strategy can be used to dissect the events controlling anther development.

1. INTRODUCTION

Approximately ten years ago a Discussion Meeting of the Royal Society was held on the subject of 'Differential gene expression and plant development' (Leaver *et al.* 1986). At this meeting, lectures were presented on a variety of topics, including the identification of cell-specific genes, gene regulation during seed development, hormone-induced gene expression, light control of plant-gene transcription, identification of cis-control regions mediating plant-gene transcription, and other gene expression-oriented topics (Leaver *et al.* 1986). The study of plant development from a mechanistic point of view was in its infancy, and most plant molecular biology laboratories were gathering the genes and probes needed to dissect the processes controlling plant-gene activity. A major issue then (and now) was the molecular events that programme the developmental-specific expression of plant genes. Genetic approaches to dissecting plant development, transposon- and T-DNA-tagging procedures to identify plant genes, and use of *Arabidopsis* as a model organism were not yet entrenched in the mainstream. The generation of transgenic plants by T-DNA transformation to investigate plant genes was just gaining momentum, and procedures such as β-glucuronidase (GUS) promoter fusions, particle-gun bombardment, antisense gene-inhibition, *in situ* mRNA localization, and the polymerase chain reaction (PCR) were either not yet invented or were not adapted for widespread use in plant molecular biology laboratories.

What a difference a decade has made! Technological advances in molecular biology procedures that can be applied to developmental studies have been spectacular (Davidson 1994), plant-gene transfer systems have become highly efficient (Gasser & Fraley 1992), and the exploitation of genetic systems in *Arabidopsis* and maize has provided new insights into the genes and regulatory pathways controlling a variety of plant developmental events. Genes have been isolated and characterized that play major roles in important plant processes, such as flowering (Coen & Meyerowitz 1991; Okamuro *et al.* 1993; Yanofsky 1995), light control (Millar *et al.* 1995), disease resistance (Mindrinos *et al.* 1994; Witham *et al.* 1994), embryogenesis (Goldberg *et al.* 1994; Jürgens *et al.* 1994; Meinke 1995), and hormone signalling (Chang *et al.* 1993). In addition, genetic screens in *Arabidopsis* and maize have identified scores of other genes that play major roles in plant development (Meinke 1995). Many of these genes will soon be isolated by using powerful tagging and/or map-based cloning strategies that are currently being employed by plant laboratories around the globe (Walbot 1992; Tanksley *et al.* 1989). Insights obtained from the molecular processes controlling plant development are being rapidly adapted for use in the applied sector and crops that have been genetically engineered for traits such as male fertility control (Mariani *et al.* 1990, 1992) and delayed fruit ripening (Oeller *et al.* 1991), among others, are being produced commercially. In fact, the future has never looked so exciting and promising with respect to understanding plant development from a mechanistic point of view and applying this knowledge to agriculture.

Plant development presents many novel processes and problems that are unique in the biological world (Walbot 1985; Goldberg 1988; Chasan & Walbot 1993). Some of these processes are listed in table 1. Plants have an alternation of spore- and gamete-forming generations, utilize a double-fertilization process, respond in remarkable ways to environmental signals such as light and temperature, have cells that remain totipotent and give rise to mature, fertile plants, undergo morphogenesis without cell movement,

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and possess meristems that differentiate continuously into major organ systems. Clearly, there is no shortage of fascinating developmental problems to tackle, investigate and solve.

One major unsolved problem is the process controlling the differentiation of plant cells. That is, how do highly specialized cells that establish the form and function of a plant acquire specific developmental fates? Not only is this process poorly understood, but, with the exception of trichomes (Oppenheimer *et al.* 1991; Hülskamp *et al.* 1994), the genes and regulatory networks mediating most plant-cell differentiation events have not yet been identified, isolated and studied. Although much progress has been made in understanding how plant organ systems are specified, especially those present in flowers (Yanofsky 1995), cell differentiation mechanisms after that point remain unknown. For example: (i) how do specific plant cells differentiate in space and time so that a three-dimensional organ is formed containing a precise pattern of tissues that perform specialized functions; (ii) what genes choreograph this process; and (iii) how do cell-specifier genes interact with the regulatory circuits that activate unique gene sets in the specialized cell types? In this article we outline how another development can be used to dissect plant cell differentiation processes and how a novel cell ablation strategy using chimeric cytotoxic and anticytoxic genes can be used to uncover the underlying mechanisms.

Table 1. *Unique features of higher plant development*

alternation of spore- and gamete-forming generations
spores produced by meiosis; gametes formed by mitosis
double fertilization resulting in a seed with a diploid zygote and triploid endosperm
major organ-forming events occur continuously from meristems after embryogenesis; embryo does not contain a germ line
embryos become dehydrated and enter a dormant state within the seed
differentiated cells can be totipotent and give rise to fertile, mature plants
cells are glued together by walls; morphogenesis occurs by differential cell division and expansion rates and by division in different cell planes; cell movement does not play a role in morphogenesis
environmental factors trigger specific developmental events

2. ANTHERS ARE AN EXCELLENT SYSTEM FOR STUDYING PLANT-CELL DIFFERENTIATION

The stamen is the male reproductive organ in higher plants. Stamens are specified autonomously from primordia that emerge on the third whorl of the floral meristem (Coen & Meyerowitz 1991; Okamuro *et al.* 1993; Yanofsky 1995). The stamen specification process is controlled by the interaction of three homeotic genes that are found in widely divergent plant taxa: AGAMOUS/PLENA, APETALA3/DEFICIENS1, and PISTILLATA/GLOBOSA (Coen & Meyerowitz 1991; Okamuro *et al.* 1993; Yanofsky 1995). Mutations in any one of these genes lead to flowers with either petals or carpels in their third whorl; that is, stamenless, male sterile plants (Coen & Meyerowitz,

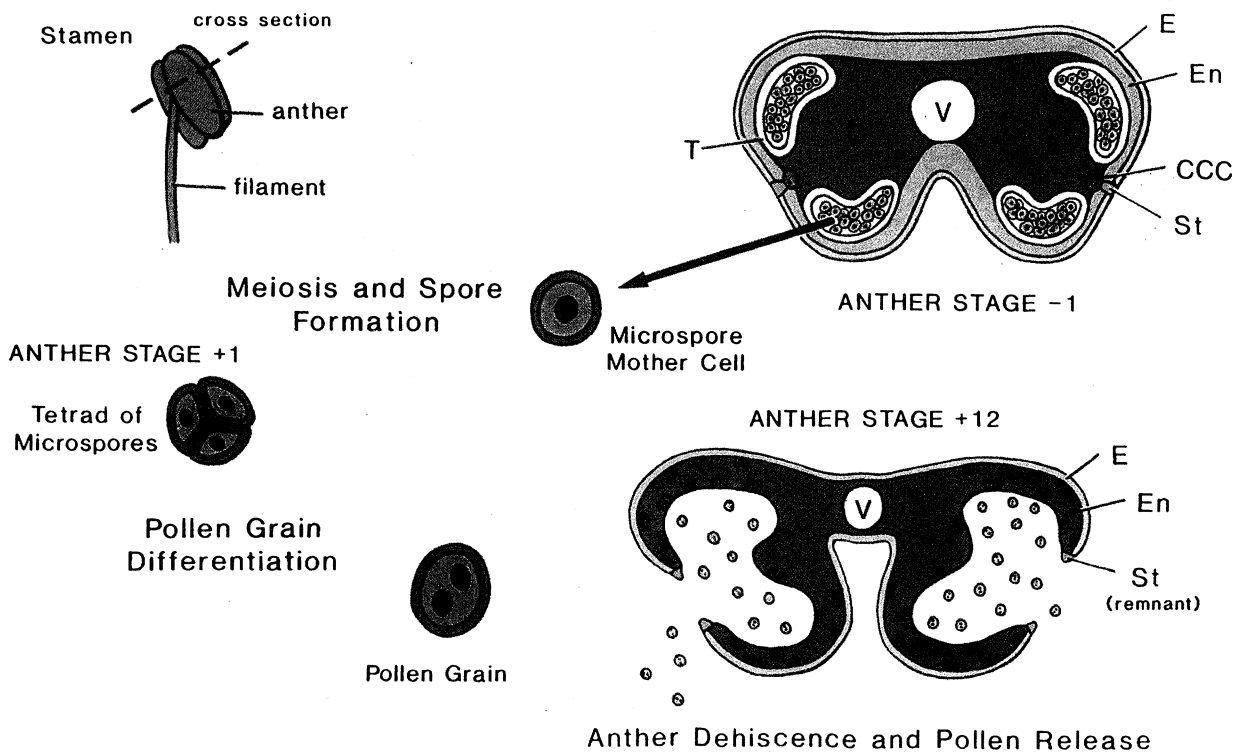

Figure 1. A generalized overview of tobacco anther structure and function. Schematic representations are based on light microscopy studies of anther development (Koltunow *et al.* 1990). C, CCC, E, En, T, St, and V refer to connective, circular cell cluster, epidermis, endothecium, tapetum and vascular bundle, respectively.

Table 2. *Tissue and cell types in a tobacco anther*

cell or tissue type[a]	function
reproduction	
microspore mother cells	meiosis; spore-formation; pollen development; sperm cell differentiation
tapetum	microspore release from tetrads; pollen wall formation; nutrients for pollen development
support and metabolism	
connective	anther structural integrity and morphology; connect anther to filament
epidermis	structure and support; gas exchange, prevent water loss
vascular bundle	connect anther to flower and filament; nutrient and water supply
support and dehiscence	
circular cell cluster[b]	dehiscence; calcium crystal formation
endothecium	structural layer of anther wall; fibrous band formation; dehiscence
middle layers	structure of anther wall; fibrous band formation; dehiscence
stomium	pollen release; dehiscence

[a] Cells and tissues present in a stage-1 tobacco anther (see figure 1). Adapted from Goldberg *et al.* (1993) and the histological studies of Koltunow *et al.* (1990).

[b] Prominent in solanaceous anthers. Not visibly detected in cruciferous anthers such as *Arabidopsis*, but may have functional counterparts. Also referred to as crystal-containing idioblasts (Trull *et al.* 1991), intersporangial septum (Bonner & Dickinson 1989) and hypodermal stomium (Horner & Wagner 1992).

1991). The stamen identity genes encode MADS-box-class transcriptional activators that set in motion a cascade of events required for stamen cell-differentiation and morphogenesis (Coen & Meyerowitz 1991; Okamuro *et al.* 1993; Yanofsky 1995). The nature of downstream genes and proteins that mediate critical events in stamen development are not yet known.

Figure 1 shows that the stamen consists of two compartments: the anther and the filament. The differentiation of the anther and filament appears to be controlled by distinct genes because mutant plants have been identified that have stamens with normal filaments but defective anthers (Chaudhury 1993; Goldberg *et al.* 1993; Chaudhury *et al.* 1994). The filament anchors the stamen to the flower and elevates the anther to a level above that of the pistil, or female reproductive system, so that self-pollination can occur. The filament consists primarily of vascular tissues that supply the anther with water and nutrients. It is not known what processes regulate filament growth and ensure that maximum elongation occurs just before flower opening and pollen release; however, growth hormones are known to play a role in this process (Biddenton & Robinson 1993).

As shown in figure 1, and summarized in table 2, the anther contains a variety of highly specialized tissues and cell types that are required for structure, metabolism and growth, microspore formation, pollen development, and pollen release at flower opening. These tissues form within specific regions, or territories, during anther development (see figure 1). The two bilateral halves of the anther, or theca, are joined together by connective tissue and are anchored to the filament by a centralized bundle of vascular tissue. Each theca contains two microsporangia, or locules, that are positioned across from each other and carry out male reproductive processes. Microspore mother cells differentiate within the microsporangia, undergo meiosis and form haploid microspores. The microspores divide by mitosis and differentiate into male gametophytes, or pollen grains, that contain the two sperm cells. Each microsporangia is surrounded by a layer of tapetal cells that is required for microspore release from tetrads, pollen wall formation, and pollen development (see figure 1). Targeted ablation of the tapetum by chimeric cytotoxic genes (Koltunow *et al.* 1990; Mariani *et al.* 1990, 1992; Paul *et al.* 1992), or mutations in critical tapetal-cell functions (Chaudhury 1993; Chaudhury *et al.* 1994), lead to pollenless, male sterile plants. Each theca also contains an outer wall of cell layers, designated as the middle layers and endothecium, that is contiguous to each microsporangia (see figure 1). These tissues provide structural support for the anther and play key roles in dehiscence and pollen release (see table 2). Positioned at the boundaries between the two microsporangia is the stomium and circular cell-cluster region that breaks at dehiscence and allows pollen grains to be released from the anther at flower opening (see figure 1). How differentiated cells and tissues form within specific anther regions and what gene regulatory networks control this process remain to be determined.

3. ANTHER DEVELOPMENT CAN BE DIVIDED INTO TWO PHASES: CELL DIFFERENTIATION AND DEHISCENCE

Anther development can be divided into two general phases (Koltunow *et al.* 1990; Goldberg *et al.* 1993). As shown in figure 2, morphogenesis and histodifferentiation events occur during phase 1 of anther development. The bilateral form of the anther is established, cell and tissue specification occur, microsporangia differentiate, and meiosis gives rise to haploid tetrads of microspores. The partioning of the stamen into anther and filament compartments also occurs during phase 1 (Koltunow *et al.* 1990; Goldberg *et al.* 1993). By the end of phase 1 a miniature anther has formed which contains all of the cells and tissues required for pollen grain development and release (see figures 1 and 2).

A dramatic switch in anther cell activities occurs during phase 2. The anther enlarges and is pushed upward in the flower by elongation of the filament (Koltunow *et al.* 1990). Microspores undergo mitosis and differentiate into pollen grains. Cell degeneration and death occur within the tapetum, connective, and

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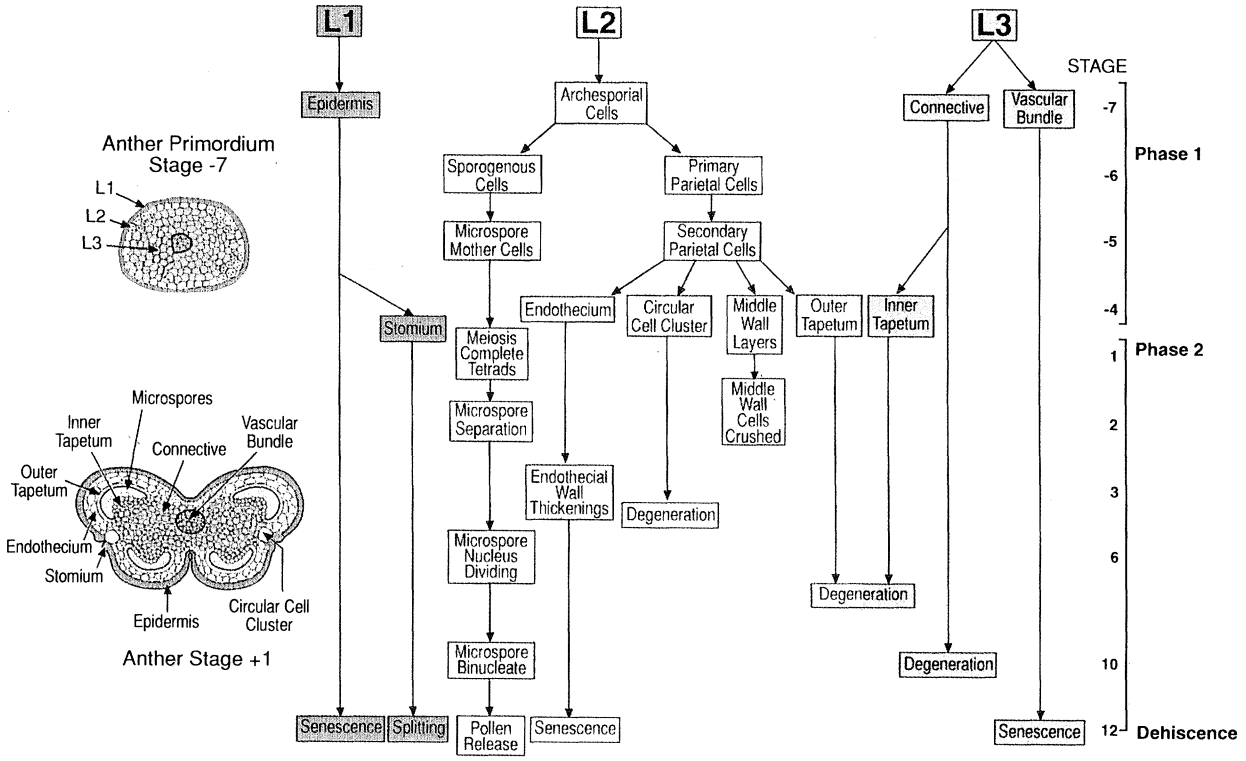

Figure 2. Cell lineages and major events that occur during tobacco anther development. Cells derived from the L1, L2, and L3 primordia layers at different anther stages were inferred from the histological studies of Satina & Blakeslee (1941), Joshi *et al.* (1967), Koltunow *et al.* (1990), and P. M. Sanders, S. H. Tu & R. B. Goldberg, unpublished results. Adapted from Goldberg *et al.* (1993).

circular-cell cluster regions, and, at flowering opening, the stomium breaks releasing the mature pollen grains (see figures 1 and 2). Pollen grain differentiation, anther cell death, filament extension, and breakage of the anther wall are exquisitely timed and coordinated so that they are complete by the end of phase 2 when the flower opens (see figure 2). The molecular events that cause the anther to switch from a histo-differentiation and morphogenesis programme (phase 1) to a cell degeneration and dehiscence programme (phase 2) are not known.

4. ANTHER CELL TYPES CAN BE TRACED TO SPECIFIC STAMEN PRIMORDIA CELL LAYERS

Stamen primordia consist of three cell layers, designated as L1, L2, and L3, that are derived from analogous layers of the floral meristem (see figure 2, and also Satina & Blakeslee 1941; Huala & Sussex 1993). Cytochimera studies with *Datura*, a close relative of tobacco, in which L1, L2, and L3 layers differed in ploidy and cell size indicated that each layer gives rise to specific anther cell and tissue types (Satina & Blakeslee 1941). Figure 2 shows the anther cell lineages that are inferred from histological observations to be derived from the L1, L2, and L3 layers (Satina & Blakeslee 1941; Koltunow *et al.* 1990; Goldberg *et al.* 1993). For example, the epidermis and stomium are derived from the L1 layer; microspores, pollen grains, and sperm cells are derivatives of the L2; the tapetum is formed from both the L2 and L3 layers; and the connective and vascular tissues differentiate from L3. Although this scheme is tentative, it shows that each layer is committed to follow a fixed differentiation pathway during anther development; that is, once the floral primordia are specified to become stamens, the L1, L2, and L3 layers have predictable fates. No information exists with respect to the precise mechanisms and genes that control the differentiation of diverse anther cell types derived from each primordial cell layer.

The developmental fate of cells within the L1, L2, and L3 layers appears to depend, in part, upon their position within the floral primordia; that is, the cells that they are contiguous to (Sussex 1989; Huala & Sussex 1993). This fate is fixed as long as the cell maintains its position within a given layer. For example, rare divisions that permit L2 cells to invade the L1 layer cause these cells to follow an L1, or epidermal pathway (Sussex 1989; Huala & Sussex 1993). Thus the cells of each layer appear to be 'conditionally specified' and have the potential to follow more than one differentiation pathway (Davidson 1989, 1991). This is in contrast to a cell that is 'autonomously specified' and follows one predetermined developmental pathway regardless of its position (Davidson 1989, 1991). Conditional specification implies that 'localized interactions' between neighbouring cells of a given layer determine the fate of an individual cell (Davidson 1991), and that intercellular signalling mechanisms operate within the floral primordia. It follows then that if the position of a cell changes with respect to primordial cell layers a new

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signal is received that directs the cell down a different developmental pathway (Davidson 1991). The nature of signalling mechanisms that operate within floral organ primordia are not yet understood. However, these signalling mechanisms are probably coupled to the action of homeotic specifier genes that direct the primordia to follow a particular organ pathway (for example, stamens).

5. CELL ABLATION STUDIES WITH CYTOTOXIC GENES CAN BE USED TO INVESTIGATE ANTHER CELL-DIFFERENTIATION

Although cell interaction processes appear to operate within the primordia L1, L2, and L3 layers, it is not known the extent to which position-dependent and position-independent events direct the differentiation of functionally diverse cell types during subsequent stages of anther development (see figure 2). Nor is it known how these specialized cells and tissues differentiate within spatially restricted territories of the anther (see figures 1 and 2). For example, what causes L2-derivatives to form archesporial cells in four specific regions of the developing anther and not in others? What directs cells within the centre of the young anther to become the vascular bundle? What mechanisms direct archesporial-daughter cells to follow two different developmental pathways: one reproductive and one non-reproductive? Finally, what events control the differentiation of parietal cells into several functionally distinct cell layers that are contiguous to each locule?

Cell ablation studies with either a microbeam laser (Sulston *et al.* 1983) or cytotoxic genes (Palmiter *et al.* 1987) can be used to distinguish between position-dependent and position-independent cell-differentiation processes. For example, if a cell type produces a signal that is required for the differentiation of a neighbouring cell, then the targeted ablation of the signal-producing cell should block and/or alter the developmental path of the neighbouring cell. On the other hand, if a given cell type develops autonomously, then the selective destruction of contiguous cells should have no effect on its development. Although cell ablation experiments *per se* cannot identify genes that direct and participate in the specification events, they can provide important clues as to the types of genes and proteins involved (for example, signal-transduction molecules versus cytoskeletal components). In addition, they can yield important information with respect to the functions carried out by specialized cell types as well as the lineages from which they are derived.

(*a*) *Targeted ablation of the tapetum leads to male sterile plants*

One example of the usefulness of cell ablation studies is the selective destruction of tapetal cells by cell-specific cytotoxic genes during anther development (Koltunow *et al.* 1990; Mariani *et al.* 1990, 1992; Paul *et al.* 1992). Figure 3 shows that a tobacco anther-specific mRNA, designated as TA29, is localized specifically within the tapetal cell layer surrounding each locule (Koltunow *et al.* 1990; Mariani *et al.* 1990). This mRNA is not present at detectable levels in other floral or vegetative organ systems (Koltunow *et al.* 1990). The TA29 mRNA accumulates within the tapetum during stages 1 to 3 of anther development (see figures 3*a*, *b*) and then is destroyed at later stages during the tapetal cell degeneration process (see figure 3*c*). The TA29 gene encodes a glycine rich protein with properties similar to cell-wall proteins suggesting that it is a component of the pollen wall (Seurinck *et al.* 1990; Koltunow *et al.* 1990). This gene is under strict transcriptional control and a 122 b.p. promoter region (−207 to −85) programmes its tapetal-specific transcription during anther development (Koltunow *et al.* 1990).

Figure 4 shows bright-field photographs of developing tobacco anther cross sections that contained a 1.5 kb TA29 promoter region (−1477 to +51) fused to either the diphtheria toxin A-chain (DTA) gene or to two different RNase genes (RNase T1 and barnase) (Koltunow *et al.* 1990; Mariani *et al.* 1990). Anthers containing the chimeric TA29/cytotoxic genes develop normally and undergo a normal dehiscence process (see figure 4). These anthers, however, do not produce pollen grains and are male sterile due to the selective destruction of the tapetal cell layer by stage 1 (see figure 4; Koltunow *et al.* 1990; Mariani *et al.* 1990). Thee results indicate that the tapetum is essential for pollen development and that it is not required for events that occur during phase 2 of anther development (for example, dehiscence, see figures 1, 2). That is, tapetal cells function autonomously after they are formed in phase 1 (see figure 2).

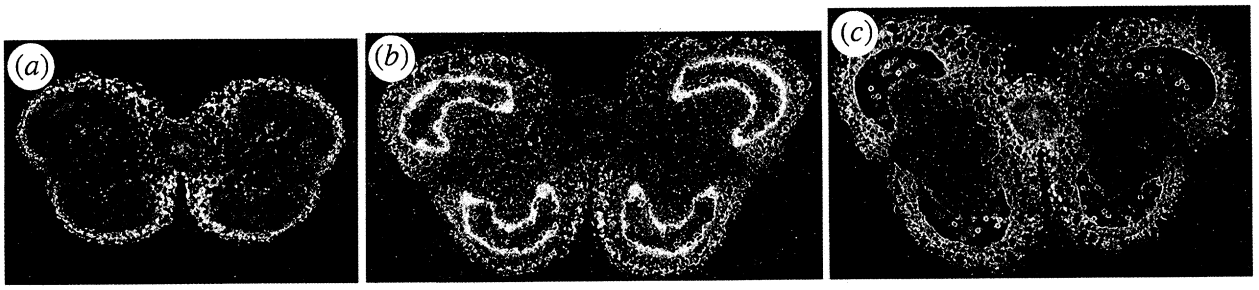

Figure 3. Localization of TA29 mRNA during tobacco anther development at stage 1 (*a*), stage 3 (*b*), and stage 6 (*c*). Data taken from Koltunow *et al.* (1990).

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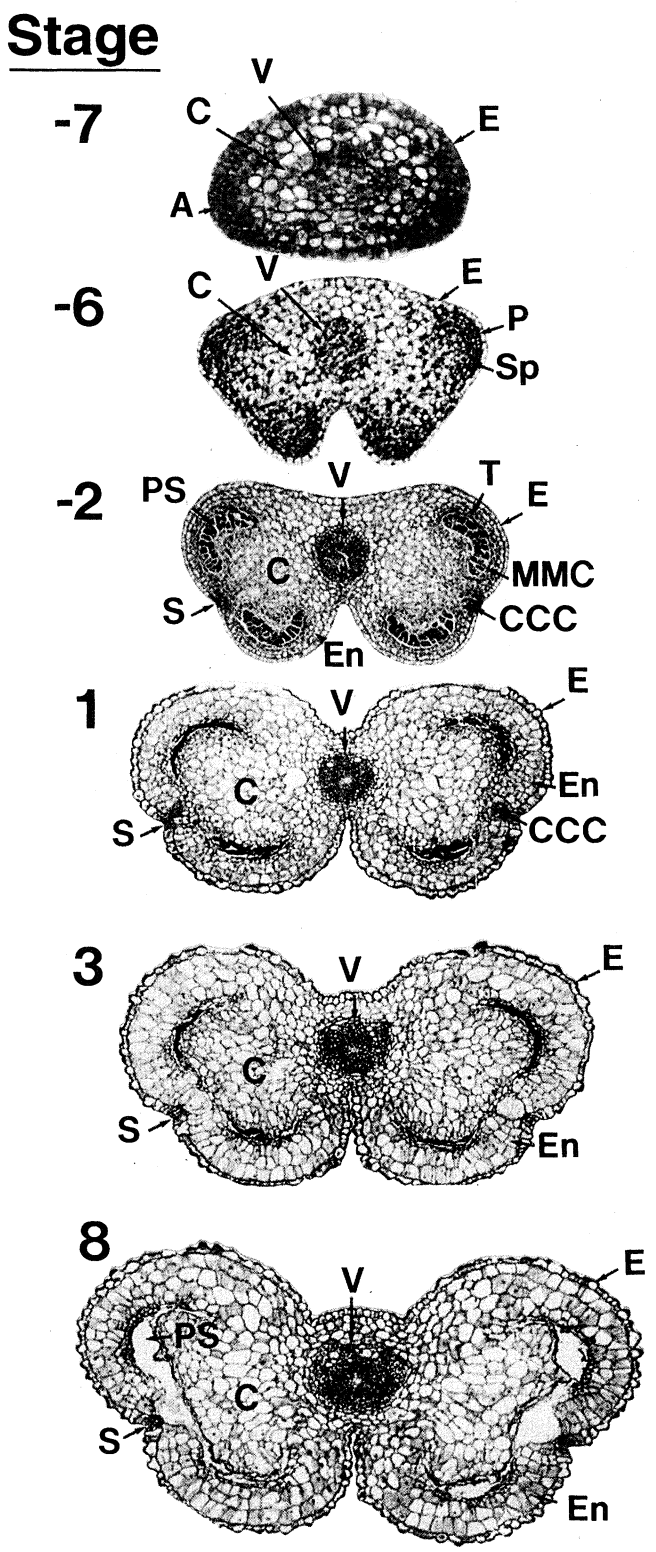

Figure 4. Selective ablation of anther tapetal cells with chimeric cytotoxic genes. The TA29 promoter was fused with either the DTA gene (Koltunow *et al.* 1990) or two different RNase genes (Mariani *et al.* 1990) and the chimeric TA29/cytotoxic genes were transferred to tobacco plants (Koltunow *et al.* 1990; Mariani *et al.* 1990). Data were taken from Koltunow *et al.* (1990) for TA29/DTA anthers; however, results were identical using TA29/RNase genes (Mariani *et al.* 1990). A, C, CCC, E, En, MMC, P, PS, S, Sp, T, and V refer to archesporial cells, connective, circular-cell cluster, epidermis, endothecium, microspore mother cells, parietal layer cells, pollen sac, stomium, sporogenous cells, tapetum and vascular bundle, respectively.

(*b*) *Cell ablation studies with cytotoxic genes require cell-specific promoters*

One advantage of using the TA29 promoter for cell ablation studies with cytotoxic genes is its absolute cell specificity (see figures 3 and 4; Koltunow *et al.* 1990; Mariani *et al.* 1990). Normal-looking transgenic plants are generated readily from tissue culture and only the tapetal cells are destroyed by the cytotoxic gene product (Koltunow *et al.* 1990; Mariani *et al.* 1990). By contrast, figure 5 shows that many tobacco anther-specific genes do not have the same exclusive cell specificity as that of the TA29 gene (Koltunow *et al.* 1990; Goldberg *et al.* 1993).

For example, the TA56 mRNA which encodes a thiol endopeptidase is represented in the stomium, circular cell cluster, and connective regions of the anther (Koltunow *et al.* 1990). Although the precise function of the TA56 gene is not known, it appears to be a marker for cell-specific degeneration events that occur before dehiscence and release of pollen grains from the anther at flowering opening (see figure 1). Figure 6 shows that the TA56 mRNA accumulates first within the circular cell cluster region (see figures 6*e*, *f*), then in the stomium (see figure 6*g*), and finally in the connective late in anther development (see figures 6*g*, *h*) (Koltunow *et al.* 1990). These temporal and spatial mRNA accumulation patterns are due to the regulated transcriptional activity of the TA56 gene within these cell types during anther development

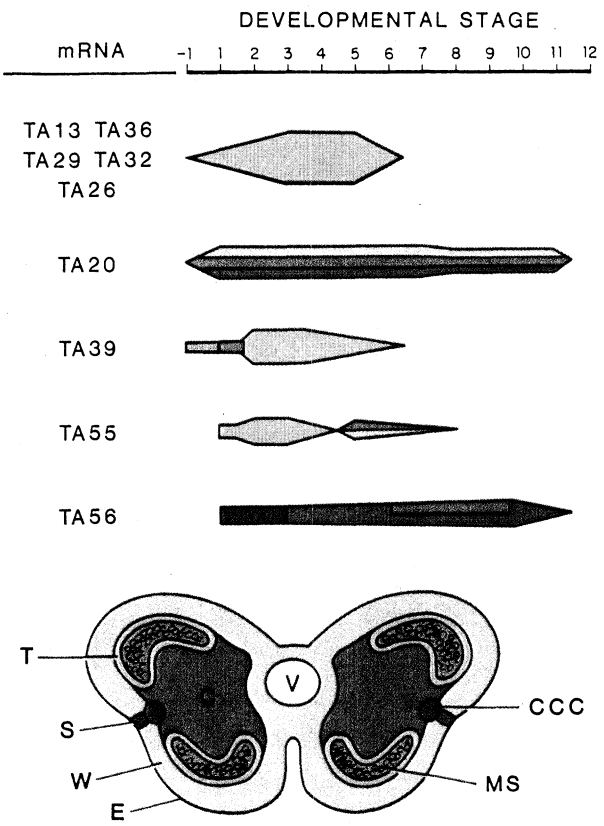

Figure 5. Regulation of gene expression during tobacco anther development. Taken from Goldberg *et al.* (1993) and the data of Koltunow *et al.* (1990) and T. P. Beals & R. B. Goldberg, unpublished results.

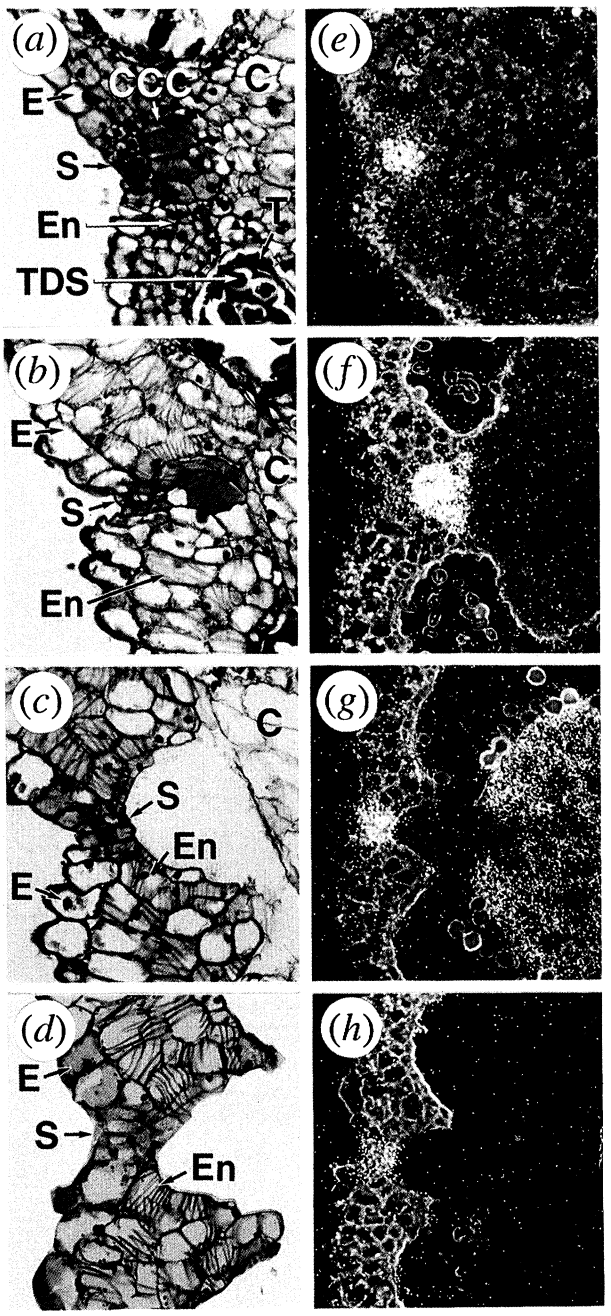

Figure 6. Localization of TA56 mRNA during tobacco anther development at stage 1 ((*a*) and (*e*)), stage 3 ((*b*) and (*f*)), stage 8 ((*c*) and (*g*)), and stage 11 ((*d*) and (*h*)). Bright-field photographs of anther cross sections are shown in (*a*) through (*d*), whereas dark-field *in situ* mRNA localization results are shown in (*e*) through (*h*). Data taken from Koltunow *et al.* (1990). C, CCC, E, En, S, and TDS represent connective, circular cell cluster, epidermis, endothecium, stomium and tetrads, respectively.

(T. P. Beals & R. B. Goldberg, unpublished results). Transformation of tobacco plants with a cytotoxic TA56/barnase gene failed to produce transgenic plants indicating that the TA56 promoter is active within regenerating callus cells as well, a result verified using a TA56/GUS marker gene (T. P. Beals & R. B. Goldberg, unpublished results). These data indicate that the lack of promoter cell specificity can cause serious problems in regenerating transgenic plants with a given chimeric cytotoxic gene. In addition, even if

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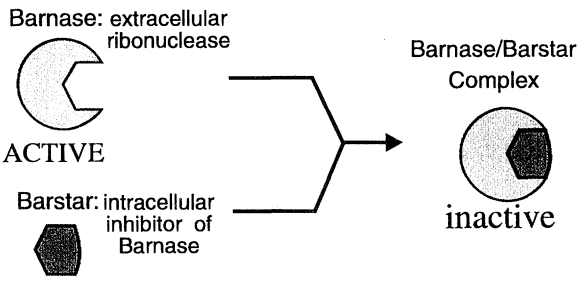

Figure 7. Schematic representation of the formation of a barnase/barstar protein complex.

transgenic plants are obtained using these promoters, ablation of multiple cell types makes it difficult to determine whether position-dependent or cell-autonomous events govern the differentiation of a specific anther cell type.

(*c*) *Use of the barnase/barstar gene system for cell ablation studies*

To overcome the difficulty in obtaining promoters with absolute cell specificity for targeted ablation studies using cytotoxic genes, we adapted a strategy used by us and our collaborators at Plant Genetic Systems (Gent, Belgium) to genetically engineer crop plants for male fertility control (Mariani *et al.* 1992). Figure 7 presents a schematic representation of two proteins, designated as barnase and barstar, that are produced by the bacteria *Bacillus amyloliquefaciens* (Hartley 1989). Barnase is a 110 amino acid extracellular RNase that is secreted by the bacteria and used either as a digestive enzyme for nutritional purposes or as a defense toxin, or both (Hartley 1989). By contrast, barstar is an 89 amino acid intracellular inhibitor of barnase that is produced constitutively by the *B. amyloliquefaciens* bacteria (Hartley 1989). Barstar binds specifically with barnase forming inactive barnase/barstar complexes (see figure 7) (Hartley 1989). The K_D of a barnase/barstar complex has been estimated to be approximately 10^{-15} M, indicating that, once formed, barnase/barstar complexes are highly stable and rarely dissociate (J. Leemans, Plant Genetic Systems, personal communication). Genetic crosses between male sterile plants containing a TA29/barnase gene and male fertile plants containing a TA29/barstar gene produce male fertile plants with both chimeric genes; that is, the TA29/barstar gene is a dominant restorer of male fertility (Mariani *et al.* 1992). This result indicates that stable barnase/barstar complexes can form specifically within tapetal cells of the anther, and that these complexes neutralize the cytotoxic effects of barnase restoring tapetal cell function and male fertility (Mariani *et al.* 1992). Hybrid oilseed rape plants generated by using parent lines containing the TA29/barnase and a TA29/barstar genes are currently in field production (J. Leemans, Plant Genetic Systems, personal communication).

Figure 8 shows how the barnase/barstar gene system can be coupled with plant promoters that are active in multiple cell types to ablate a specific target cell. Suppose two gene promoters have been identified, designated as promoter 1 and promoter 2, that programme transcription within distinct, but overlapping, sets of cell types. Promoter 1 is active within blue-coloured cells. By contrast, promoter 2 is active in a subset of these cells (red-coloured) as well as in a different cell type (brick-red-coloured). Cell ablation studies can be used to determine the function of brick-red-coloured cells and whether these cells provide a signal needed for the differentiation of their red-coloured cell-neighbours. However, if a plant is transformed with a cytotoxic promoter 2/barnase gene alone, both the red-coloured and brick-red-coloured cells will be destroyed. Thus no information will be obtained with respect to the interaction between these

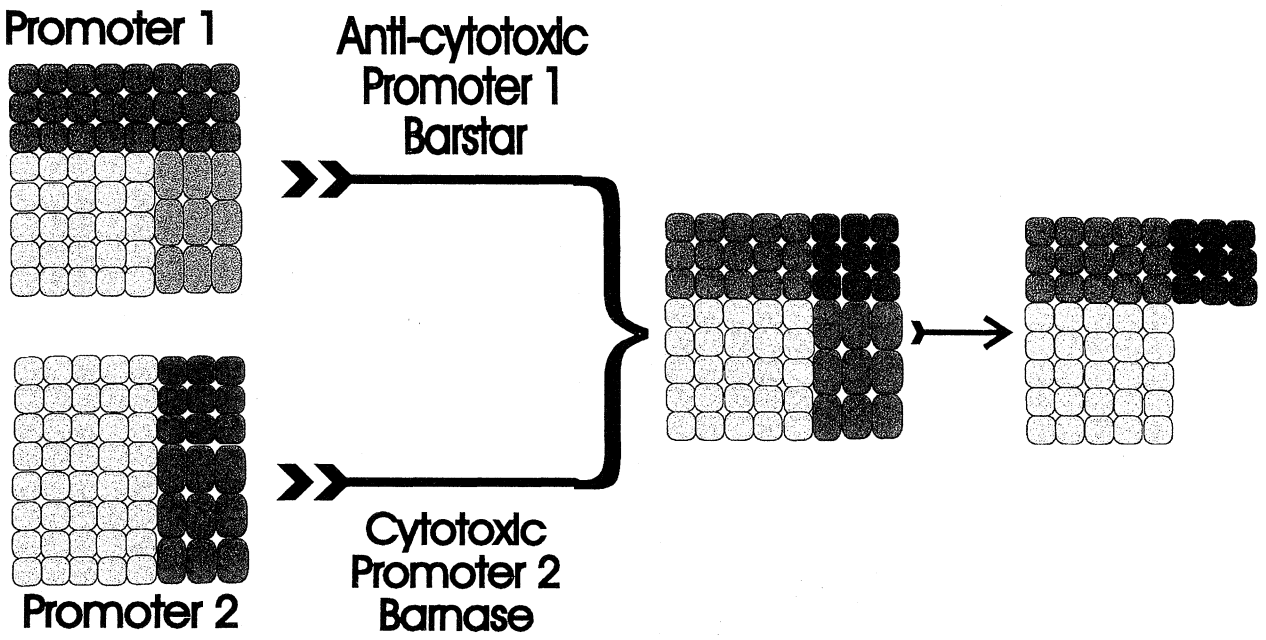

Figure 8. A cell ablation strategy using promoters with overlapping cell specificities.

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cell types or the function of the brick-red-coloured cells. On the other hand, if the cytotoxic promoter 2/barnase gene is introduced into a plant together with an anticytotoxic promoter 1/barstar anticytotoxic gene, only the brick-red-coloured cells will be ablated. Red-coloured cells will be protected from the cytotoxic effects of barnase by the formation of barnase/barstar complexes in these cells (dark-coloured cells). This strategy suggests that a combination of cytotoxic and anticytotoxic genes driven by promoters with multiple cell-specificities can be used to ablate specific cell types.

There are several advantages of using the barnase/barstar system for cell ablation studies. First and foremost, it is not necessary to use promoters that are active exclusively within a target cell type. The target cell only has to be included within the set of cell types the promoter is active in. Second, the range of potential target cells that can be studied using ablation experiments is increased significantly. In principle, any cell type that a promoter is active in can be ablated selectively, if other promoters are available with overlapping, but distinct, cell specificities. Third, anticytotoxic barstar genes can be used as 'prophylactics' to protect regenerating transgenic plant cells and/or other cell types from the toxic effects of a potentially 'leaky' cytotoxic barnase gene. Finally, the interpretation of cell ablation results is simplified because non-target cells can be protected from being ablated by the presence of barstar. Thus if a non-target cell type fails to function and/or differentiate normally the most likely explanation is that a critical signal required for these processes was lost by destruction of the target cell type.

6. THE STOMIUM AND CIRCULAR-CELL CLUSTER REGIONS CAN BE USED TO DISSECT ANTHER CELL DIFFERENTIATION PROCESSES

One of the most important events in plant reproduction is the release of pollen grains from the anther when the flower opens. Plants which have anthers that fail to dehisce are functionally male sterile (Goldberg *et al.* 1993; Dawson *et al.* 1993). The anther splits open at dehiscence within the stomium region that is located between the two locules of each theca (see figure 1). Figure 9*a* shows that the stomium differentiates from founder cells derived from the L1 primordia cell layer (see figure 2). A fully differentiated tobacco stomium consists of a notch-like structure only three to four cells layers thick embedded in the interlocular wall (see figure 6*c*). The electron micrograph presented in figure 9*b* shows that stomium cells can be distinguish from epidermal neighbours by their small size and reduced vacuoles. The stomium is the weakest point of the anther wall and breaks as a consequence of cell degeneration, water loss, and mechanical forces that are timed to occur within this region precisely as the flower opens (Bonner & Dickenson 1989). What mechanisms cause stomium cells to differentiate from L1 founder cells within the interlocular region of the anther remains to be determined.

A unique island of highly specialized cells, designated as the circular-cell cluster (see Table 2; Goldberg *et al.* 1993), also differentiates within the interlocular region of the tobacco anther (see figure 1). The circular-cell cluster differentiates from L2 layer cells that are adjacent to L1 cells destined to become the stomium (see figure 9). Cells of the circular-cell cluster divide and elongate into large, column-like cells, accumulate calcium oxalate crystals, and then undergo a cell degeneration and death process (see figures 4, 6, and 9). The destruction of the circular-cell cluster unites both locules into a single pollen chamber (see figure 4). This enables pollen grains produced by each locule to be released from a common stomium region at dehiscence (see figure 1). The precise function of the circular-cell cluster is not known; however, it probably plays a major role in events that cause anther rupture at the stomium (Bonner & Dickinson 1989). The processes that control circular-cell cluster differentiation and trigger its cell death programme are not known.

The stomium and the circular-cell cluster provide a novel opportunity to dissect the events responsible for the differentiation of two functionally related cell types that are contiguous to each other within the anther (see figures 6 and 9). Cells destined to become the stomium and circular-cell cluster are in intimate contact with each other and are specified from adjacent L1 and L2 founder cells at approximately the same time during phase 1 of anther development (see figures 2, 9). Development of the stomium and circular-cell cluster is not complete, however, until the early stages of phase 2 (see figure 9). Although division and expansion of cells within the circular-cell cluster occur just before the completion of stomium development, the differentiation of these two highly specialized cell types appears to be coordinated in space and time (see figure 9). This suggests that these cells might be interacting with each other as well as with other contiguous cell types in their immediate neighbourhood. For example, it is possible that founder cells of the circular-cell cluster produce a signal that induces the differentiation of the adjacent stomium region, or vice versa. Targeted ablation of the circular-cell cluster should provide important clues as to the extent to which position-dependent and/or cell-autonomous events are involved in the development of the circular-cell cluster and stomium regions. Selective destruction of the circular-cell cluster should also provide insight into the functional role this unique collection of cells plays in the anther dehiscence process.

7. ABLATION OF THE CIRCULAR-CELL CLUSTER AND STOMIUM PRODUCES ANTHERS THAT FAIL TO DEHISCE

As pointed out above, the TA56 thiol endopeptidase gene is expressed within the circular-cell cluster as well as in the stomium and connective at different times of anther development (see figures 5 and 6). The activity of the TA56 promoter in anther cell types is listed in table 3. Using a cytotoxic TA56/barnase gene in combination with anticytotoxic barstar genes with

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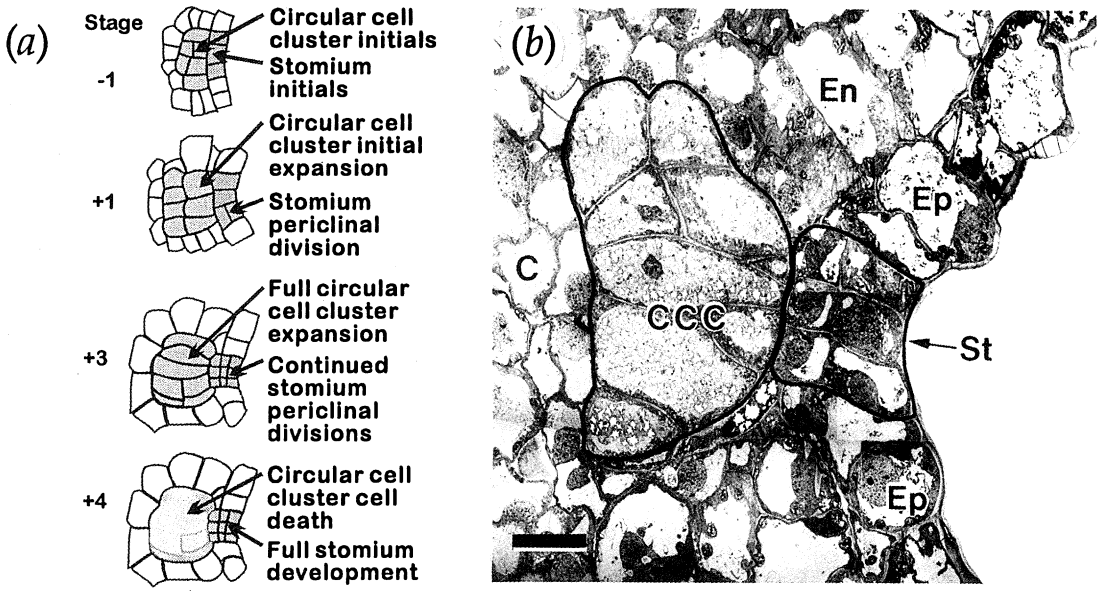

Figure 9. Differentiation of stomium and circular-cell cluster regions during tobacco anther development. (*a*) A schematic representation of stomium and circular-cell cluster differentiation. Cartoons represent anther cross sections observed at the light and electron microscope levels (Koltunow *et al.* 1990; P. M. Sanders, T. P. Beals, S. H. Tu and R. B. Goldberg, unpublished results). Bright-field photographs showing stomium and circular-cell cluster differentiation events are presented in figure 6. (*b*) An electron micrograph of the stomium/circular-cell cluster region in a stage 2 anther. C, CCC, En, Ep, and St represent connective, circular-cell cluster, endothecium, epidermis, and stomium, respectively. Bar equals 20 μm.

Table 3. *Gene promoters used in anther cell ablation studies*

(S, CCC, C, En, and T refer to stomium, circular-cell cluster, connective, endothecium and tapetum, respectively.)

			activity in anther cell types[b]					
promoter	plant	length[a]	S	CCC	En	C	T	references
TA56	tobacco	0.83	+ + +	+ + + +	+ +	+ +	+	Koltunow *et al.* (1990) T. P. Beals & R. B. Goldberg, unpublished results
TP12	tobacco	2	+ + +	+ + + +	+ + +	+ +	+ + +	Drews *et al.* (1992) T. P. Beals & R. B. Goldberg, unpublished results
Lectin	soybean	4	+	+	+ / −	+	+	Goldberg *et al.* (1983) Okamuro *et al.* (1986) R. Yadegari, T. P. Beals & R. B. Goldberg, unpublished results

[a] kb.

[b] Promoter activities were assayed using chimeric GUS reporter genes in transgenic tobacco plants. These promoters are also active in other organ systems and/or developmental stages at varying levels (T. P. Beals, R. Yadegari & R. B. Goldberg, unpublished results).

different promoter specificities and/or strengths should permit the selective ablation of either the circular-cell cluster, the stomium, or both (see figure 8). In addition, because the TA56 promoter is active within callus cells, anticytotoxic barstar genes can be used to protect regenerating transgenic plants from the toxic effects of barnase.

(*a*) *Barnase/barstar complexes form within diverse anther cell types*

We knew from previous experiments with TA29/barnase and TA29/barstar genes that barnase/barstar complexes could form within the tapetum (Mariani *et al.* 1992). However, we wanted to determine whether these complexes could form within other anther cell types such as the connective, stomium, and circular-cell cluster. As shown in figure 10, we introduced the TA56/barnase gene into tobacco plants using an

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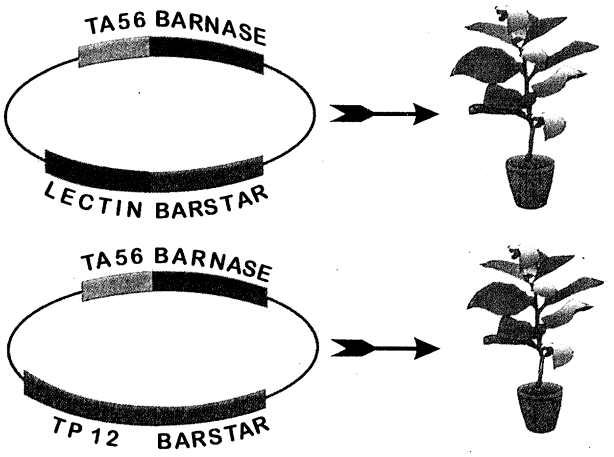

Figure 10. Transformation of tobacco plants with *Agrobacterium* Ti-plasmids containing chimeric cytotoxic and anticytotoxic genes. The transcriptional activities of the TA56, TP12, and lectin promoters in tobacco anther cells are listed in table 3.

Agrobacterium Ti-plasmid that also contained a TP12/barstar gene. TP12 is the tobacco homologue of the patatin gene expressed in potato tubers (Drews *et al.* 1992). TP12 is expressed primarily in petals but is also active within the anther (Drews *et al.* 1992). Table 3 shows that the TP12 promoter is highly active in most anther cell types. In addition, this promoter is active within callus cells (T. P. Beals & R. B. Goldberg, unpublished results).

Transgenic plants were generated that contained both the TA56/barnase and TP12/barstar genes (see Figure 10). Thus the anticytotoxic TP12/barstar gene neutralized the toxic activity of the TA56/barnase gene in regenerating callus cells. As shown schematically in figure 11, the development of the circular-cell cluster and stomium region in these plants was normal and identical to that which occurred in wild-type plants (see figure 9*a*). Figures 12*a* and *b* show that wild-type tobacco anthers split along the stomium at dehiscence and released mature pollen grains. Figures 12*c*, *d* show that the dehiscence process also occurred normally in plants containing the TA56/barnase and TP12/barstar genes, except that these anthers lacked pollen grains and were male sterile. We conclude from this experiment, that barnase/barstar complexes can form in several different anther cell types and that anther development in plants with the TA56/barnase and TP12/barstar genes is normal.

(*b*) *Barnase selectivity ablates the stomium and circular-cell cluster*

In the second experiment we introduced the TA56/barnase gene into tobacco plants together with a lectin/barstar gene (see figure 10). The soybean lectin promoter is highly active in tobacco embryos (Okamuro *et al.* 1986; R. Yadegari & R. B. Goldberg, unpublished results). However, as shown in table 3, it is also active at weak to moderate levels in tobacco anthers (T. P. Beals, R. Yadegari & R. B. Goldberg, unpublished results). Transgenic plants were generated that contained both the TA56/barnase and lectin/barstar genes (see figure 10). Thus like the TP12/barstar gene, the lectin/barstar gene can protect regenerating callus cells from the cytotoxic effects of TA56/barnase gene activity. By contrast with the TA56/barnase + TP12/barstar plants, figure 11 shows that both the circular-cell cluster and the stomium were ablated in the anthers of TA56/barnase + lectin/barstar plants. As shown in figures 12*e* and *f*, the selective ablation of stomium and circular-cell cluster region resulted in anthers that failed to dehisce. These results indicate that the stomium/circular cell-cluster region is essential for anther dehiscence, and that the targeted ablation of specific anther cell types can be accomplished using the barnase/barstar gene system coupled with promoters that have overlapping cell specificities (see figure 8).

(*c*) *Selective ablation of the circular cell cluster*

Figure 5 shows that the tobacco TA20 gene is expressed in most anther cell types except the circular-cell cluster (Koltunow *et al.* 1990). We are currently using the TA56/barnase gene in combination with a TA20/barstar gene to specifically ablate the circular-cell cluster. Transgenic plants containing both of these genes should provide clues as to the function of the circular-cell cluster and whether cell–cell interaction processes play a role in the differentiation of the stomium and circular-cell cluster regions.

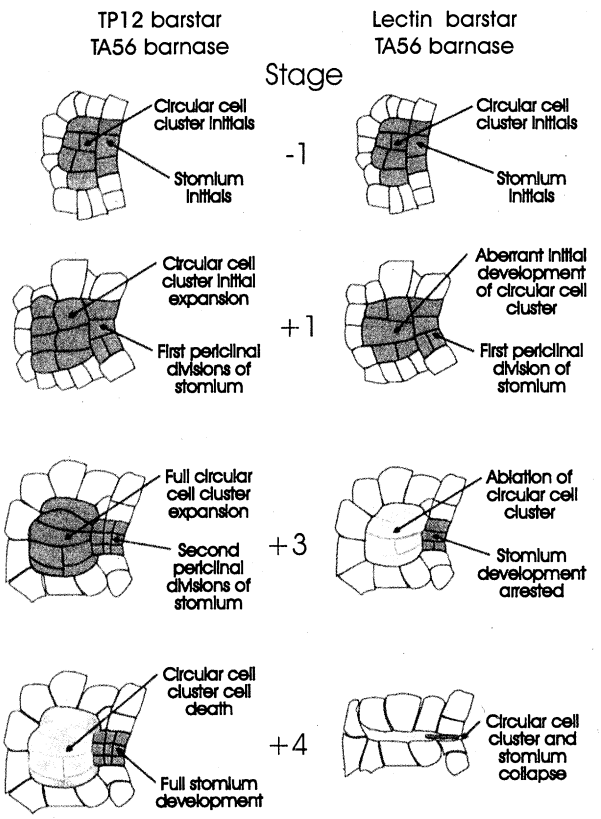

Figure 11. A schematic representation of stomium and circular-cell cluster differentiation in anthers containing a cytotoxic TA56/barnase gene and either an anti-cytotoxic TP12/barstar gene or an anti-cytotoxic lectin/barstar gene.

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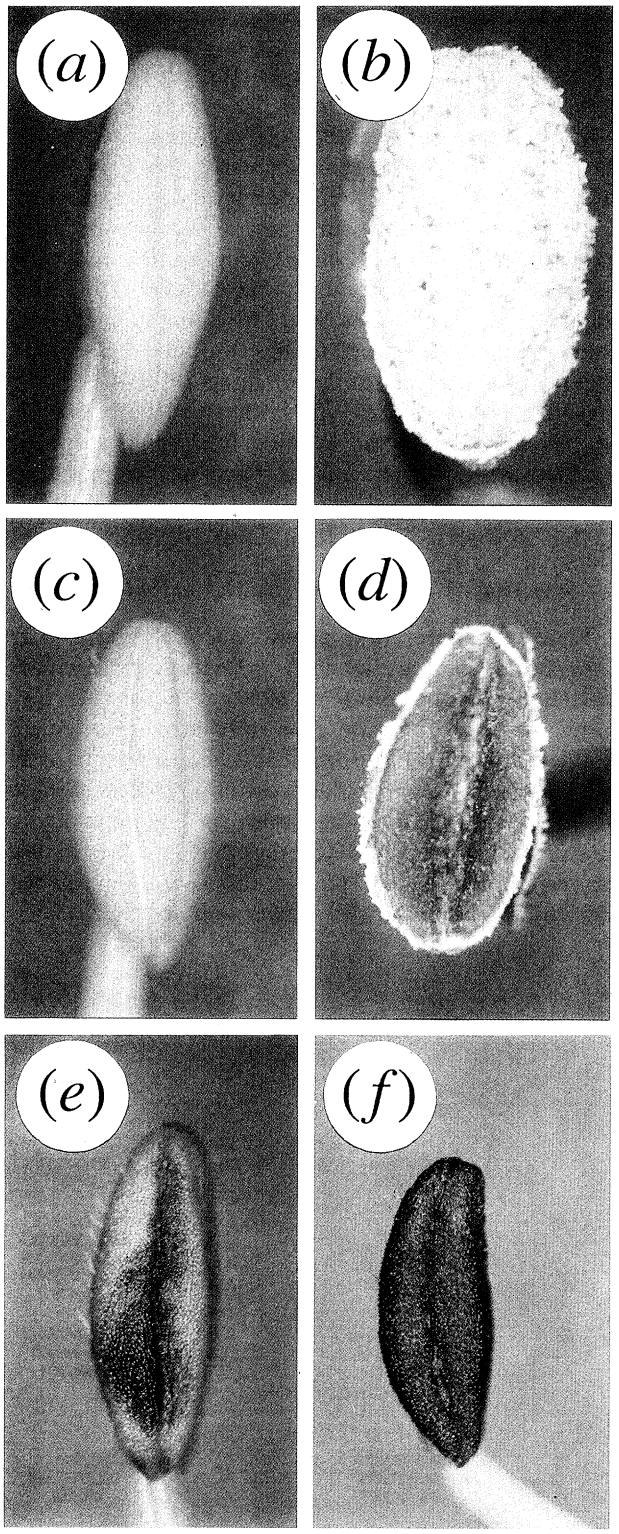

Figure 12. Dehiscence of tobacco anthers in wild-type plants ((*a*) and (*b*)), transgenic plants containing the TA56/barnase+TP12/barstar genes ((*c*) and (*d*)), and transgenic plants containing the TA56/barnase+lectin/barstar genes ((*e*) and (*f*)). Stage 6 anthers ((*a*), (*c*) and (*e*)), stage 12 anthers ((*b*), (*d*) and (*f*)).

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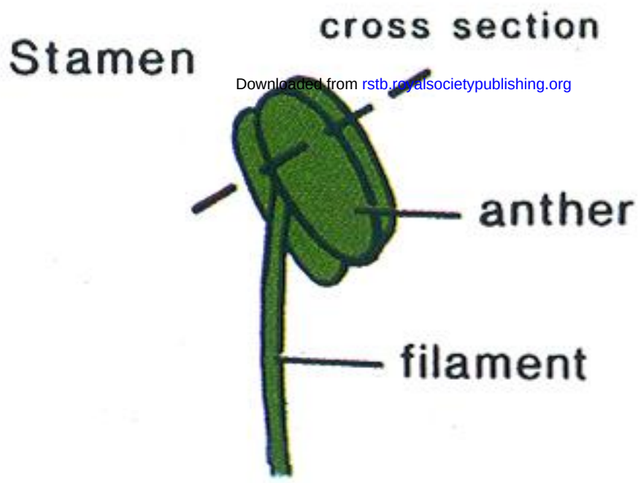
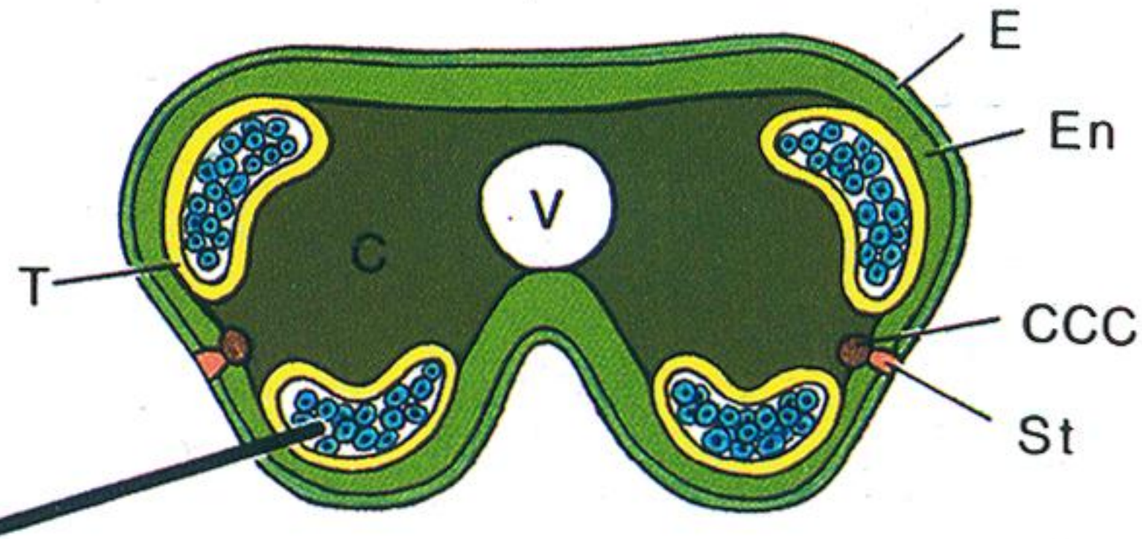




ANTHER STAGE -1

Meiosis and Spore Formation

Microspore Mother Cell

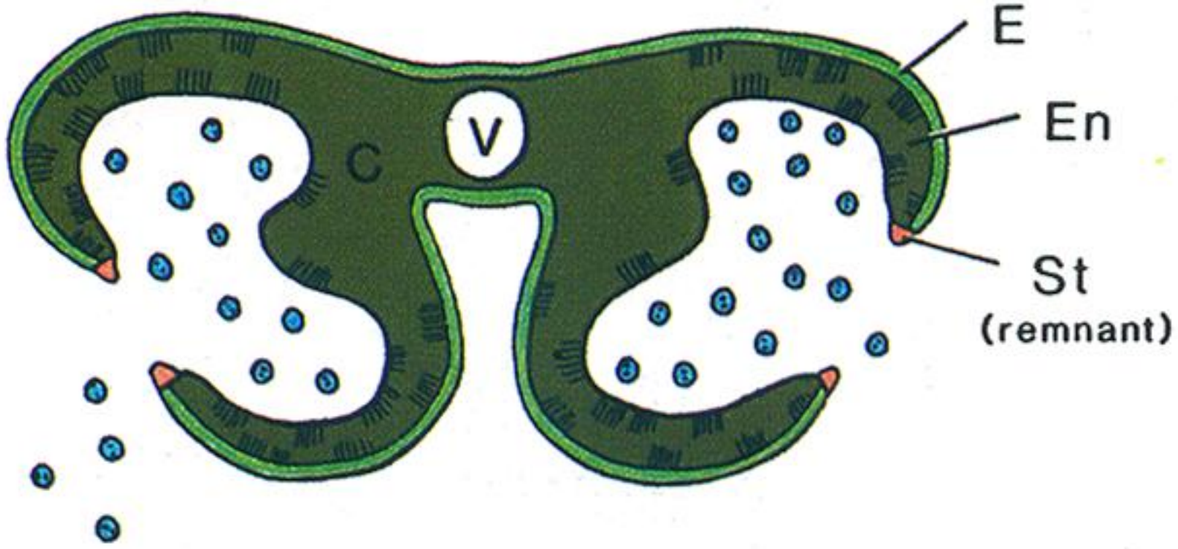
ANTHER STAGE +1

Tetrad of Microspores

Pollen Grain Differentiation

Pollen Grain

ANTHER STAGE +12



Anther Dehiscence and Pollen Release

Figure 1. A generalized overview of tobacco anther structure and function. Schematic representations are based on light microscopy studies of anther development (Koltunow *et al.* 1990). C, CCC, E, En, T, St, and V refer to connective, circular cell cluster, epidermis, endothecium, tapetum and vascular bundle, respectively.

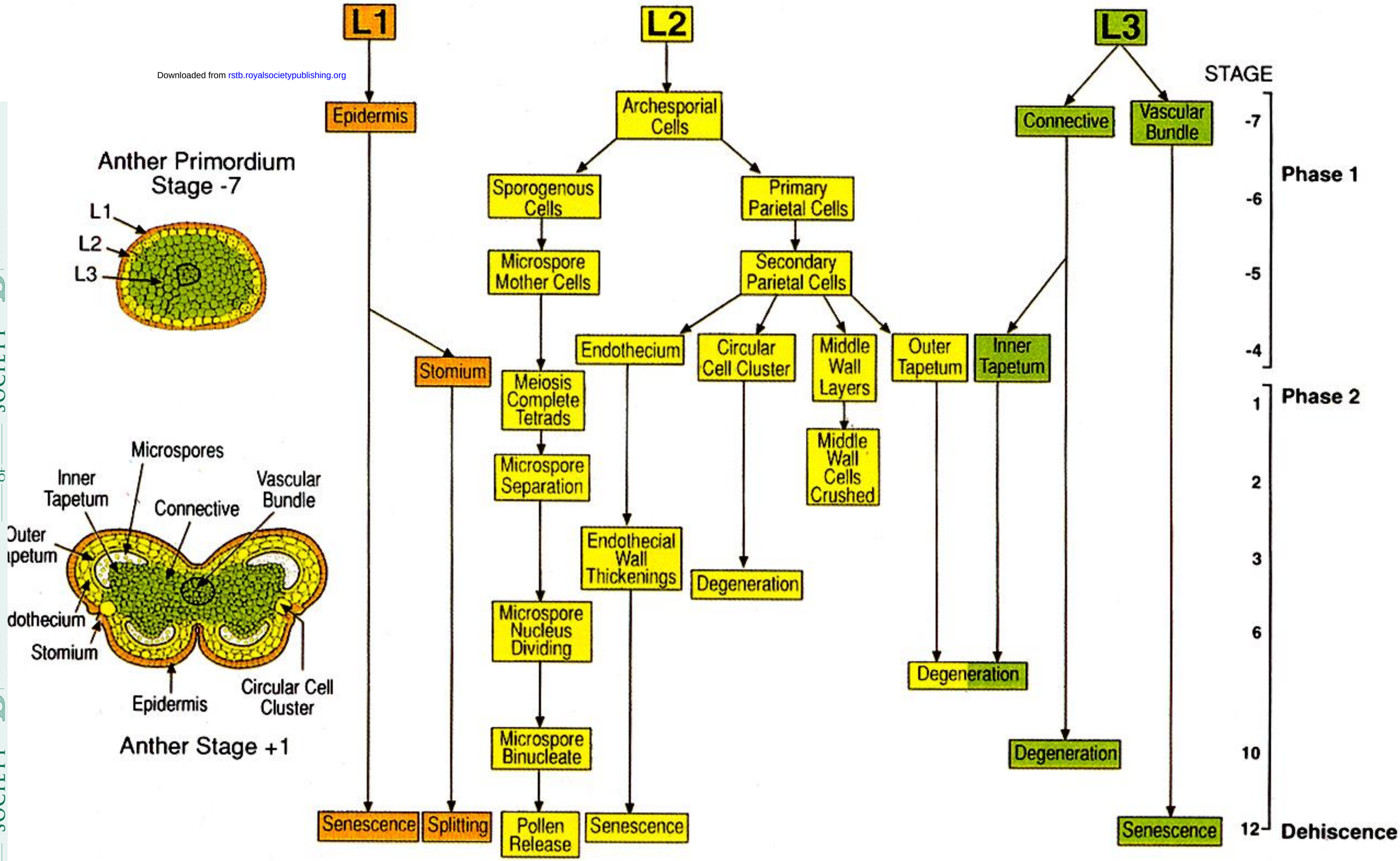


Figure 2. Cell lineages and major events that occur during tobacco anther development. Cells derived from the L1, L2, and L3 primordia layers at different anther stages were inferred from the histological studies of Satina & Blakeslee (1941), Joshi *et al.* (1967), Koltunow *et al.* (1990), and P. M. Sanders, S. H. Tu & R. B. Goldberg, unpublished results. Adapted from Goldberg *et al.* (1993).

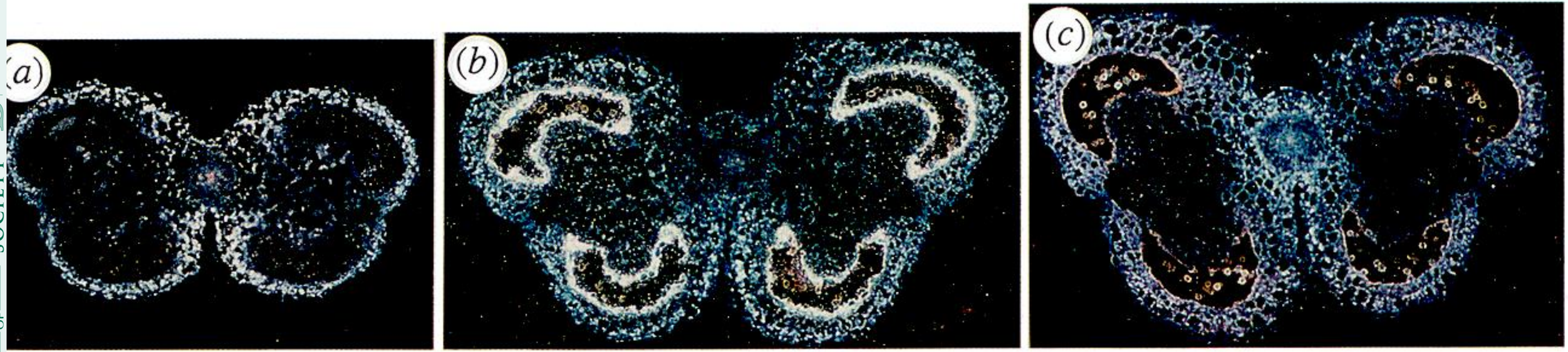


Figure 3. Localization of TA29 mRNA during tobacco anther development at stage 1 (*a*), stage 3 (*b*), and stage 6 (*c*). Data taken from Koltunow *et al.* (1990).

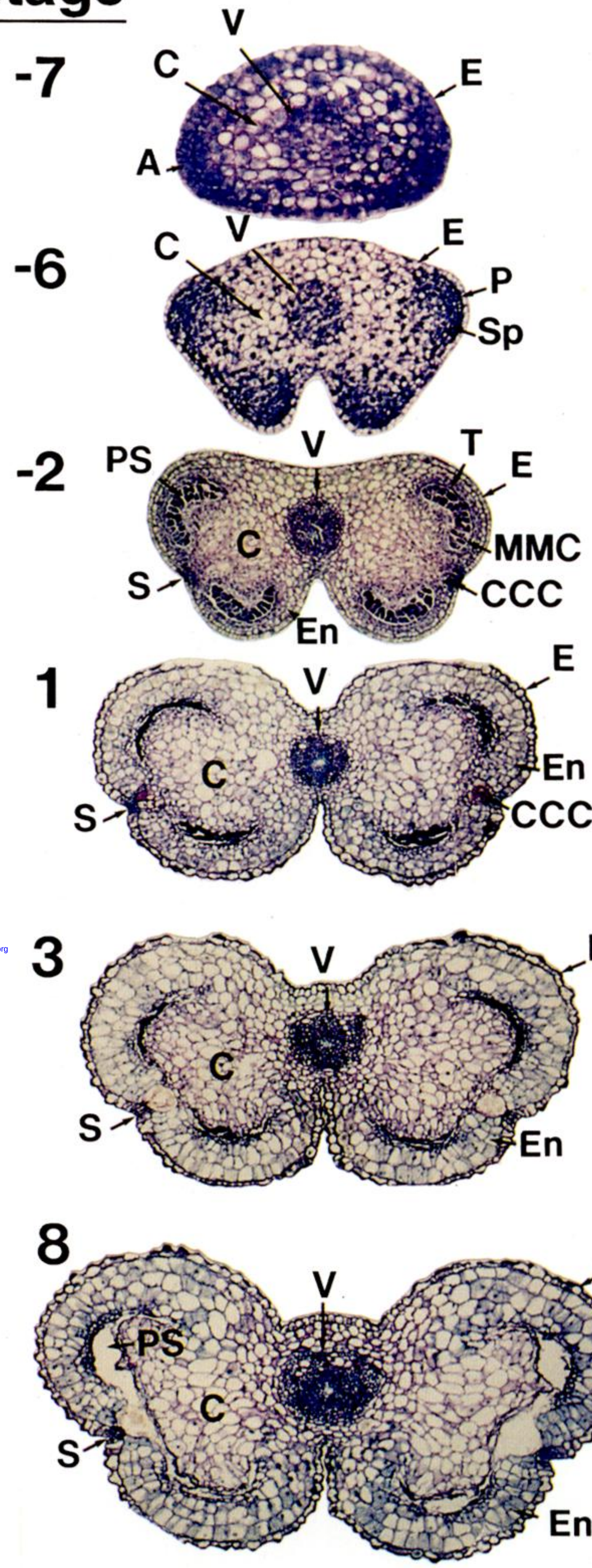
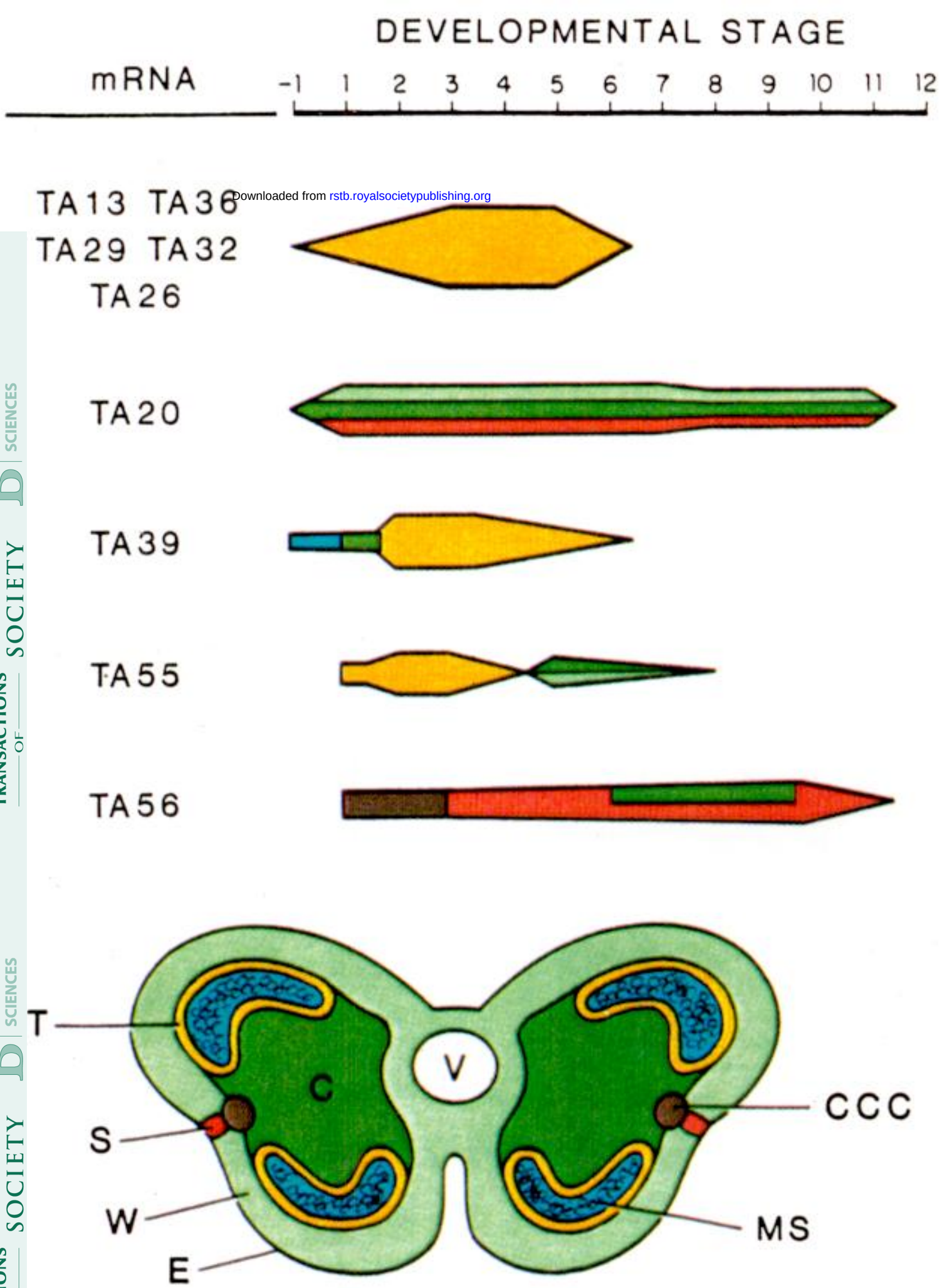


Figure 4. Selective ablation of anther tapetal cells with chimeric cytotoxic genes. The TA29 promoter was fused with either the DTA gene (Koltunow *et al.* 1990) or two different RNase genes (Mariani *et al.* 1990) and the chimeric TA29/cytotoxic genes were transferred to tobacco plants (Koltunow *et al.* 1990; Mariani *et al.* 1990). Data were taken from Koltunow *et al.* (1990) for TA29/DTA anthers; however, results were identical using TA29/RNase genes (Mariani *et al.* 1990). A, C, CCC, E, En, MMC, P, PS, S, Sp, T, and V refer to archesporial cells, connective, circular-cell cluster, epidermis, endothecium, microspore mother cells, parietal layer cells, pollen sac, stomium, sporogenous cells, tapetum and vascular bundle, respectively.



gure 5. Regulation of gene expression during tobacco
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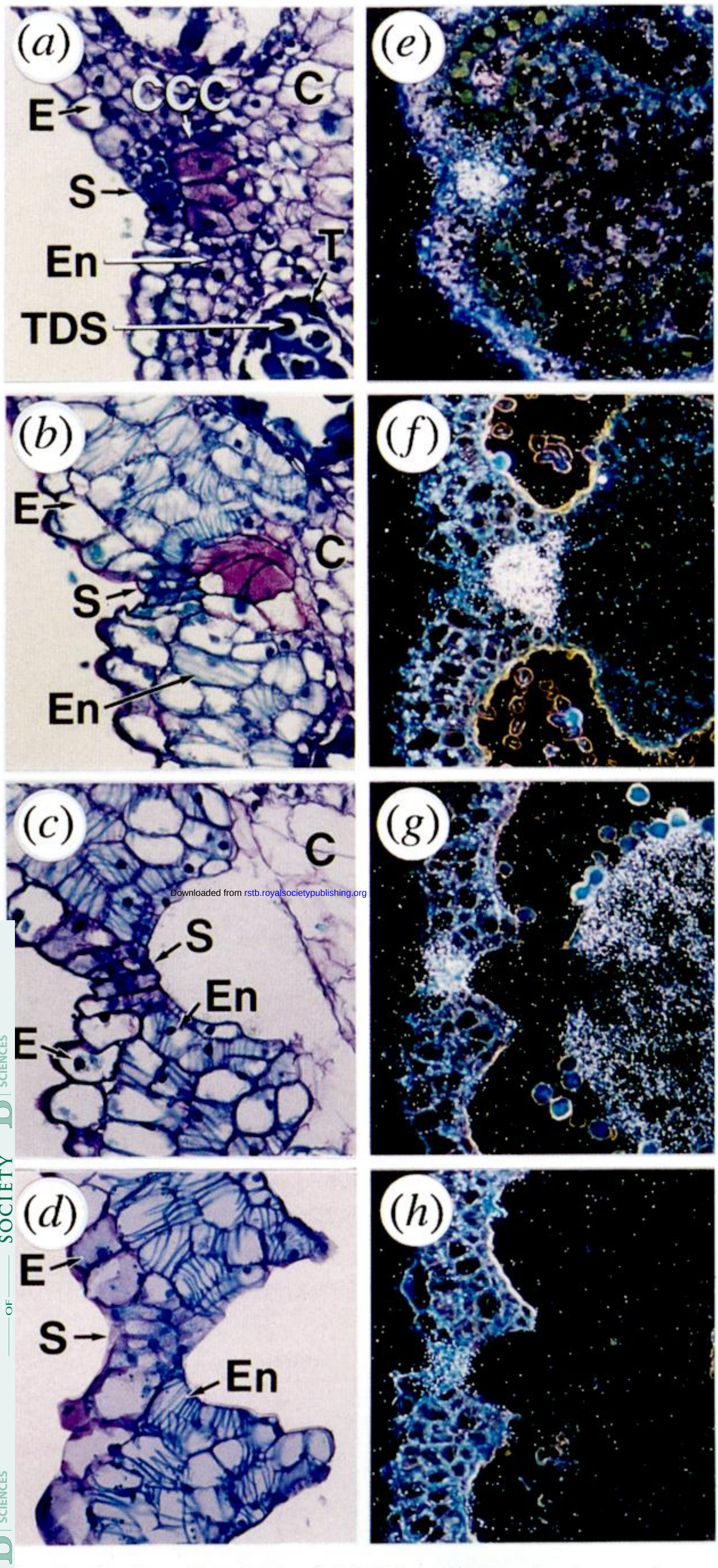
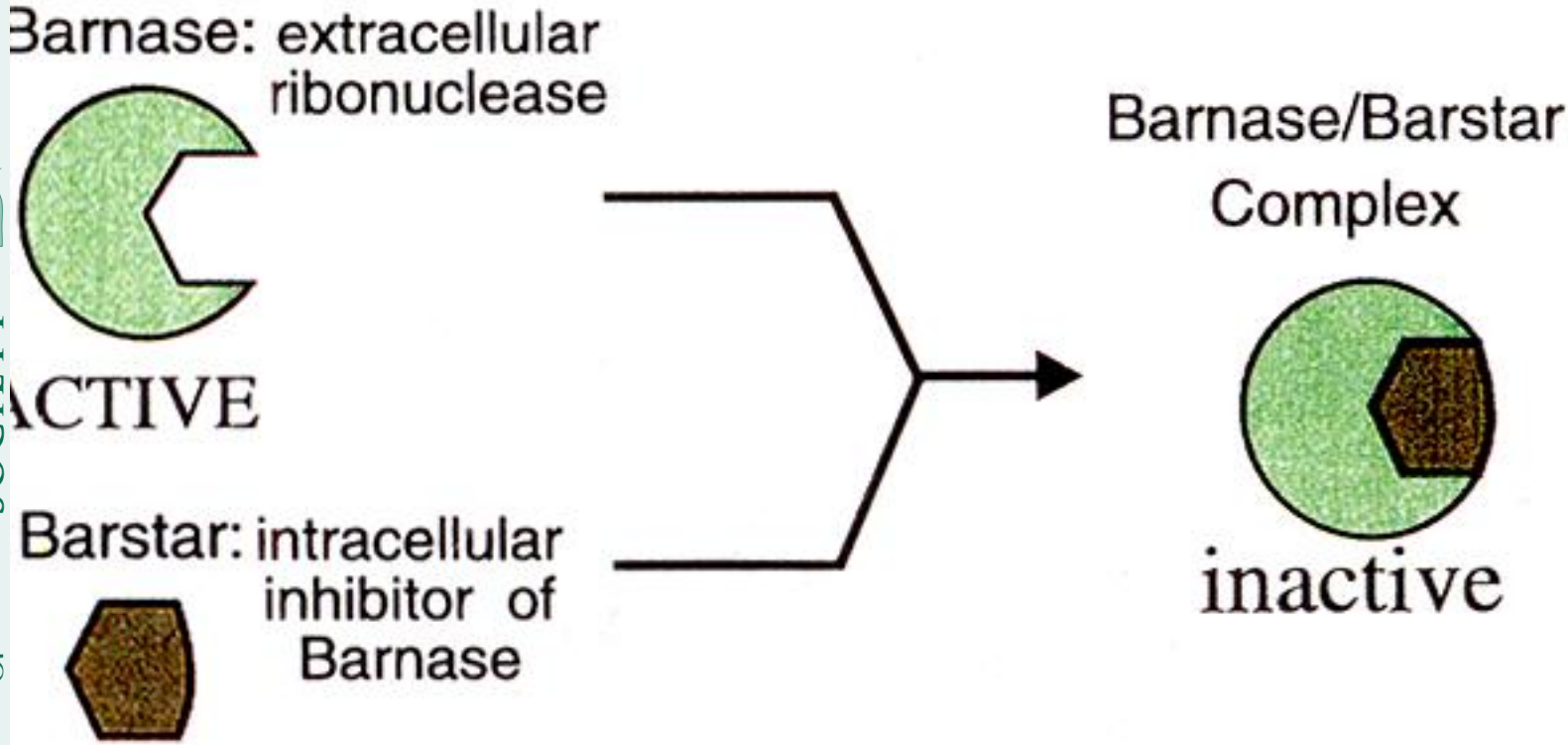


igure 6. Localization of TA56 mRNA during tobacco nther development at stage 1 ((*a*) and (*e*)), stage 3 ((*b*) and *f*)), stage 8 ((*c*) and (*g*)), and stage 11 ((*d*) and (*h*)). Bright-eld photographs of anther cross sections are shown in (*a*) rough (*d*), whereas dark-field *in situ* mRNA localization sults are shown in (*e*) through (*h*). Data taken from oltunow *et al.* (1990). C, CCC, E, En, S, and TDS represent nnective, circular cell cluster, epidermis, endothecium, omium and tetrads, respectively.



gure 7. Schematic representation of the formation of a rnase/barstar protein complex.

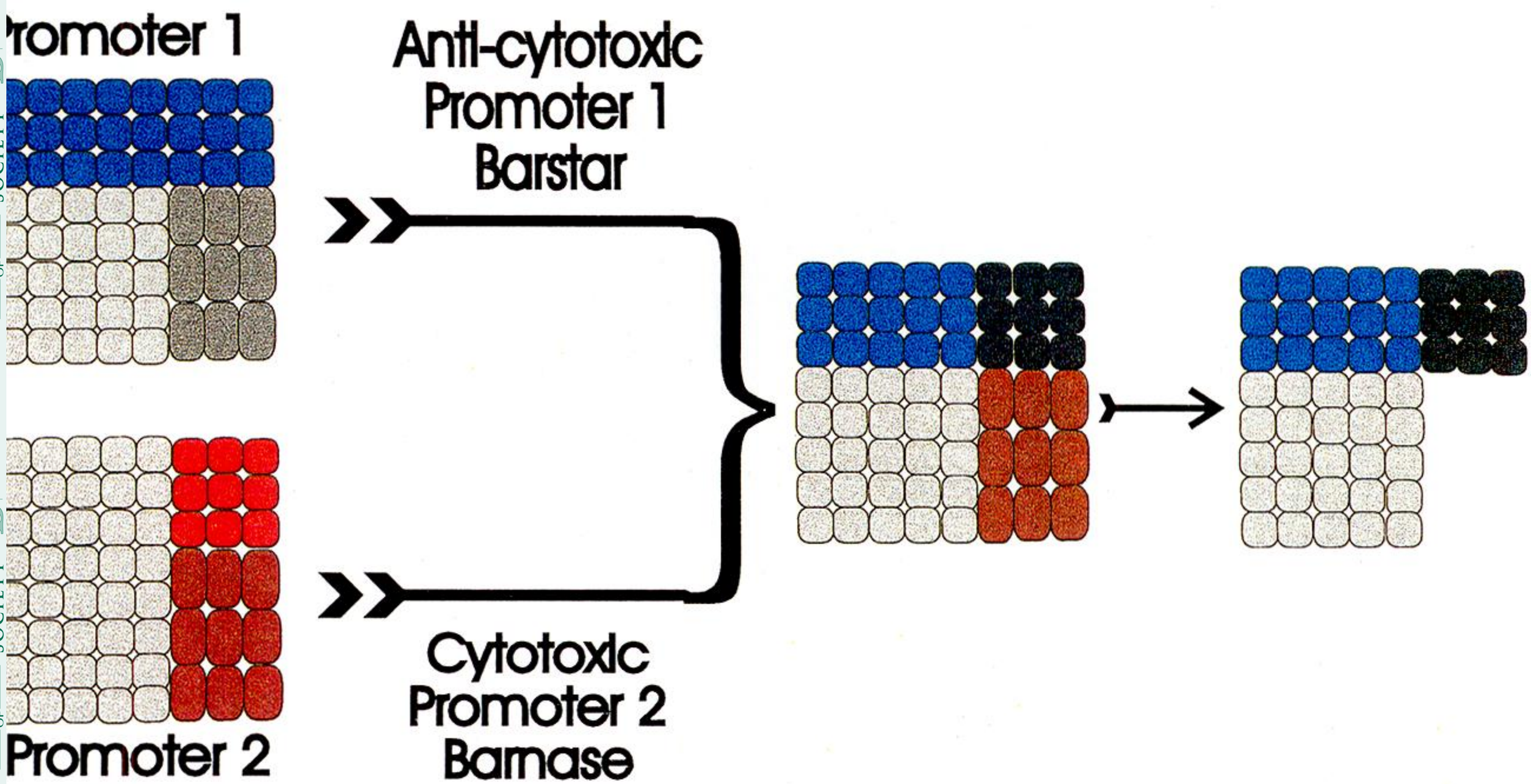
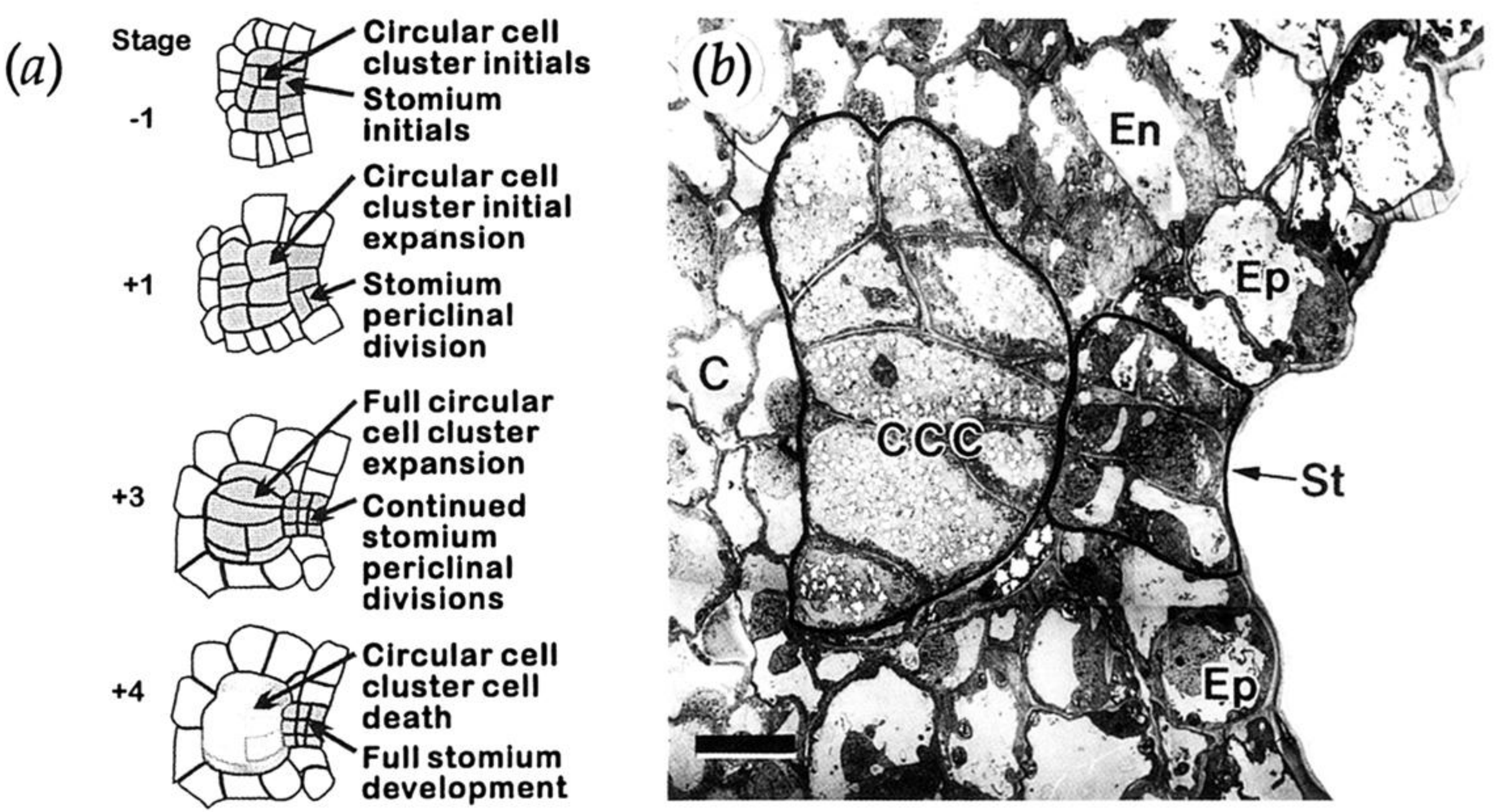
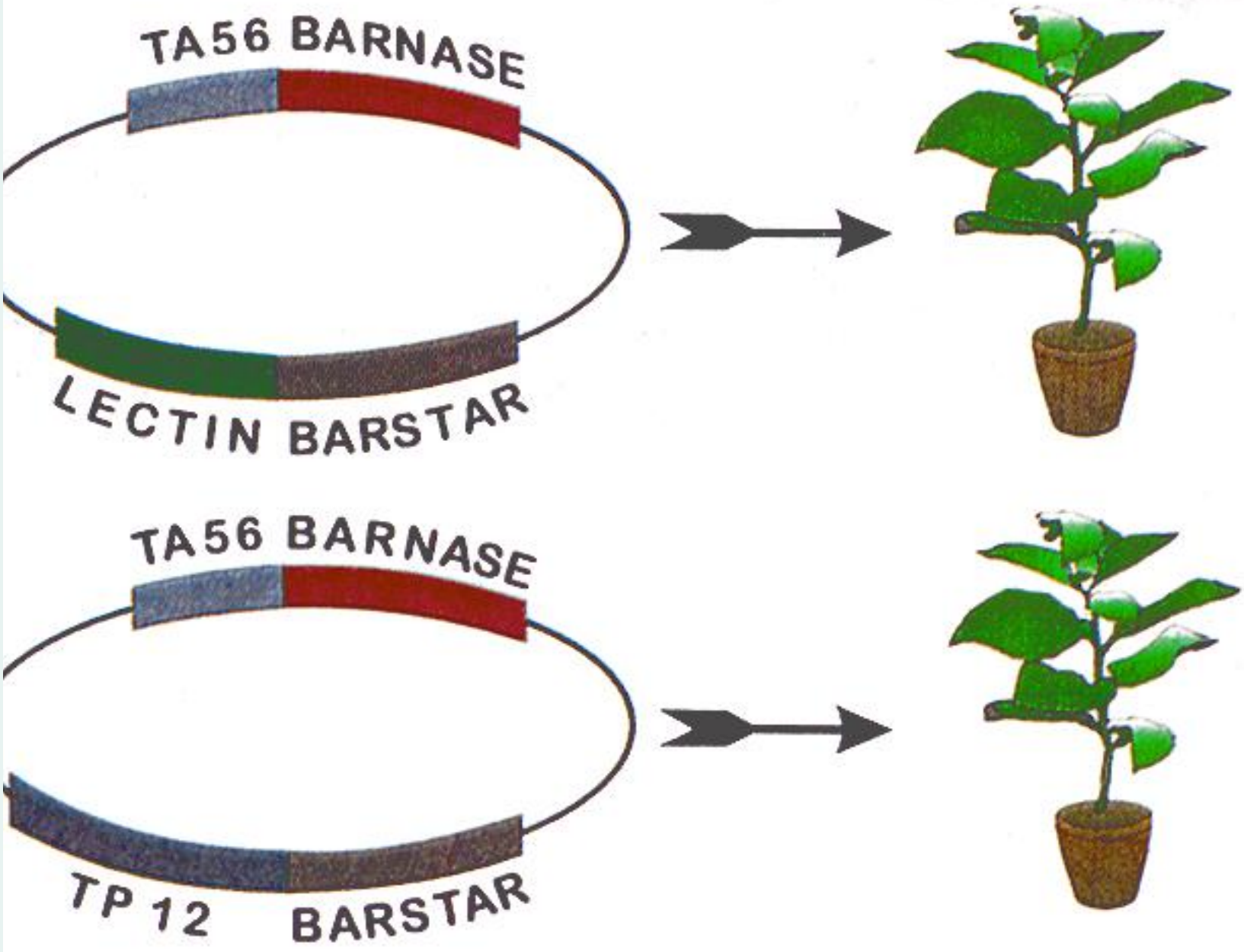


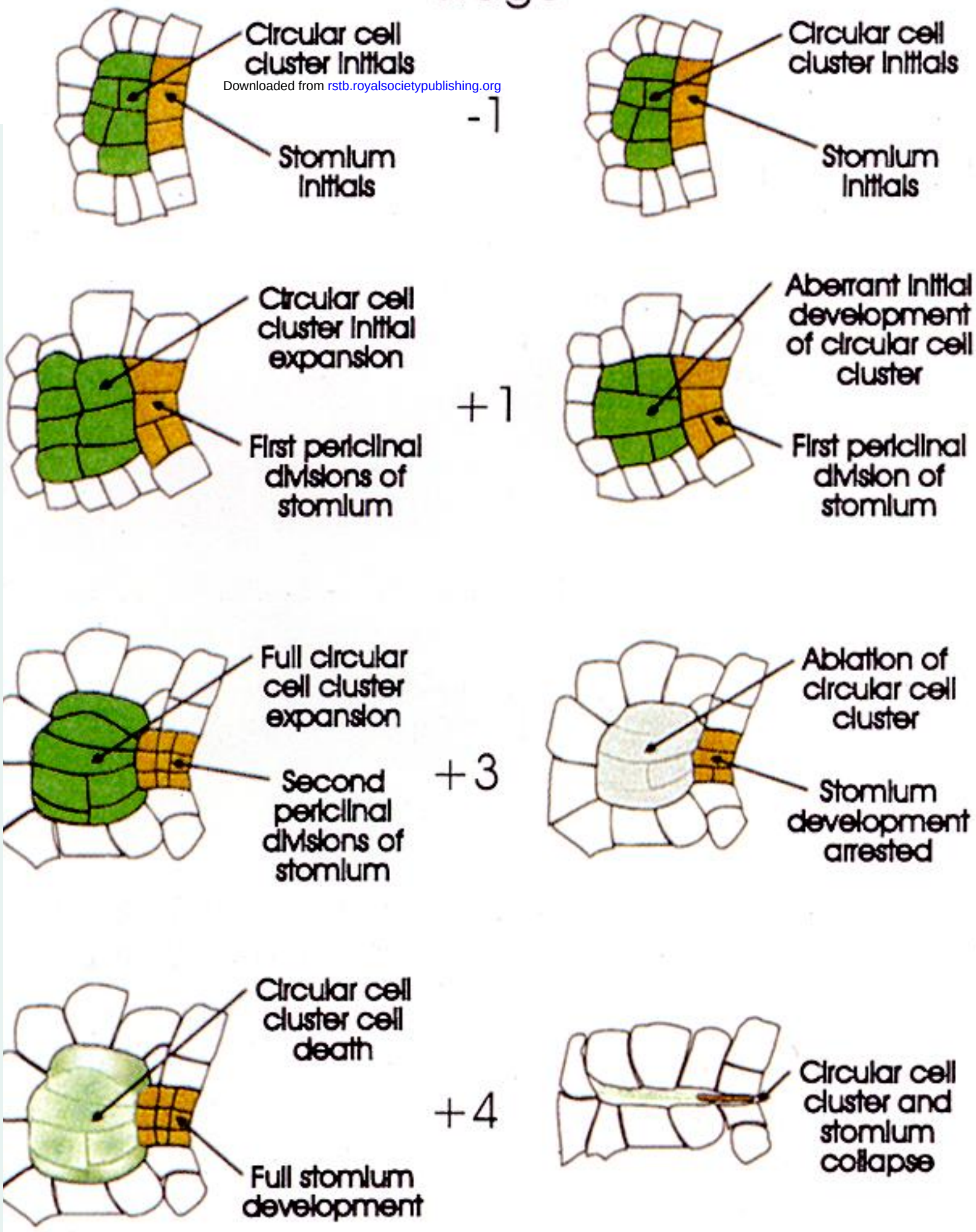
Figure 8. A cell ablation strategy using promoters with overlapping cell specificities.



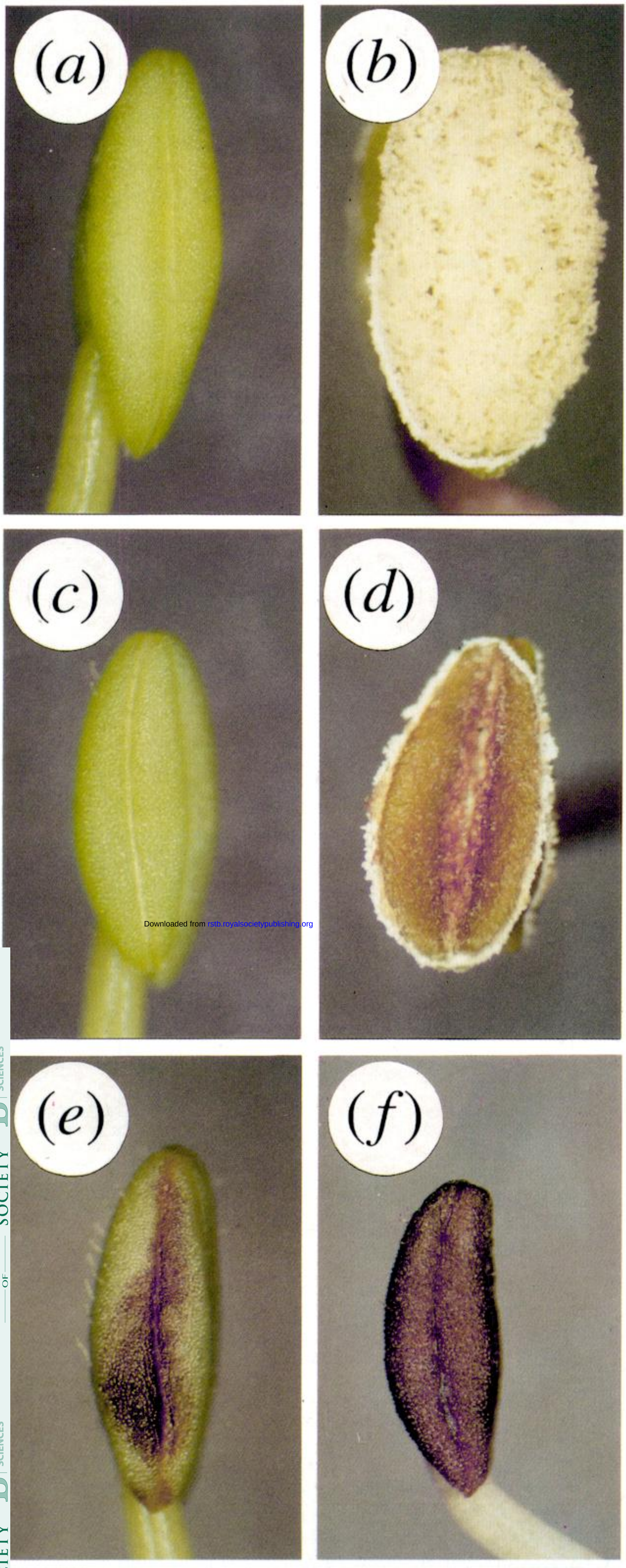
gure 9. Differentiation of stomium and circular-cell cluster regions during tobacco anther development. (*a*) A nematic representation of stomium and circular-cell cluster differentiation. Cartoons represent anther cross sections served at the light and electron microscope levels (Koltunow *et al.* 1990; P. M. Sanders, T. P. Beals, S. H. Tu and B. Goldberg, unpublished results). Bright-field photographs showing stomium and circular-cell cluster fferentiation events are presented in figure 6. (*b*) An electron micrograph of the stomium/circular-cell cluster region a stage 2 anther. C, CCC, En, Ep, and St represent connective, circular-cell cluster, endothecium, epidermis, and omium, respectively. Bar equals 20 μm.



gure 10. Transformation of tobacco plants with *Agro-cterium* Ti-plasmids containing chimeric cytotoxic and nticytotoxic genes. The transcriptional activities of the A56, TP12, and lectin promoters in tobacco anther cells are ted in table 3.



gure 11. A schematic representation of stomium and rcular-cell cluster differentiation in anthers containing a totoxic TA56/barnase gene and either an anti-cytotoxic P12/barstar gene or an anti-cytotoxic lectin/barstar gene.



'igure 12. Dehiscence of tobacco anthers in wild-type plants (*a*) and (*b*)), transgenic plants containing the TA56/ arnase + TP12/barstar genes ((*c*) and (*d*)), and transgenic lants containing the TA56/barnase + lectin/barstar genes (*e*) and (*f*)). Stage 6 anthers ((*a*), (*c*) and (*e*)), stage 12 nthers ((*b*), (*d*) and (*f*)).