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Combinatorial and synergistic properties of CaMV 35S enhancer subdomains

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We have analyzed expression conferred by five subdomains of the cauliflower mosaic virus (CaMV) 35S enhancer in mature transgenic plants. Expression was detected from subdomains that gave no expression at earlier stages of development indicating developmental regulation of expression and confirming the modular organization of the enhancer. In several cases the expression patterns are highly restricted in cell type, providing useful markers for developmental studies. Comparison of expression patterns conferred by various combinations of 35S enhancer *cis*-elements suggests that synergistic interactions among *cis*-elements may play an important role in defining tissue-specific expression. This has implications for the nature of a *cis*-element combinatorial code that could define expression throughout development.

Key words: *cis*-elements/developmental regulation/GUS reporter gene/histochemical localization/tissue specificity

Introduction

Tissue-specific gene expression is frequently controlled by DNA sequences that bind specific *trans*-acting factors. In several cases the DNA sequences have been shown to have a modular organization (reviewed in Dynan, 1989; Mitchell and Tjian, 1989). The question of how a cell integrates the information contained in a linear array of transcription factors bound to DNA remains unanswered. The modular organization of promoters also suggests that there may be a combinatorial code that can define gene expression throughout development (Yamamoto, 1985). To understand the properties of this code we are analyzing the organization of the cauliflower mosaic virus (CaMV) 35S enhancer. Our approach is to identify the modules that make up the enhancer and define expression conferred by the individual modules and by combinations of the modules. Since understanding the code requires an accurate determination of expression patterns throughout development we have used histochemical localization to analyze tissue-specific expression in transgenic plants at various developmental stages. In the accompanying paper (Benfey *et al.*, 1990) we have described the tissue-specific expression patterns conferred by subdomains of the 35S enhancer in seeds and seedlings of transgenic plants. In these early stages of plant development expression conferred by the subdomains was strikingly varied and, in some cases, changed with developmental stage. Combinations of *cis*-elements were able to specify expression in tissues in which the isolated *cis*-elements had no detectable activity.

In this paper we analyze expression from the 35S subdomains in different organs of mature plants. We are able to detect expression from subdomains that gave no expression at earlier stages of development indicating developmental regulation of expression. We detect complementary and redundant expression patterns from different subdomains confirming the modular organization of the enhancer. Perhaps most significantly, the expression patterns in the mature plant allow us to more precisely define the nature of the synergistic interactions among subdomains. Our results indicate that synergistic interactions between heterologous *cis*-elements may be able to specify cell-specific expression. Comparison of the expression patterns generated by different combinations of *cis*-elements suggests that not all *cis*-elements are equivalent in their ability to mediate synergistic interactions.

Results

Analysis of expression in mature plants

The constructs containing various combinations of 35S enhancer subdomains fused to either a minimal promoter vector (−46 to +8 of the 35S promoter, referred to as the TATA vector) or to a vector containing the A domain (−90 to +8, referred to as the A domain vector), are described in the accompanying paper. Both vectors have transcriptional fusions to the *Escherichia coli* β-glucuronidase (GUS) coding sequence (Jefferson *et al.*, 1987). Transgenic R1 tobacco plants containing the 35S enhancer subdomain constructs were maintained in tissue culture on media that contained antibiotics for selection. Fresh sections were cut at 7−10 weeks after germination as previously described (Benfey *et al.*, 1989). We analyzed expression in sections of the stem apex, of upper and lower stems, of younger and older leaves, and of roots. After initially analyzing expression from the R1 plants we generated additional primary transformants (R0 plants) containing constructs for which we had less than six R1 plants. These constructs contained subdomains B1, B3 and B4 fused to the TATA vector. For these plants we analyzed cuttings from the primary transformants.

Expression in stem

In stem tissue we were able to detect expression from four of the five subdomains fused to the minimal TATA vector. One of the subdomains, B5, had never shown expression in any tissue at earlier stages of development. Another subdomain, B2, had not shown any expression in stem tissue. A third subdomain, B4, had shown only extremely weak expression in the stem tissue of a single germinating plant. Synergistic interactions were observed with three of the subdomains, B1, B2 and B5, fused to the A domain vector. No staining in any tissue was detected in 11 independent transgenic plants containing the TATA vector alone.

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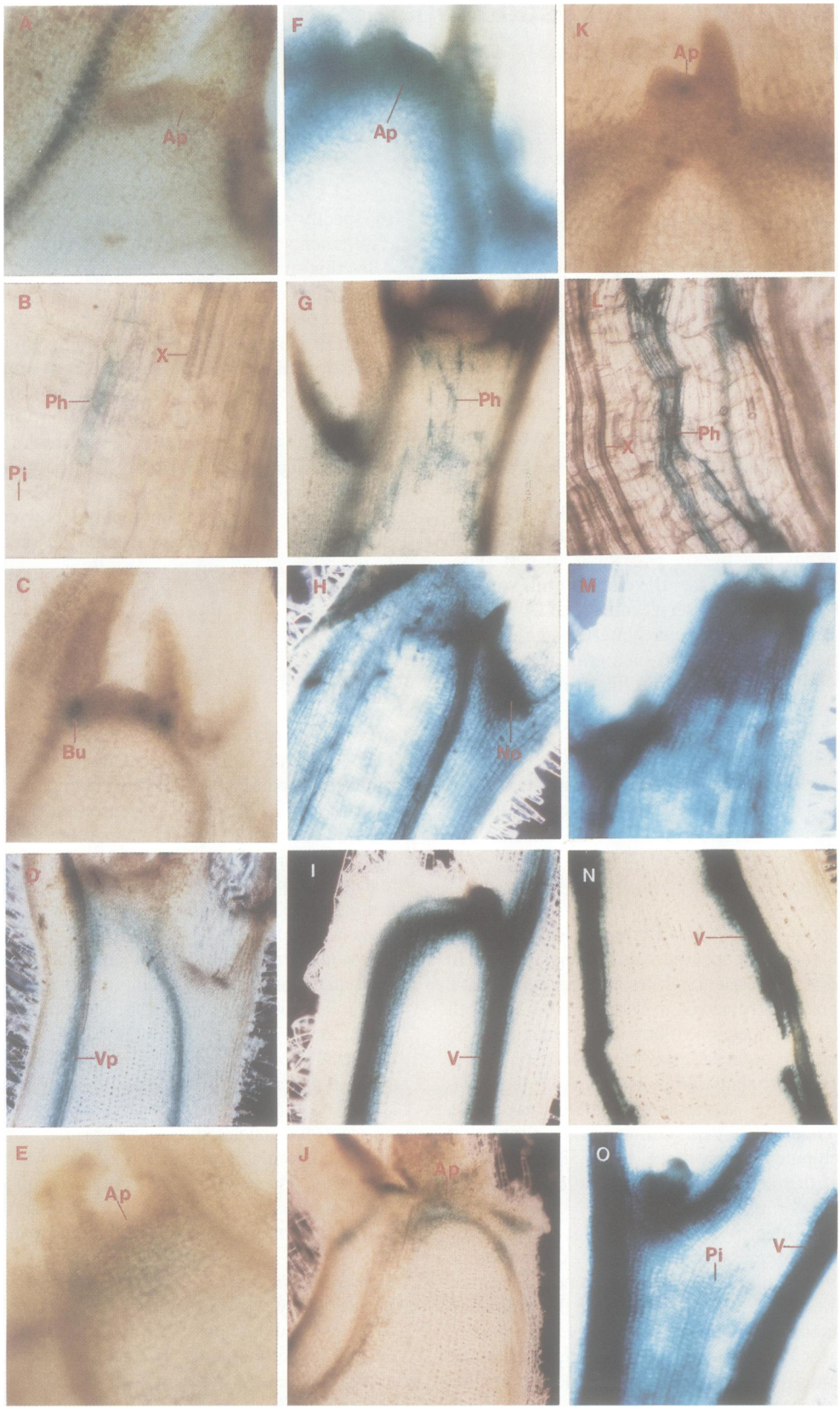

Fig. 1. Histochemical localization of expression in longitudinal sections at the stem apex of representative plants containing subdomain constructs. (In Figures 1–4 the first two columns of sections follow the same order.) (**A**) 4×B1; (**B**) 4×B2; (**C**) 4×B3; (**D**) 4×B4; (**E**) 4×B5; (**F**) 4×B1+A; (**G**) 4×B2+A; (**H**) 4×B3+A; (**I**) 4×B4+A; (**J**) 4×B5+A; (**K**) A domain; (**L**) higher magnification of 4×B2+A; (**M**) B domain; (**N**) 4×(B4+B5); (**O**) (B4+B5)+A. Abbreviations: Ap, apical meristem; Bu, axillary bud; No, node; Ph, phloem; Pi, pith; V, vascular tissue; Vp, vascular parenchyma; X, xylem.

In longitudinal sections of the stem apex, weak expression from subdomain B1 (4×B1) was detected in only one plant in the region of the apical meristem (Figure 1A). However, when B1 is fused to domain A (4×B1+A) it reproducibly confers expression in cortex and vascular tissue in the region of the stem apical meristem (Figure 1F). When fused to the TATA vector (4×B2), subdomain B2 confers expression that appears to be highly restricted in cell type. Expression in the stem is only detected in isolated cells found between the pith and the xylem (Figure 1B). These cells appear to be part of the phloem elements. In combination with domain A, expression from B2 (4×B2+A) is observed in cells that appear to make up the network of sieve tubes (Figure 1G). These cells are shown in higher magnification in Figure 1L.

Two distinct expression patterns were observed for plants containing subdomain B3 fused to the TATA vector (4×B3). In four plants, expression was detected principally at the site of emerging leaves in the stem apex (Figure 1C). In four other plants, expression in nearly all cells of the apex was observed. Possible reasons for this variation are discussed below. In combination with domain A, expression from B3 (4×B3+A) is consistently detected in most cells throughout the stem apex (Figure 1H). When fused to the TATA vector (4×B4), subdomain B4 confers expression principally in immature vascular cells (vascular parenchyma and phloem) (Figure 1D). When combined with domain A (4×B4+A), expression appears to be significantly enhanced in these immature vascular cells (Figure 1I). Subdomain B5 (4×B5) confers weak expression only in cells that are just below the apical meristem. Some enhancement of expression in this region is observed with the addition of domain A (4×B5+A) (Figure 1J).

The extent of the synergistic interaction mediated by the A domain is indicated by the expression pattern of the A domain vector alone in the stem apex. This domain confers only very weak expression in cells that appear to be part of the apical meristem and that flank the apex (Figure 1K). In contrast, the B domain fused to the TATA vector confers strong expression in nearly all cells of the stem apex (Figure 1M). The combination of subdomains B4 and B5 as a monomer gives expression that is very similar to that of the tetramer of subdomain B4—staining is principally in the vascular parenchyma. As a tetramer B4+B5 shows enhanced expression in the vascular parenchyma tissue (Figure 1N). When fused to domain A the monomer of B4+B5 confers strong expression in vascular tissue as well as some expression in pith and cortex (Figure 1O).

Transverse sections through the upper and lower stem revealed expression in certain tissues not detected at the apex. In the lower stem of the one plant that shows easily detectable expression in the stem apex, 4×B1 confers weak expression in phloem at the leaf trace (Figure 2A). When fused to domain A, weak expression from B1 (4×B1+A) is frequently detected in cortex and pith as well as in vascular tissue (Figure 2F). The highly restricted expression pattern of 4×B2 is seen in transverse stem sections where only a single cell type stains (Figure 2B). These cells appear to be part of the phloem elements and may be sieve tube cells at a particular stage of development. In combination with domain A strong expression from 4×B2 is observed in phloem elements (Figure 2G). Four plants with subdomain B3 fused to the TATA vector (4×B3) show expression in nearly all cell types in lower stem (Figure 2C). In the four other plants in which B3 confers expression in emerging leaves in the upper stem, relatively strong expression in vascular tissue is detected at the leaf trace in lower stem. Expression is also detected in the cells at the stem−leaf junction (data not shown). B3, in combination with domain A (4×B3+A), reproducibly confers expression in most cells of the stem (Figure 2H). Subdomain B4 (4×B4) confers weak expression in what appears to be the developing vascular tissue including vascular parenchyma and phloem element cells (Figure 2D). This expression is strongly enhanced when 4×B4 is combined with domain A (Figure 2I). Expression from 4×B5 is only detected in a small set of cells within buds emerging from the stem (Figure 2E). Fusion to domain A results in expression in the entire bud as well as in cells at the stem−leaf junction (Figure 2J). In some plants expression in vascular parenchyma tissue is also observed with this combination.

In transverse stem sections domain A alone confers very weak expression in vascular parenchyma and phloem cells, particularly at the leaf trace (Figure 2K). This expression is only occasionally detectable. In contrast, the intact B domain gives strong expression in nearly all cells of the stem (Figure 2L). As a monomer the combination of subdomains B4+B5 confers expression in vascular parenchyma and phloem cells (shown in high magnification in Figure 2M) which is similar to the expression of B4 alone (Figure 2D). Expression from the tetramer appears to be somewhat enhanced in the same cells (Figure 2N), while expression from the monomer fused to the A domain is observed in some pith and cortex cells in addition to strong expression in the vascular parenchyma and phloem (Figure 2O).

Expression in leaf

In leaf we detected expression from two subdomains (B2 and B4) that had shown no expression in leaf tissue at earlier developmental stages. Synergistic interactions resulting in enhanced or novel expression patterns were seen with all five subdomains in combination with domain A.

Expression in leaf tissue was observed from subdomain B1 (4×B1) only in the one plant that shows easily detectable expression in the stem and only in young leaves. Mesophyll and vascular cells in the lamina of the leaf show weak staining (Figure 3A). In combination with domain A, subdomain B1 reproducibly gives expression in most cells of the leaf (Figure 3F). As in the stem, 4×B2 confers expression only in isolated cells within the phloem elements in the leaf (Figure 3B). When fused to domain A, 4×B2 gives strong expression in phloem elements (Figure 3G). In the four B3 plants that have high expression in the stem, different expression patterns in young leaves and old leaves are observed. In young leaves expression is principally in the lamina and is particularly strong in mesophyll and trichome cells. In the midrib of these young leaves expression is observed primarily in the trichomes (Figure 3C). In older leaves expression is detected in the vascular tissue of the midrib, and only faint staining of mesophyll and trichome cells in the lamina is observed (data not shown). In the other four B3 plants only weak expression in vascular tissue of the midrib in older leaves was detected. When combined with domain A, strong expression in nearly all cell types of the leaf is consistently observed. Strong expression in all cells is seen in both young (Figure 3H) and old leaves. 4×B4 confers expression in vascular parenchyma cells of the leaf

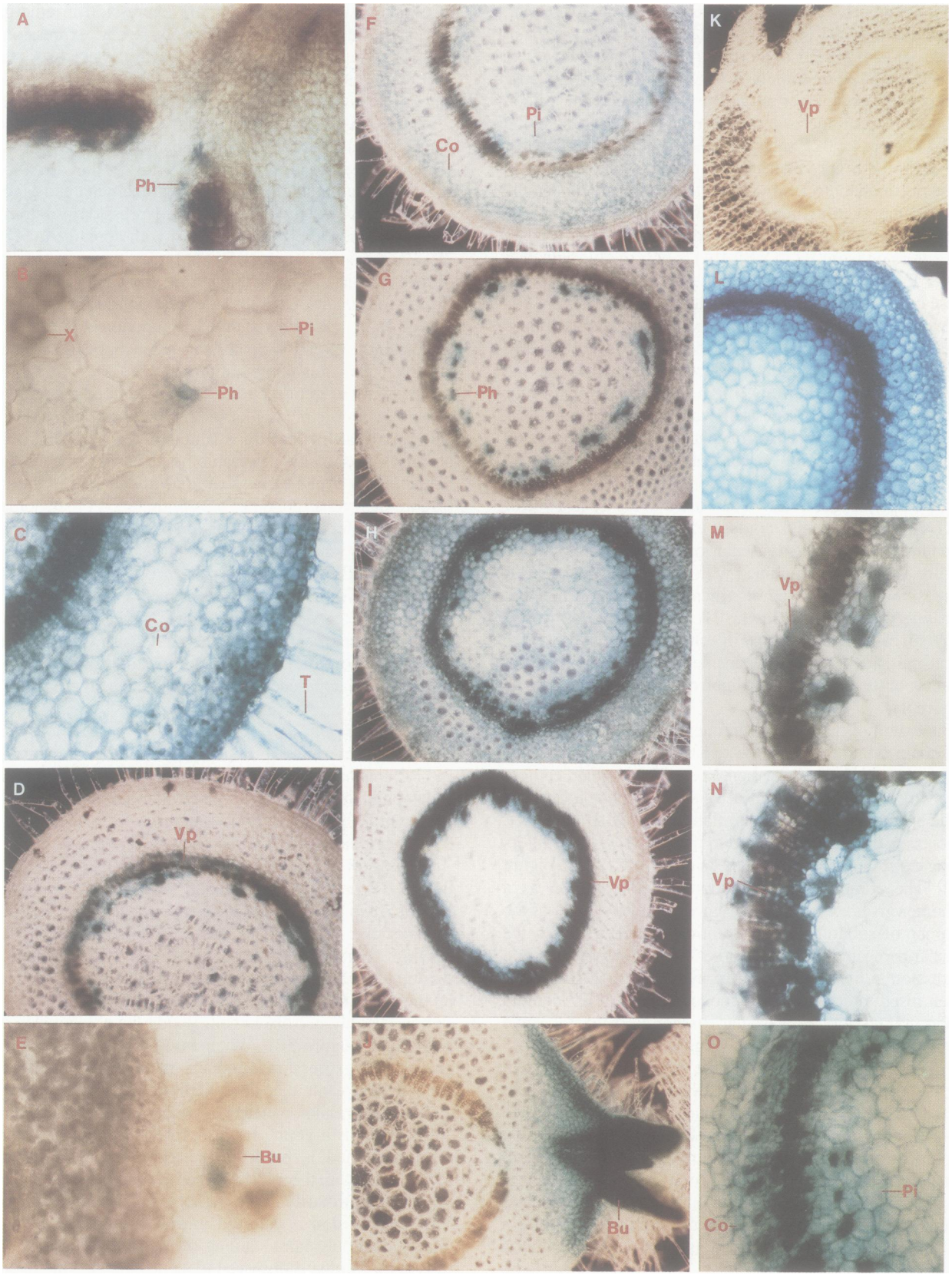

Fig. 2. Histochemical localization of expression in stem of representative plants containing subdomain constructs. (**A**) 4×B1; (**B**) high magnification of 4×B2; (**C**) 4×B3; (**D**) 4×B4; (**E**) 4×B5; (**F**) 4×B1+A; (**G**) 4×B2+A; (**H**) 4×B3+A; (**I**) 4×B4+A; (**J**) 4×B5+A; (**K**) A domain; (**L**) B domain; (**M**) high magnification of B4+B5; (**N**) high magnification of 4×(B4+B5); (**O**) high magnification of (B4+B5)+A. Abbreviations as in Figure 1 and: Co, cortex; T, trichome.

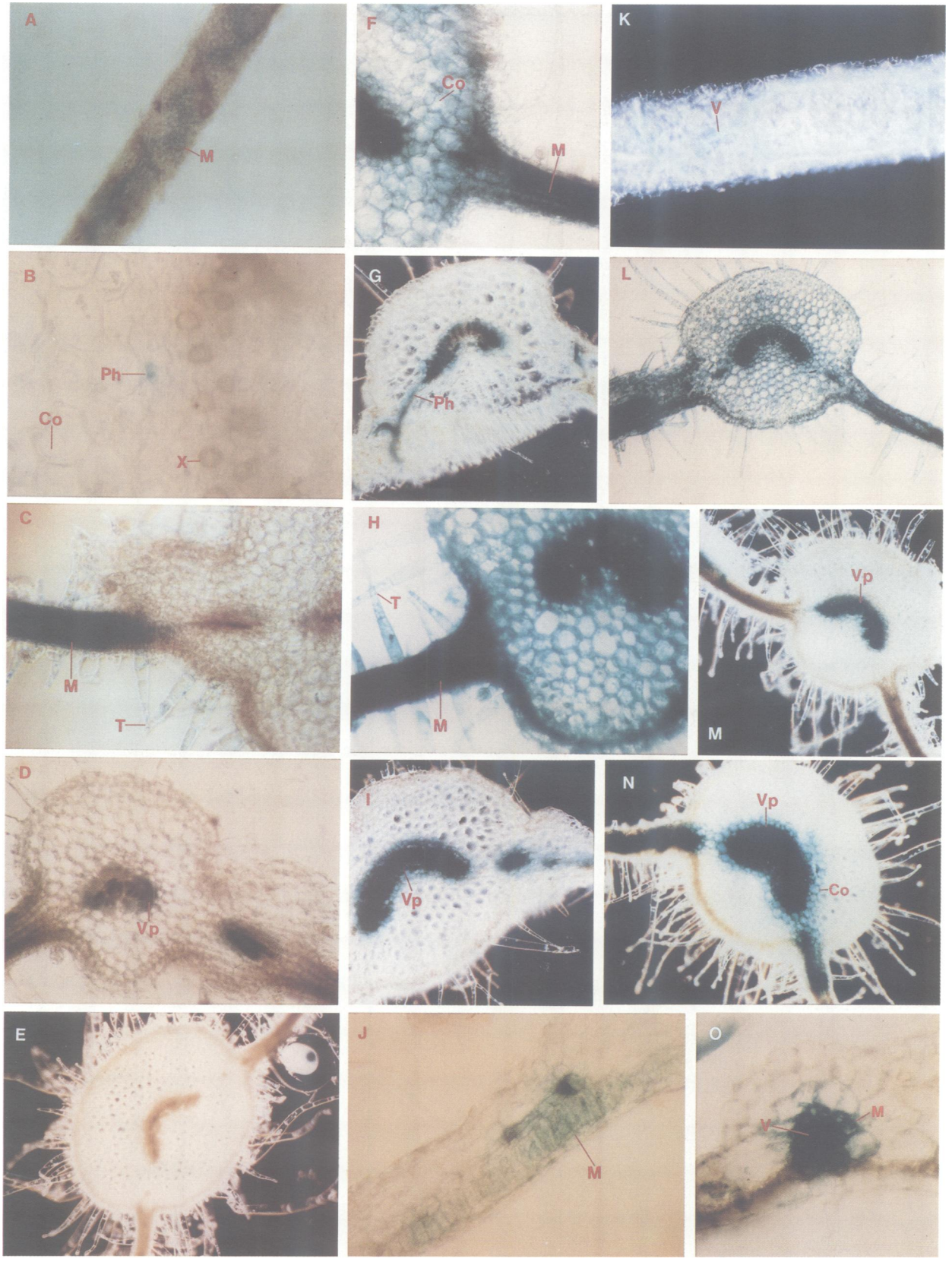

Fig. 3. Histochemical localization of expression in leaf of representative plants containing subdomain constructs. (**A**) flat section of 4×B1; (**B**) high magnification of 4×B2; (**C**) 4×B3; (**D**) 4×B4; (**E**) 4×B5; (**F**) 4×B1+A; (**G**) 4×B2+A; (**H**) 4×B3+A; (**I**) 4×B4+A; (**J**) high magnification of lamina of 4×B5+A; (**K**) flat section of A domain; (**L**) B domain; (**M**) (B4+B5); (**N**) (B4+B5)+A; (**O**) high magnification of (B4+B5)+A in lamina. Abbreviations as Figures 1 and 2 and: M, mesophyll.

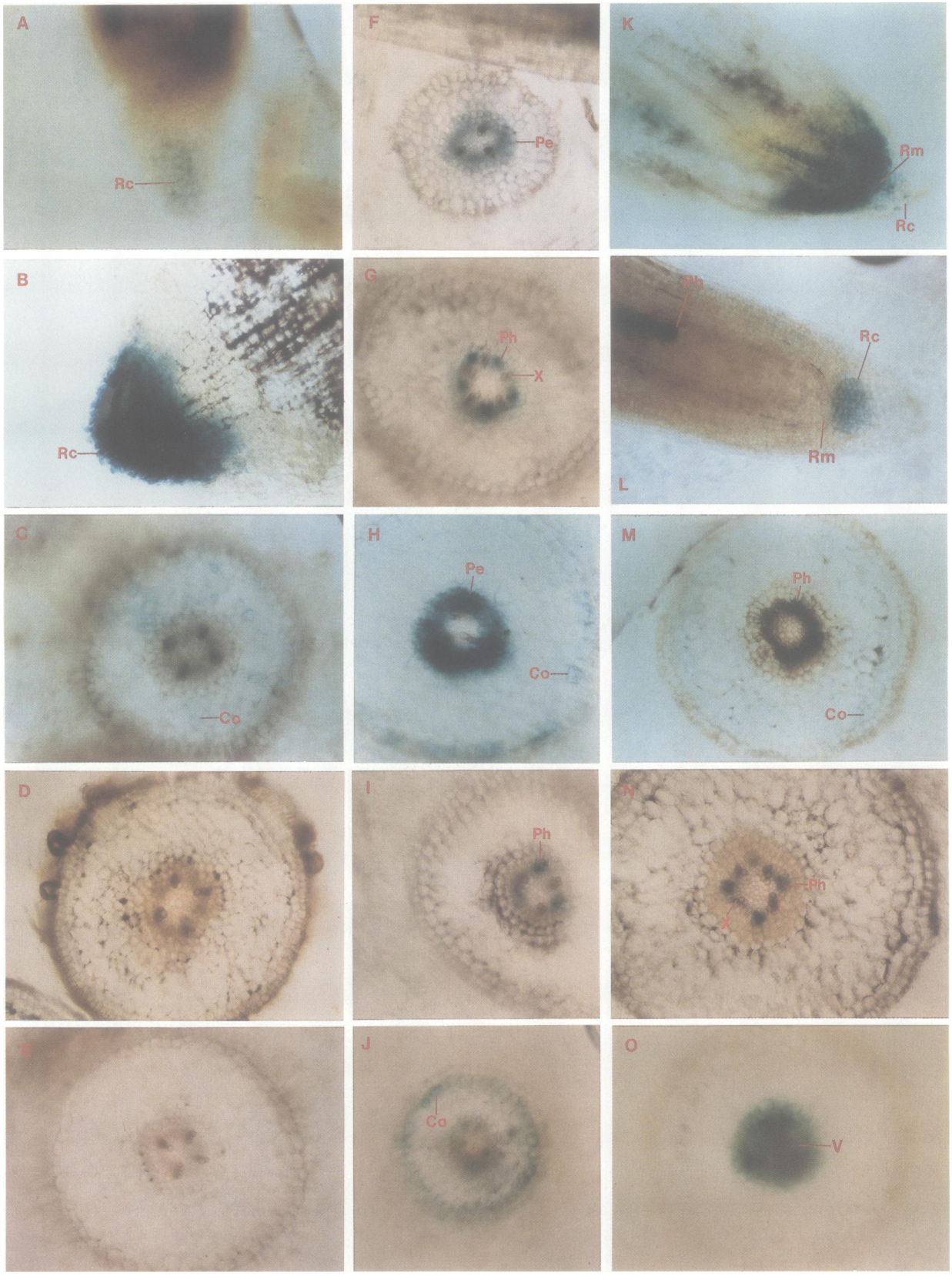

Fig. 4. Histochemical localization of expression in root of representative plants containing subdomain constructs. (**A**) longitudinal section at tip of 4×B1; (**B**) longitudinal section at tip of 4×B2; (**C**) transverse section of 4×B3; (**D**) transverse section of 4×B4; (**E**) transverse section of 4×B5; (**F**) transverse section of 4×B1+A; (**G**) transverse section of 4×B2+A; (**H**) transverse section of 4×B3+A; (**I**) transverse section of 4×B4+A; (**J**) transverse section of 4×B5+A; (**K**) longitudinal section at tip of A domain; (**L**) longitudinal section at tip of B domain; (**M**) transverse section of B domain; (**N**) transverse section of 4×(B4+B5); (**O**) transverse section of (B4+B5)+A. Abbreviations as Figures 1–3 and: Pe, pericycle; Rc, root cap; Rm, root meristem.

(Figure 3D). Enhancement of expression in these cells appears to occur when 4×B4 is combined with domain A (Figure 3I). In one plant containing 4×B5 we detected very weak expression in mesophyll tissue while all other plants had no detectable expression in the leaf (Figure 3E). When combined with domain A, weak expression from 4×B5 in mesophyll and vascular tissue is often observed (Figure 3J).

Domain A alone confers barely detectable expression in vascular tissue of the leaf (Figure 3K). Domain B alone gives expression in nearly all cells of the leaf (Figure 3L). The combination, B4+B5 as a monomer (Figure 3M) or as a tetramer confers expression in vascular parenchyma and phloem cells similar to that found with the tetramer of B4. When fused to domain A, the monomer of B4+B5 gives strong expression in the vascular tissue (Figure 3N). The cells immediately adjacent to the vascular elements also stain (Figure 3O). Due to the intense staining in the vascular elements it is difficult to rule out diffusion of the histochemical dye as the reason for the staining of the cells surrounding the vascular tissue.

Expression in root

In root tissue we detected reproducible expression from only two of the isolated subdomains (B2 and B3). A particularly informative tissue for detecting synergistic interactions appears to be root phloem. No isolated subdomain reproducibly confers expression in this tissue. However, four different combinations of *cis*-elements (B2+A, B4+A, B4+B5 and the intact B domain) consistently give expression in these cells.

In root tissue, subdomain B1 (4×B1) gives weak expression in cells that appear to be part of the root cap in the only plant that shows easily detectable expression in stem (Figure 4A). Expression from domain A alone is principally observed in the meristematic region and cortex of the root tip (Figure 4K) as well as in the pericycle (see Benfey *et al.*, 1989). Less frequently, some expression has been detected in the root cap, in root hairs and in cortex. No expression has been observed in vascular tissue. Expression from 4×B1+A usually resembles that of domain A alone with expression at the meristematic region of the tip and in the pericycle (Figure 4F). 4×B2 confers strong expression that appears to be restricted to the cells in the root cap (Figure 4B) as well as faint expression in the root hairs closest to the root tip. No expression is detected in other root tissue. However, when fused to domain A, expression in phloem tissue is observed (Figure 4G) as well as expression in the root tip and pericycle, characteristic of domain A. In the four 4×B3 plants that show high stem expression, staining was consistently observed in root cortex (Figure 4C). Very weak expression in phloem tissue was also occasionally detected. In the other four 4×B3 plants, expression in some cortex cells was occasionally observed. When combined with domain A, expression in cortex in addition to expression characteristic of domain A was frequently observed (Figure 4H). We have not detected any expression in root from subdomain B4 (4×B4) when fused to the TATA vector (Figure 4D). When combined with domain A, however, expression in phloem tissue is reproducibly observed (Figure 4I). For 4×B5 also, no expression was detected in the root (Figure 4E). In combination with domain A, there was occasionally some apparent enhancement of expression in cortex tissue (Figure 4J), and some weak expression in vascular tissue.

Domain B alone confers expression in phloem tissue and in cells that appear to be part of the root cap (Figure 4L). Weak cortex expression in addition to phloem expression is detectable in root cross-sections (Figure 4M). The combination B4+B5 as a monomer gives no expression in root, but as a tetramer consistently confers expression in phloem tissue of the root (Figure 4N). When fused to domain A, B4+B5 confers expression in most of the cells of the vascular cylinder (Figure 4O).

The effect of subdomain multimerization on expression

The effect of multimerization of the subdomains on expression was analyzed by fusion of monomers of four of the subdomains to the A domain vector. For subdomain B3, B4 or B5, the monomer confers the same qualitative expression pattern as the tetramer when fused to domain A. There is some indication from staining intensity, particularly in the case of B3, that there are higher levels of expression with the tetramer than with the monomer. There also appears to be less variation among independent transformants with the tetramer than with the monomer. The fact that the monomer of a subdomain is sufficient to confer the same expression pattern as the tetramer when fused to domain A indicates that multimerization is not a necessary condition to produce a synergistic interaction.

In one case, the same qualitative pattern was not generated with the monomer and tetramer. The monomer of B2 combined with domain A gives an expression pattern that is similar to that of domain A alone. (However, there does appear to be some slight enhancement of expression in stem vascular tissue.) When the monomer is inserted upstream of the minimal promoter (−72 to +8) used in our previous analysis (Benfey *et al.*, 1989) we do not detect any reproducible expression. In the case of this subdomain it would appear that multimerization is a necessary condition for generating the pattern of expression we describe. The fact that multimerization is necessary to produce detectable expression raised the possibility that new *cis*-elements had been created during the multimerization process of this subdomain. While we cannot rule out this possibility entirely, one piece of evidence suggests that the expression pattern that we observe comes from *cis*-elements found within the 35S enhancer. The intact B domain confers expression in cells of the root cap. The only subdomain that reproducibly confers expression in these cells is B2. In addition, the B2 subdomain has at least one of the characteristics of an enhancer. As a tetramer, there is no change in the expression pattern when B2 is inserted in either orientation in the TATA vector or the A domain vector.

Discussion

Complementary and redundant expression patterns

In the mature plant we are able to detect expression in at least one organ from four of the five B subdomains when fused to the minimal promoter vector. In all cases, analysis of the mature plant revealed expression that had not been detected in earlier stages of development. This suggests that the expression conferred by the 35S B subdomains is developmentally regulated. In addition, expression from several subdomains is quite restricted in cell type. Subdomain B4 gives detectable expression only in vascular tissue. This expression appears to be principally in the immature vascular

cells of the vascular parenchyma and phloem. The ability of B4 to confer expression in developing vascular tissue is also evident in the finding that, in combination with domain A or with subdomain B5, expression is consistently observed in vascular tissue in the hypocotyl of young seedlings (see Benfey *et al.*, 1990). Subdomain B2 also confers expression in cells that appear to be part of the vascular tissue. However in this case expression is only detected in isolated cells that appear to be a constituent part of the phloem. Comparison of the expression patterns of B4 and B2 suggests that the former confers expression primarily in immature vascular tissue and the latter gives expression in more mature cells of the vascular elements. This result indicates that within the B domain there are modules that confer complementary expression patterns. Expression from B2 is also found in two other tissues, the root cap and root hair.

The three other subdomains are able to confer expression in non-vascular tissue of the leaf and stem. For subdomains B1 and B5 the expression is only readily apparent when they are fused to domain A. The expression pattern of subdomain B3 is the most complex. In young leaves of some plants, expression from the subdomain fused to the TATA vector is strong only in non-vascular tissues. In older leaves of these plants, however, the situation is reversed, with strong staining in vascular tissue and only weak staining in other tissues. In stem, expression in nearly all cells is observed. In addition, the variation among independent transformants was greatest for this subdomain. When combined with domain A, nearly all cells show staining in both young and old leaves. The complexity of the expression pattern conferred by B3 suggests there may be several active *cis*-elements within this subdomain. The presence of more than one active *cis*-element within this subdomain may also account for the qualitative differences in expression among independent transformants observed in the early stages of development (Benfey *et al.*, 1990).

The ability of these three subdomains to confer expression in non-vascular leaf tissue indicates that there is some functional redundancy among the subdomains that make up the 35S enhancer. However, this redundancy is generally restricted to a particular organ at one stage of development. When the expression patterns are compared in other organs and throughout development it becomes clear that substantial differences exist among the patterns conferred by the subdomains. The tissue-specific expression patterns conferred by some of the subdomains can serve as useful markers for cells at particular developmental stages. These restricted expression patterns raise the possibility that the factors that interact with these sequences regulate endogenous plant genes containing cognate binding sites that perform important roles in plant development.

Synergistic interactions and the combinatorial code

Evidence for synergistic interactions among *cis*-elements within the 35S enhancer comes from the combination of each one of the subdomains with domain A, which generates expression that is detectable in tissues that do not show expression with the isolated subdomain or with domain A alone. In Figure 5 we have summarized in schematic form the salient features of the expression patterns of the subdomains fused to either the TATA vector or the A domain vector in mature plants. Although expression in more tissues was observed from the isolated subdomains in the mature plant than in the earlier stages of development, the patterns

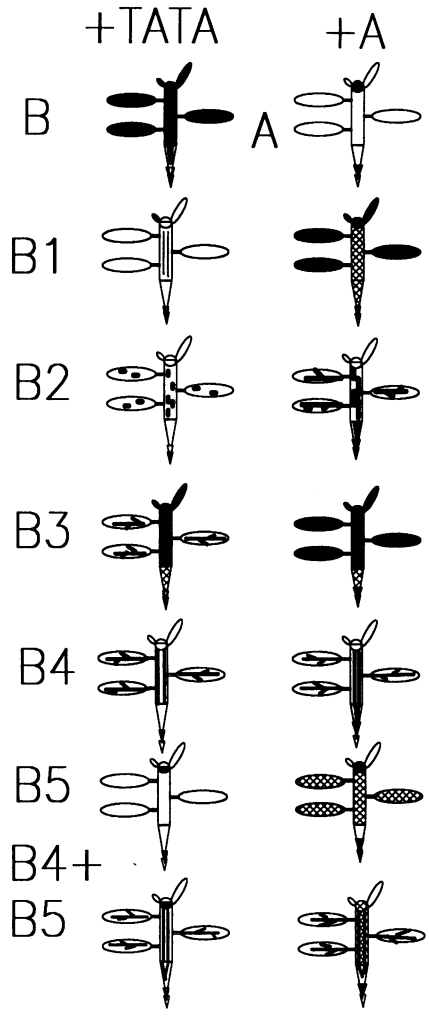

Fig. 5. Schematic representation of expression patterns of subdomain constructs. Expression in mature plants is represented by the darkened areas in the schematic representations. On the left, expression conferred by the intact B domain (at top) and by the subdomains in the TATA vector is represented. On the right, expression conferred by the domain A vector alone (at top) and by the subdomains in the domain A vector is represented. Only the major features of the expression pattern for each construct have been shown. Cross-hatching represents low level expression in that region.

from the isolated subdomains still do not add up to the expression pattern of the intact B domain. (For example, no subdomain confers expression in mesophyll tissue of older leaf.) As noted in the accompanying paper it is possible that important *cis*-elements were interrupted when the subdomains were isolated. Given the evidence for synergistic interactions between the subdomains and domain A we consider it more likely that synergistic interactions among the B subdomains are responsible for the missing expression. Further evidence for synergistic interactions among B subdomains comes from the observation that the combination of B4 and B5 confers expression in root phloem tissue, while neither subdomain alone is able to confer any expression in this tissue. The fact that B4 is also able to give expression in root phloem tissue when combined with domain A suggests that the synergistic interaction between B4 and B5 may be functionally equivalent to the synergistic interaction between B4 and domain A (see below).

The finding that synergistic interactions among *cis*-

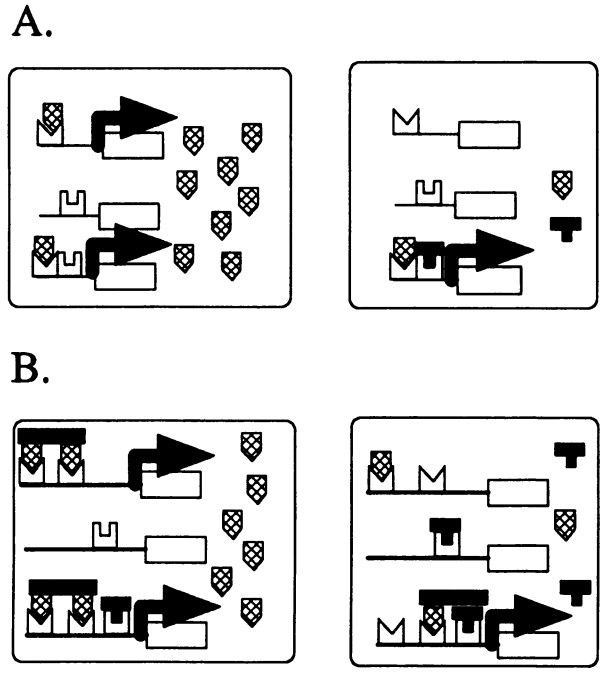

Fig. 6. Models for synergistic interactions among *cis*-elements. **(A)** Transcriptional activation when synergism is dependent on cooperative binding of factors to *cis*-elements. On the left, when factor concentration is high (cross-hatched polygons), transcriptional activation occurs if there is a binding site for the factor. On the right, when factor concentration is low, transcriptional activation only occurs from a promoter that contains sites for heterologous factors (solid T-shaped polygons) that are able to cooperatively bind to the DNA. **(B)** Transcriptional activation when synergism is mediated by an interaction with a target factor. On the left, when factor concentration is high enough to fill the number of sites needed for productive interaction with the target, transcriptional activation occurs from promoters that have enough bound sites. On the right, when factor concentration is low, transcriptional activation only occurs when the target factor interacts with heterologous factors that bind independently to the DNA.

elements within the 35S enhancer can produce cell-specific expression has implications for a combinatorial code. Two types of models can explain how synergistic interactions among *cis*-elements can result in tissue-specific expression. The first postulates that cooperative interactions between heterologous factors enable binding to *cis*-elements when both factors are in low concentration in the cell (see e.g. Wahli and Martinez, 1989 and references therein). This type of model predicts that a cell that contains a high concentration of an active *trans*-factor should be able to activate transcription from genes that contain a binding site for the factor (Figure 6A, left). In the situation when active factor concentration is low, binding to the *cis*-element would not occur unless there is a cooperative interaction with another factor (Figure 6A, right). Thus, the synergism between two *cis*-elements (e.g. B4 and domain A) that results in expression in a cell type not detected with either *cis*-element alone (e.g. root vascular tissue) would be the result of an interaction between heterologous factors allowing cooperative binding to a promoter that contains both *cis*-elements. No expression would be detected with either isolated *cis*-element because the concentration of either factor in that cell is not high enough to allow for productive binding without the cooperative interaction.

An alternative model postulates that synergism is mediated by an interaction between bound *trans*-factors and a target factor (Kakidani and Ptashne, 1988; and described in Berk and Schmidt, 1990). In this model the target factor must interact with more than one bound factor at a time. Synergism arises from the necessity to have a minimum number of sites filled to obtain a productive interaction. Below this minimum number a productive interaction does not occur. According to this model, when the concentration of active factor is high enough to allow binding to the correct number of sites on the DNA, then activation of transcription occurs (Figure 6B, left). However, when there is an insufficient concentration of factor to consistently occupy the sites then no expression occurs from the promoters that contain only those sites. A promoter that contains a combination of different binding sites can confer expression because a heterologous factor can bind and interact with the target factor to produce a productive interaction (Figure 6B, right).

The key features of the second model are that binding to *cis*-elements is not mediated by cooperative interactions between heterologous factors and that different *trans*-factors interact with the target factor in a functionally equivalent manner. According to the second model the synergism between B4 and domain A that results in expression in root vascular tissue would be due to an insufficiently high concentration of the factors that bind the *cis*-elements within these modules. Therefore, with either B4 or the A domain alone there are not enough sites occupied to activate transcription. However, with the combination of B4+A, there are enough partially filled sites to obtain a productive interaction with the target factor and activate transcription.

There are several predictions concerning combinations of *cis*-elements made by these models. The first model predicts that in order to effect cooperative factor binding, the distance between *cis*-elements is likely to be critical. In the second model, the distance between *cis*-elements is not particularly important, since it is postulated that the DNA between the bound factors is flexible enough to allow the target to contact two non-adjacent factors. Our results only indirectly address the question of spacing between *cis*-elements. Since we do not know exactly where factors bind on some of the *cis*-elements within the subdomains, we either produced the correct spacing that allows cooperative binding of heterologous factors by chance or spacing is not critical for synergistic interactions. We can further test this prediction by relocating one of the subdomains (e.g. B1) that shows synergistic interactions with domain A further away and observing if synergism still occurs.

A second prediction concerns the functional equivalence of *cis*-elements. The first model proposes that *cis*-elements bind factors that must interact with each other. Since these protein−protein interactions are likely to be highly specific there are probably constraints as to what *cis*-elements can interact to produce a synergistic effect. In the second model, the target factor must be able to interact with a variety of different factors through their similar activation domains (Ptashne, 1988), so that *cis*-elements are functionally equivalent.

The prediction from this second model is that if expression can be detected from a combination of two *cis*-elements in a particular cell, then multimerization of either of the *cis*-elements should also produce expression. If expression is not produced when one of the elements is multimerized then this model predicts that the concentration of factor is so low that increasing the number of sites does not increase

the likelihood of binding that factor.

Most of our results can be readily explained by the second model. The advantage of this model is that the sort of synergistic interaction we observe with various subdomains fused to the A domain does not require that the factor that binds the A domain must be able to physically interact with each of the factors that bind the subdomains. In addition, multimerization of the as-1 site that is found within the A domain results in expression in most of the tissues in which combinations of subdomains and the A domain give expression (E.Lam and N.-H.Chua, in preparation).

However, there is one case that suggests there may be qualitative differences among *cis*-elements. One copy of subdomain B4 in combination with domain A is sufficient to confer expression in vascular tissue of young seedlings and mature root. However, four copies of subdomain B4 do not confer expression in either tissue. In addition, domain A alone does not confer expression in either tissue. Our results also show that the combination of subdomains, B4+B5 as a tetramer reproducibly gives expression in seedling and root vascular expression. However, four copies of B5 fused to domain A do not confer expression in seedling vascular tissue (there is a low amount of expression from this combination in root vascular tissue). If *cis*-elements are functionally equivalent then one would predict that since B4+B5 gives the same expression as B4+A then B5+A should also give the same expression pattern. While further experiments with different numbers of *cis*-elements need to be performed, our results suggest that there may be qualitative differences in the abilities of *cis*-elements to mediate synergistic interactions. This suggests that domains A and B5 have a similar character that differs from B4. A modification of the second model to include a target factor able to distinguish different classes of bound factors could explain our results. Although synergistic interactions have been observed in other model systems (e.g. Fischer and Maniatis, 1988; Crenshaw *et al.*, 1989) we note that transgenic plants provide a particularly useful system to test models of synergistically mediated gene expression since expression can be easily analyzed in a variety of cells at different stages of development.

Variation among independent transformants

In Table I we have listed the number of plants analyzed for each construct and the number that gave the expression pattern reported. Except where noted, when a plant did not show the reported expression pattern, no expression in any tissue was observed. In the case of B3, there were two types of expression patterns in mature plants. This may indicate the presence of several *cis*-elements or it may be an extreme example of quantitative variation due to position effect. For B1 in the TATA vector, expression was detected only in a single plant. This may be due to insertion into the chromosome near an enhancer that confers this expression pattern. It is also possible that the insertion site is particularly favorable for the expression mediated by the *cis*-element within the B1 subdomain.

Cis-elements and trans-factors

One of the reasons for undertaking this analysis was to identify the tissues in which factors that bind to the B domain are likely to be found in the highest abundance. Our results

Table I. Variation among transgenic plants containing 35S subdomain constructs

Construct	Generation	Stage	Expressing/ analyzed	Variation
4×B1	R1	P	0/4	
	R0	P	1/10	
4×B1+A	R1	P	10/10	variable amount of mesophyll expression in leaf and of pith and cortex expression in stem
1×B2+ (−72 to +8)	R1	P	0/7	
4×B2(F)	R1	P	3/4	
	R0	P	1/1	
4×B2(R)	R1	P	3/3	
1×B2+A	R1	P	5/6	
4×B2(F)+A	R1	P	3/6	
4×B2(R)+A	R1	P	8/9	
4×B3	R1	P	3/6	only in buds and lower stem
	R0	P	5/12	1 only in buds, 4 strong in lamina of young leaves (see text)
1×B3+A	R1	P	5/6	1 young leaf like 4×B3, variable amount of pith and cortex expression in stem
4×B3+A	R1	P	8/10	
4×B4	R1	P	4/4	
	R0	P	6/8	1 only light spot at apex
1×B4+A	R1	P	6/6	1 some pith and cortex expression in stem
4×B4+A	R1	P	9/9	2 some pith and cortex expression in stem
4×B5	R1	P	3/6	
1×B5+A	R1	P	10/10	3 some mesophyll expression in leaf
4×B5+A	R1	P	8/10	8 show mesophyll expression in leaf
1×(B4+B5)	R1	P	3/7	
4×(B4+B5)	R1	P	8/10	5 light pith and cortex expression in stem
1×(B4+B5)+A	R1	P	10/10	
B domain	R1	P	7/8	1 no vascular expression in midrib of young leaf, no non-vascular stem expression
−46 vector	R1	P	0/11	

Abbreviations: P, mature plant; (F), forward orientation; (R), reverse orientation.

provide this information as well as allowing us to make some predictions as to the possible physiological role of the factor interactions with endogenous plant genes. For example, expression from subdomain B4 appears to be in a particular cell type, suggesting that genes expressed within the same cell type in the plant may interact with the factor or factors responsible for this expression pattern. However, expression from subdomain B2 is in three different cell types. In this case there may be three *cis*-elements or a single *cis*-element that confers expression in these three tissues because they are in a similar developmental stage or are responding to the same environmental cue or endogenous physiological effectors. The number of active *cis*-elements within the 35S enhancer is difficult to predict. The fact that nearly the entire B domain is within open reading frame (ORF) VI of CaMV suggests that there may be strong constraints on the number and placement of *cis*-elements within this region. Characterization of non-viral plant gene promoters has shown that they also have several *cis*-elements which bind different factors (Green *et al.*, 1988; Giuliano *et al.*, 1988). Therefore this modular structure is not confined to viral promoters. It will be interesting to see whether cellular constitutive promoters have this sort of organization.

We have previously described the characterization of a factor that binds to the as-1 *cis*-element within domain A (Lam *et al.*, 1989; Katagiri *et al.*, 1989). As discussed in the accompanying paper we cannot conclusively attribute the synergistic properties of domain A to the presence of the as-1 *cis*-element. However, the cloned factor that binds to this element has one of the properties expected of a factor able to mediate expression in root tissue and interact synergistically to generate expression in other tissues. The RNA homologous to the cDNA for the factor is 5- to 10-fold more abundant in root but it is present in easily detectable amounts in leaf tissue. A sequence element named 'activation sequence' (as)-2 has been identified within subdomain B1 which interacts with a factor present in leaf extracts, but which is undetectable in root extracts (Lam and Chua, 1989).

We note that it is difficult to rigorously rule out the possibility that we have created new sites for factor interactions in the process of making the various constructs. We have attempted to control for the creation of new sequences in the multimerization process by analyzing expression of both the monomer and the multimer in the A domain vector. Even with the monomers it remains possible that factor binding sites were created at the junction between the subdomains and the sequences added to provide cloning sites. However, several of our constructs can be viewed as simple internal deletions. These include the monomer of B4+B5 in the TATA vector, and each of the B subdomain monomers in the A domain vector. In all these cases the expression we detected was less than that of the intact region, suggesting the loss of information rather than the gain of information. The combination of subdomains B4+B5 as a monomer gives expression in most tissues that is similar to that of B4 as a tetramer indicating that multimerization is not a necessary condition to obtain this expression pattern. Although no similar result has been observed in any other system we also cannot rule out the possibility that merely increasing the distance between the subdomain and TATA sequence is responsible for the synergistic interactions observed. We note that we have analyzed a total of 27 independent transformants that carry either the TATA vector alone or the −72 to +8 promoter vector (Benfey *et al.*, 1989) and we have not detected expression in any tissue at any stage of development. In addition, several other putative sequence elements from other genes (e.g. ABA inducible genes) have not shown any expression when inserted in either the A domain or the TATA vector (M.Mino and N.-H.Chua, unpublished observations).

Conclusion

In our attempt to understand the combinatorial code that determines developmentally regulated expression in higher plants we have found evidence for both additive and synergistic interactions among subdomains of the 35S enhancer. Monitoring expression throughout development and in various tissues allowed us to detect differences in expression that would have been obscured had we analyzed a single organ at one developmental stage. As we identify the *cis*-elements within the 35S promoter we will be able to introduce new combinations of these elements into the plant in order to precisely define the combinatorial properties of *cis*-elements that control developmentally regulated and tissue-specific expression in plants.

Materials and methods

Constructs

The constructs containing the 35S subdomains are described in Benfey *et al.*, 1990.

Transgenic plants

The constructs were mobilized into *Agrobacterium tumefaciens* and transgenic tobacco plants were generated as previously described (Benfey *et al.*, 1989). R1 plants were handled as previously described (Benfey *et al.*, 1989). For the R0 plants, cuttings were made from the plant that emerged from callus. The cuttings were transplanted into media containing the antibiotics used for selection (kanamycin and carbenicillin) and allowed to root for 4–6 weeks before analysis.

Histochemical staining

Histochemical staining was performed as previously described (Jefferson *et al.*, 1987; Benfey *et al.*, 1989).

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