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Chitinase, β-1,3-Glucanase, Osmotin, and Extensin Are Expressed in Tobacco Explants during Flower Formation

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Sequence analysis of five gene families that were isolated from tobacco thin cell layer explants initiating floral development [Meeks-Wagner et al. (1989). Plant Cell 1, 25-35] showed that two encode the pathogenesis-related proteins basic chitinase and basic β-1,3-glucanase, while a third encodes the cell wall protein extensin, which also accumulates during pathogen attack. Another sequence family encodes the water stress-induced protein osmotin [Singh et al. (1989). Plant Physiol. 90, 1096-1101]. We found that osmotin was also induced by viral infection and wounding and, hence, could be considered a pathogenesis-related protein. These genes, which were highly expressed in explants during de novo flower formation but not in explants forming vegetative shoots [Meeks-Wagner et al. (1989). Plant Cell 1, 25-35], were also regulated developmentally in day-neutral and photoresponsive tobacco plants with high expression levels in the roots and moderate- to low-level expression in other plant organs including flowers. An unidentified gene family, FB7-4, had its highest level of expression in the basal internodes. Our findings indicate that these genes, some of which are conventionally considered to encode pathogen-related proteins, also have a complex association with normal developmental processes, including the floral response, in healthy plants.

INTRODUCTION

The morphogenetic changes evident in plants during the transition from vegetative to reproductive development are accompanied by the appearance of new gene products (Pierard et al., 1980). The identification of these gene products and the molecular mechanism of their induction endure as a major challenge to understanding the regulation of angiosperm development. The in vitro tobacco thin cell layer (TCL) explant system offers the opportunity to compare gene expression during vegetative and floral development in tissues at equivalent developmental stages. TCL explants taken from the floral branches of the day-neutral tobacco *Nicotiana tabacum* cv samsun *nn* can be induced to form either floral buds or leafy vegetative shoots by altering the cytokinin used in the culture medium (Tran Thanh Van et al., 1974). Medium containing the cytokinin kinetin directs flower formation, whereas medium containing zeatin (a related cytokinin) directs vegetative shoot formation. The type of cytokinin used is the only variable in the culture medium.

The TCL system was used to isolate genes expressed at an early stage of TCL floral but not vegetative development by differential screening of a cDNA library prepared from floral bud day 7 (FB7) explants after 7 days of culture on kinetin medium (Meeks-Wagner et al., 1989). These clones represent transcripts expressed abundantly in the mRNA population of FB7 explants and rarely in the mRNA population of vegetative shoot day 7 (VS7) explants. The 52 clones isolated were assigned by sequence homology to six gene families designated FB7-1 to FB7-6.

During normal development of whole *N. tabacum* cv samsun *nn* plants grown from seed, expression of FB7-1 and FB7-2 was detected in the prefloral and floral shoot apices with very little expression evident in vegetative/juvenile shoot apices (Meeks-Wagner et al., 1989).

In this paper we report on the results obtained from gene analysis undertaken on five of the six FB7 gene families. We identified four of the FB7 gene families as encoding gene products that are also induced by environmental and physiological stress, including pathogen attack. The fifth family showed no significant homology to any previously reported gene sequence. We also extended the analysis of the expression patterns of these genes in healthy plants, both in the day-neutral samsun tobacco

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and the photoperiodic plants *N. sylvestris* and Maryland Mammoth tobacco. Our findings indicate that these genes are developmentally regulated during normal plant morphogenesis in other than floral-initiating tissues.

RESULTS

The expression of FB7 genes isolated from TCL explants during in vitro floral initiation is not confined solely to the meristematic tissue. These genes are expressed in many cell types throughout the explant at the time of cell division and elongation that accompanies floral initiation (Meeks-Wagner et al., 1989). In whole plants FB7 gene expression is also observed in nonfloral tissues with the highest levels found in the roots. The activity of the FB7-1, FB7-2, and FB7-5 genes in the roots of the day-neutral *N. tabacum* cv samsun peaks immediately before the transformation of the shoot apex to its reproductive state (Meeks-Wagner et al., 1989). Gene analysis, undertaken in an attempt to define the association of these genes with floral induction events, revealed that four of five FB7 cDNA families encode gene products known to be induced by environmental and physiological stress.

Several Chitinase Genes Are Expressed in TCL Explants during Floral Development

The FB7-1 cDNA isolates revealed three different classes of sequence belonging to the chitinase family. In tobacco, this gene family comprises both acidic and basic chitinases (Legrand et al., 1987). All FB7-1 sequences show extensive homology to the basic chitinase sequence isolated from the tobacco Havana 425 (Shinshi et al., 1987). Figure 1 illustrates that two of the classes, FB7-$1_{(1)}$ and FB7-$1_{(2)}$, show complete identity to the published Havana sequence in both the 5′- and 3′-terminal regions. The differences in the FB7-$1_{(1)}$ and FB7-$1_{(2)}$ sequences involve only four codons within the domain which is believed to code for an amino-terminal leader sequence and may represent allelic variation.

The third sequence, FB7-$1_{(3)}$ (see Figure 1), is approximately 95% homologous to the coding region of the other two sequence classes with the majority of nucleotide changes resulting in silent substitutions. In addition, this sequence contains a 12-bp insert in the coding region which adds four amino acid residues to the protein, two of which are prolines. The homology between the 3′-noncoding region of this third sequence class and that of the other two sequence classes is only 85%, suggesting that the third sequence class is not an allele of the other two but represents a sequence from a different gene.

Tobacco is an amphidiploid with one diploid genome being derived from *N. sylvestris* and the other from *N. tomentosiformis* (Gerstel, 1976). The presence of a KpnI restriction enzyme site (Figure 1) unique to the FB7-$1_{(3)}$ sequence enabled the relationships of the sequences to the progenitor species to be determined. As seen in Figure

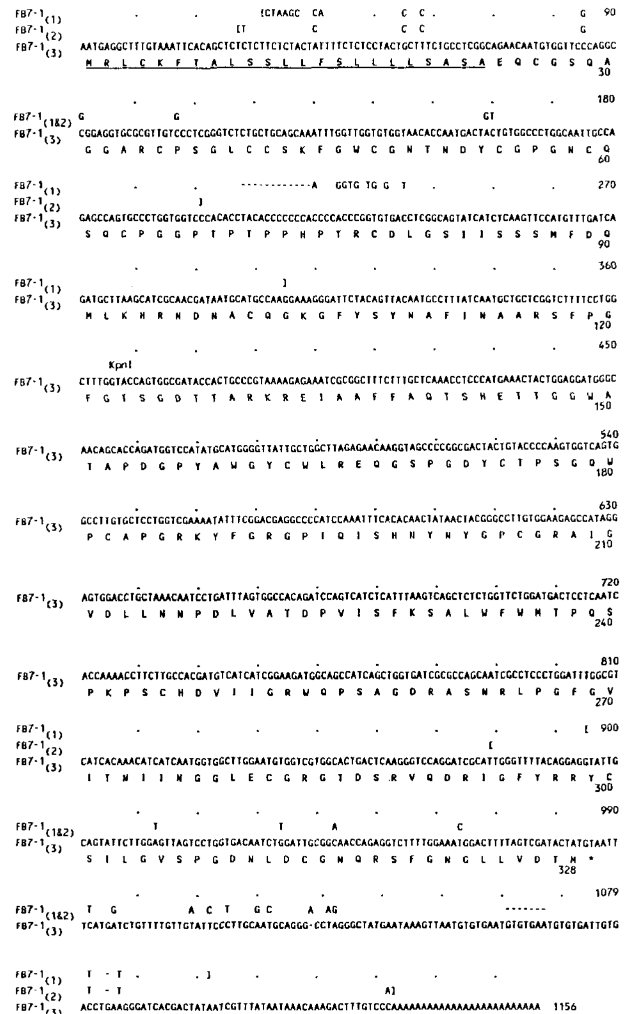

Figure 1. Nucleotide Sequences of Basic Chitinase cDNAs in *N. tabacum* cv Samsun *nn*.

The nucleotide sequence of the FB7-$1_{(3)}$ cDNA clone is shown. The amino acid sequence deduced from the FB7-$1_{(3)}$ sequence is shown below the cDNA sequence with the putative amino-terminal leader sequence underlined. Sequences FB7-$1_{(1)}$ and FB7-$1_{(2)}$ have been partially sequenced in regions as indicated by enclosed brackets. [FB7-$1_{(1)}$ nucleotides 32 to 304 and nucleotides 897 to 1101; FB7-$1_{(2)}$ nucleotides 28 to 200 and nucleotides 880 to 1131.] The nucleotide mismatches that occur in FB7-$1_{(1)}$ and FB7-$1_{(2)}$ are indicated above the FB7-$1_{(3)}$ sequence. Dashes indicate gaps introduced into the sequences to maximize alignment. The recognition site for KpnI is indicated.

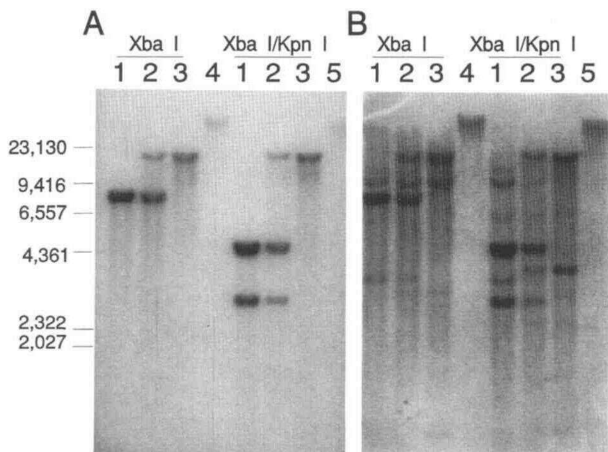

Figure 2. Tobacco DNA Digests Probed with the Chitinase FB7-$1_{(3)}$ cDNA.

(A) Autoradiogram of ^{32}P-labeled FB7-$1_{(3)}$ hybridization to XbaI and XbaI plus KpnI digests of genomic DNA from *N. tomentosiformis* (lane 1), *N. tabacum* cv samsun *nn* (lane 2), and *N. silvestris* (lane 3). Undigested DNA from *N. tomentosiformis* (lane 4) and *N. tabacum* cv samsun *nn* (lane 5) was included.

(B) The same filter-hybridization probed and washed at lower stringency as described in Methods.

2A, DNA gel blot analysis of restricted DNA from samsun tobacco and its two progenitors showed that the FB7-$1_{(3)}$ chitinase sequence is derived from *N. tomentosiformis*. Complementary experiments with other unique restriction enzyme sites showed that the FB7-$1_{(1)}$ and FB7-$1_{(2)}$ sequences are derived from *N. sylvestris*.

Hybridizations performed under less stringent conditions suggested that two or more basic chitinase genes are present in each progenitor genome, as shown in Figure 2B.

FB7-2 Encodes Osmotin, Which Is Related to the PR-S Protein

FB7-2 encodes the protein osmotin. Osmotin has been shown to accumulate in plant cells under both water and salt stress but not as a result of heat shock or anoxia (Singh et al., 1985). The deduced amino acid sequence of FB7-2 differs in 7 residues from that of an osmotin cDNA clone isolated from osmotically stressed cells of *N. tabacum* cv Wisconsin 38 (Singh et al., 1989). No differences are observed in the N-terminal signal peptides of the gene products isolated from the two tobacco cultivars. The calculated pI for the mature FB7-2 protein is 7.5 and the predicted molecular size is 24,285 D. Where the sequence deviates from the Wisconsin 38 tobacco sequence, FB7-2 shows identity with the homologous NaCl-induced protein from tomato (King et al., 1988).

The amino acid sequence of the mature form of FB7-2 also shows 65% homology to the tobacco mosaic virus (TMV)-induced thaumatin-like protein PR-S from samsun *NN* (Van Kan et al., 1989), as shown in Figure 3, and the equivalent PR-R protein from CV Xanthi-nc tobacco (Pierpoint et al., 1987; Payne et al., 1988). The 65% level of homology between FB7-2 and PR-S does not extend to the 25-amino acid N-terminal sequence of PR-S, which is believed to represent a signal peptide involved in secretion of this TMV-induced protein into the intercellular space of the tobacco leaf (Bol, 1988). However, neither the tomato

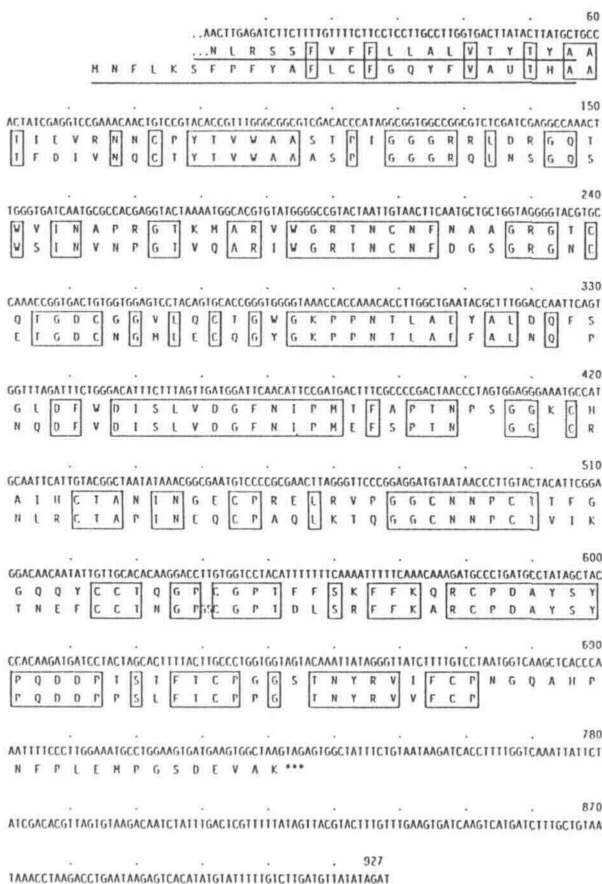

Figure 3. Comparison of the Deduced Amino Acid Sequence of Osmotin with PR-S from *N. tabacum* cv Samsun *NN* (Cornelissen et al., 1986).

The nucleotide sequence of the FB7-2 cDNA clone from *N. tabacum* cv samsun *nn* is shown with the deduced amino acid sequence directly beneath. Amino acids that are identical in the PR-S sequence, shown below the FB7-2 amino acid sequence, are boxed. The underlines indicate the regions corresponding to the N-terminal signal peptides.

nor the tobacco salt-stress proteins have been shown to be secreted. The majority of the cellular osmotin protein is localized in the vacuole of salt-stressed cells (Singh et al., 1985).

Extensin Is Expressed during Floral and Vegetative Development when Explants Are Cultured on Kinetin but not in Explants Cultured on Zeatin

In contrast to the other FB7 cDNA gene families, FB7-3 shows significant expression in kinetin-cultured TCL explants originating from a basal region of the tobacco stem (Meeks-Wagner et al., 1989). These explants undergo vegetative shoot formation instead of the floral development exhibited by kinetin-cultured explants derived from floral branches higher on the stem. The fact that FB7-3 expression occurs in both basal and floral branch explants cultured on kinetin but not in floral branch explants cultured on zeatin suggests that FB7-3 may be kinetin induced. The FB7-3 cDNA clone shows 96.5% homology throughout its 285 bp to the 3′-noncoding region of a tobacco extensin cDNA clone isolated by Memelink et al. (1988). This extensin clone was isolated from transgenic tobacco shoots containing elevated cytokinin levels due to the expression of the *Agrobacterium tumefaciens* T-DNA isopentenyltransferase gene. However, these researchers found that spraying with cytokinin did not result in extensin mRNA accumulation in tobacco leaves and suggest that its accumulation is an ethylene-mediated stress response to abnormal growth. Because FB7-3 mRNA was present in kinetin cultures of both inflorescence explant tissue, which develops florally, and basal explant tissue, which develops vegetatively, the expression of this particular FB7 gene could not be correlated specifically with floral morphogenesis of explant tissue, and the analysis was not pursued further.

β-1,3-Glucanase Is also Expressed in Explants during de Novo Flower Formation

Both FB7-5 cDNA clones isolated from explants undergoing floral development show extensive homology to a basic β-1,3-glucanase isolated from *N. tabacum* cv Havana 425 (Shinshi et al., 1988). Shinshi et al. (1988) reported that this β-1,3-glucanase undergoes both N-terminal processing as well as C-terminal processing in which a glycosylated 22-amino acid C-terminal peptide is removed. Figure 4 shows that the longer of the two clones has complete homology with the Havana 425 sequence in the coding region and includes the COOH extension. However, the clone extends further in the 3′-noncoding region at nucleotide position 398 (1310 of the Havana sequence). The start of the extension is marked by a run of 22 adenine residues and continues for a further 172 nucleotides before terminating in what appears to be a poly(A) tail.

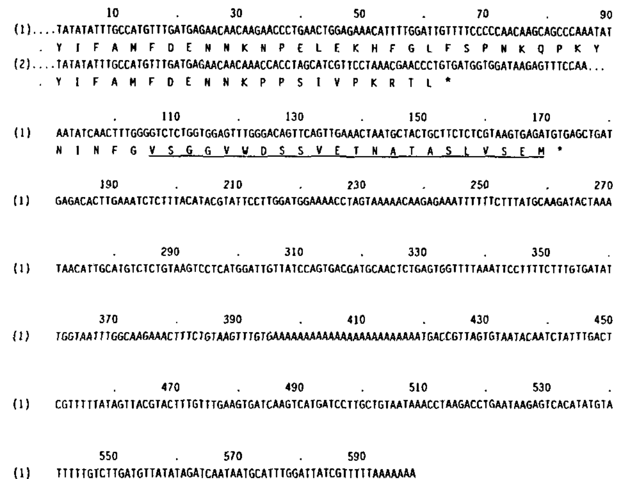

Figure 4. Divergent COOH Termini of Two Basic β-1,3-Glucanases from *N. tabacum* cv Samsun *nn*.

The 3′ ends of the two nucleotide sequences from the FB7-5 cDNA gene family are shown. The reading frame corresponding to each of the amino acid sequences was determined by the homology with the β-1,3-glucanase clones isolated by Shinshi et al. (1988). The COOH-terminal extension that is cleaved from the mature protein (Shinshi et al., 1988) is underlined.

The shorter clone, which also exhibits homology with the clones isolated by Shinshi et al. (1988), has a translation termination codon upstream of the region encoding the 22-amino acid COOH-terminal peptide present in these other sequences. Thus, this shorter clone lacks the COOH-terminal peptide that has been suggested to have a role in targeting the intracellular location of this protein (Van den Bulcke et al., 1989). The absence of this COOH-terminal peptide in one of the cDNAs isolated in this study suggests that the products of the two β-1,3-glucanase genes isolated here may be transported to different cellular locations.

The Function of FB7-4 Is Unknown

A database search with both the nucleotide sequence and the predicted amino acid sequence of FB7-4 revealed no significant similarity to published sequences. Figure 5 presents the FB7-4 nucleotide sequence of 691 bases. RNA gel blot analysis detected a message of 700 to 800 nucleotides. An open reading frame extends 438 nucleotides from position 23 at the 5′ end of the cDNA sequence. This open reading frame encodes a putative protein of 146 amino acid residues (see Figure 5) with a predicted molecular mass of 16.5 kD and a pI of 6.7. A possible glycosylation site occurs at position 41. A region characteristic of

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        10    .    30    .    50    .    70    .    90
GAAAATCCCAGACTGCTAACAAATGGGTCTCAAAGGCAAATTAATCGCTCAAATAGAGATGAAGTGTGCTGGAGATTTGCTTCATGAACA
                        M  G  L  K  G  K  L  I  A  Q  I  E  M  K  C  A  G  D  L  L  H  E  H
                                                                                         23

     .   110    .   130    .   150    .   170    .
CTTCAAATCAAATCCACACCAAACCTCCACCATGTCTCCTAATAAGATAACAAATTTCACGTTACATGAGGGTCAGTTGGGCAGTACTGG
 F  K  S  N  P  H  Q  T  S  T  M  S  P  N  K  I  T  N  F  T  L  H  E  G  Q  L  G  S  T  G
                                                                                         53

       190    .   210    .   230    .   250    .   270
TTCTGTTGTTAGCTGGAAGTTTGTTCTCGGAGGAAAAGAGAGGCATGCGAAGCAGGTCCTGCACATAGATGATGCAAAAAAATCAATCAC
 S  V  V  S  W  K  F  V  L  G  G  K  E  R  H  A  K  Q  V  L  H  I  D  D  A  K  K  S  I  T
                                                                                         83

     .   290    .   310    .   330    .   350    .
CTTCAATTTTGTTGAAGGTGATATGAATGAATTATACAAGTCCATGACAGCTACTTTGACTGCAGAGGGAAACTGGATGACTTGGACCTT
 F  N  F  V  E  G  D  M  N  E  L  Y  K  S  M  T  A  T  L  T  A  E  G  N  W  M  T  W  T  F
                                                                                        113

       370    .   390    .   410    .   430    .   450
TGTGTATGAGAAGTTGAATGAAAACATACCAGAGCCCCTTGATATTTTTGAATTAGCTATTTGCCTCCTCAAAGACCTTGAGCCTCACCA
 V  Y  E  K  L  N  E  N  I  P  E  P  L  D  I  F  E  L  A  I  C  L  L  K  D  L  E  P  H  H
                                                                                        143

     .   470    .   490    .   510    .   530    .
TGTTGGGAAATAGATATTTCTTCCCCCTCCCATCAGTATGATGAAGTATTATGGCCATCCTAAATCCTTATGAATAAATAAACTTATGGA
 V  G  K  *

       550    .   570    .   590    .   610    .   630
TAAATAAAACTATTTGGTGTGATATATATTCCCTATGTGTTCTATGTTGCGTTTCCAATGGTAATAATATTATATTAATGAATATGATGT

     .   650    .   670    .   690
ACTTTTGGATCGACTCTATGTAATAATCTAACCTCTCTTTTGGTAAAAAAAAAAAAAAAA
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Figure 5. The Nucleotide Sequence of the FB7-4 cDNA Clone.

The amino acid sequence corresponding to the open reading frame extending from position 23 to 461 is shown beneath the nucleotide sequence.

a signal or transit sequence (Verner and Schatz, 1988) is not present in the deduced FB7-4 amino acid sequence.

The Highest Level of Developmental Expression of Basic Chitinases, Osmotin, and Basic β-1,3-Glucanases in Samsun Is Found in Roots

Few studies have been conducted into the spatial expression patterns of pathogen-induced genes in uninfected plants. To help define the biological role of these genes in normal plant development, chitinase, osmotin, FB7-4, and β-1,3-glucanase probes were hybridized to RNA from various tissues of the day-neutral (DN) tobacco samsun *nn*. The hybridization patterns to mRNA for chitinase (as shown in Figure 6), osmotin, and β-1,3-glucanase were essentially identical. Consistent with our previous results (Meeks-Wagner et al., 1989) and those of Felix and Meins (1986) and Shinshi et al. (1987), all three genes displayed the strongest hybridization signal with total RNA from roots, from 2-week-old seedlings, and from the basal leaves of those plants with floral buds. The mRNA levels for these genes, in both basal leaves (Figure 6, data shown for chitinase mRNA only) and roots (Figure 7, lanes a, b, c), tended to increase marginally in intensity with the age of the plant. The chitinase mRNA levels also tended to increase in most floral parts as the flower matured. At the early bud stage (Figure 6), the level of expression in the bud as a whole was not sufficient to be detected above the nonspecific background binding that is typically obtained with dot-blot analysis using total RNA.

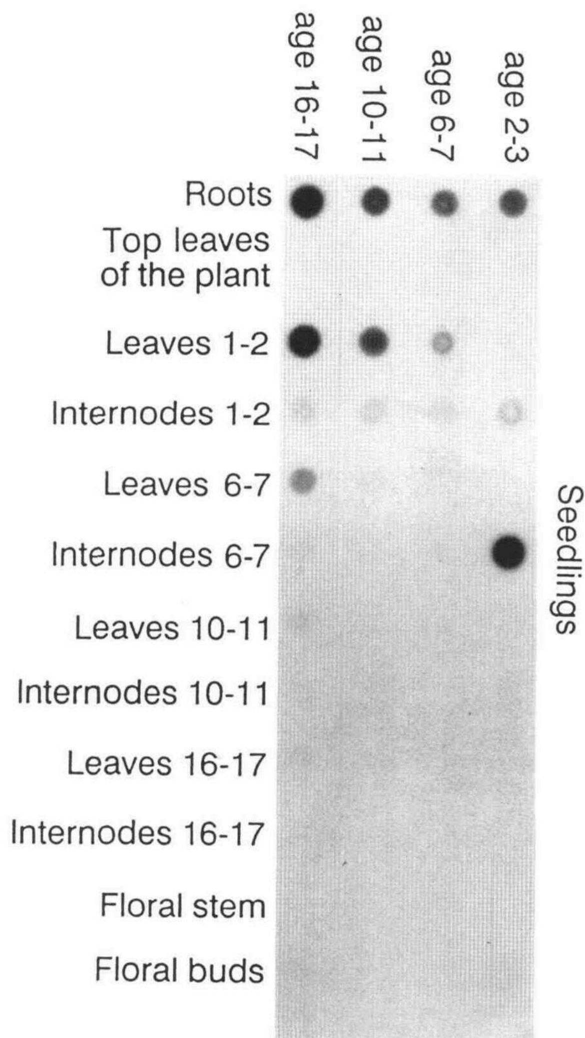

Figure 6. Developmental Pattern of FB7-1 (Chitinase) mRNA Levels in *N. tabacum* cv Samsun *nn*.

Autoradiograph of hybridization of FB7-1 to a dot blot with 3 μg of total RNA isolated from various organs of samsun *nn* plants at four different developmental ages. The numbering of leaves (and internodes) begins with the basal-most leaf (or internode) as number 1 and continues to the apex. Each column consists of RNA samples harvested from a set of similarly aged plants, as labeled. Homologous organs are aligned by rows, as labeled. The last dot in the righthand column contains total RNA from 2-week-old seedlings.

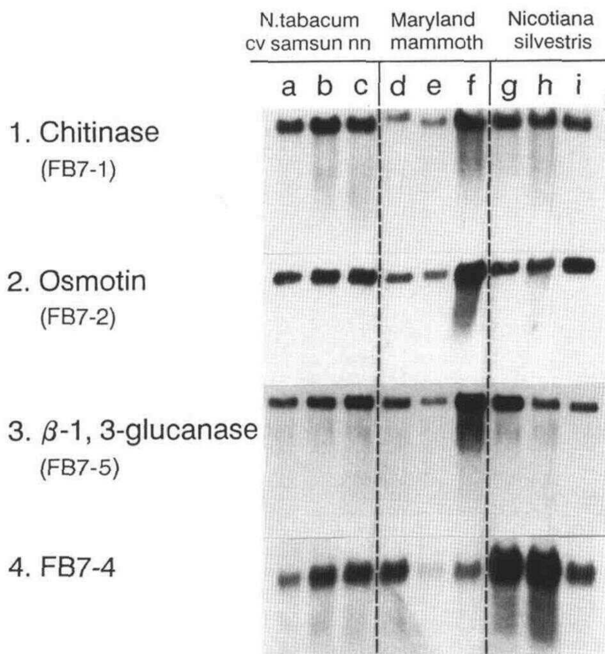

Figure 7. Hybridization of FB7-1, FB7-2, FB7-4, and FB7-5 to Samples of Total RNA from Root Tissue of the Day-Neutral Tobacco *N. tabacum* cv Samsun, and the Two Photoperiodic Tobaccos Maryland Mammoth and *N. silvestris*.

Five micrograms of total RNA from root tissues was electrophoresed through a formaldehyde-1.5% agarose gel, transferred to a nylon membrane, and hybridized with the FB7 probes as indicated in the left column. The FB7-4 gel blot contained 30 μg of RNA/lane. Total RNA was harvested from the following root tissues. a, juvenile *N. tabacum* cv samsun; b, *N. tabacum* cv samsun with small, unopened floral buds on the primary shoot apex; c, *N. tabacum* cv samsun with opened flowers on primary shoot apex; d, juvenile Maryland Mammoth; e, Maryland Mammoth induced for 4 weeks; f, uninduced Maryland Mammoth control; g, juvenile *N. sylvestris*; h, *N. sylvestris* induced for 4 weeks; i, uninduced *N. sylvestris* control.

RNA gel blot analyses (data not shown) indicated that a relatively low level of expression of chitinase, β-1,3-glucanase, and osmotin can be found in all other plant tissues. In addition, a moderate to high level of expression of osmotin occurred in floral organs at various stages during their development. Osmotin was also found to be expressed moderately in basal internodes. RNA gel blot hybridization analysis showed that FB7-4 expression was regulated differently to the other FB7 genes within whole samsun *nn* tobacco plants and that its mRNA was less abundant than that of the other genes. The strongest hybridization signal was seen in those gel lanes containing tissue from basal internodes (data not shown). A lower level of expression for FB7-4 was also detected in root tissue, and no expression was found in leaves or in seedlings. The spatial distribution of four of the five FB7 gene transcripts in flowering samsun tobacco plants is summarized in Figure 8A.

FB7 Gene Expression Levels in the Roots, but not in the Leaves, of the Photoperiodic Tobacco Maryland Mammoth Decrease after Photoperiodic Induction

To determine whether the regulation of expression of the FB7 genes was correlated to the photoperiodic control of floral induction, the pattern of expression of these genes in the short day-induced tobacco Maryland Mammoth and the long day-induced *N. sylvestris* was examined before and after photoperiodic induction (see Methods). Preliminary hybridization analysis using RNA from leaves did not

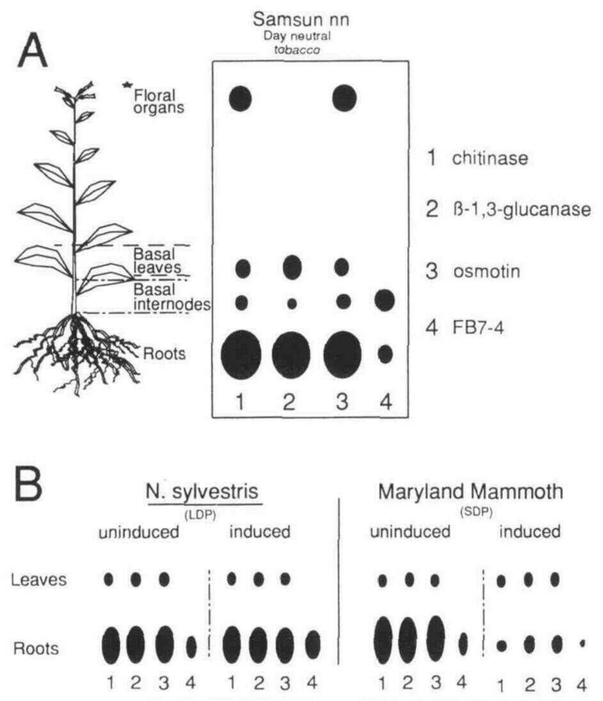

Figure 8. Schematic Diagram Depicting the Distribution of mRNAs for the FB7 Genes Chitinase, β-1,3-Glucanase, Osmotin, and FB7-4 in Healthy Tobacco Plants.

(A) Comparison of the levels of each of four FB7 mRNA species in various tissues of flowering samsun tobacco plants.

(B) Comparison of the levels of each of four FB7 mRNA species in the leaves and roots of photoperiodically induced and uninduced Maryland Mammoth tobacco and *N. sylvestris*.

The symbols used in **(A)** and **(B)** are defined in the righthand column of **(A)**. The absence of a symbol within a row indicates low-level expression. The figure is not drawn to scale and symbol height is an approximation of the relative abundance within and between particular mRNA species. An asterisk (*) indicates that expression occurs at some developmental stages.

reveal any significant differences in FB7-1, FB7-2, FB7-4, and FB7-5 transcript levels between induced and uninduced plants for both species of photoperiodic plants (data not shown). However, as seen in the DN samsun plants, developmental expression of the chitinase, β-1,3-glucanase, and osmotin genes appeared to be highest in root tissue in both Maryland Mammoth and *N. sylvestris*.

RNA gel blot hybridization analysis was performed to further quantify FB7 gene expression levels in roots of 4-week induced and uninduced photoperiodic plants (Figure 7). In the roots of Maryland Mammoth plants, chitinase, osmotin, and β-1,3-glucanase mRNAs showed similar patterns of regulation; expression was highest in the roots of uninduced plants and was significantly reduced after photoperiodic induction (Figure 7, FB7-1, FB7-2, and FB7-5, lanes e and f). The FB7-4 message was most prevalent in the roots of juvenile Maryland Mammoth plants, but, as seen with the other three genes, there was a decrease in mRNA levels concurrent with induction (Figure 7, FB7-4, lanes d, e, and f).

The expression of the chitinase, osmotin, and β-1,3-glucanase genes in root tissue of *N. sylvestris* plants was not significantly altered during photoperiodic induction. Nor did the abundance of these gene transcripts vary greatly between roots at juvenile and adult (induced and uninduced) stages (Figure 7, FB7-1, FB7-2, and FB7-5, lanes g, h, and i). The highest level of expression of FB7-4 for any of the root tissues examined was found in *N. sylvestris* plants (Figure 7, FB7-4, lanes g and h). This high level of expression was seen in all other *N. sylvestris* root samples except in those of the uninduced adult plants used as controls for the 4-week induction treatment. These plants displayed a reduced level of expression. The levels of FB7 gene transcripts in leaf and root tissue of uninduced and induced photoperiodic plants are summarized in Figure 8B.

The effect of photoperiod induction on the FB7-4 transcript levels in the basal internode tissue of Maryland Mammoth plants was investigated by RNA gel blot analysis using 20 μg of total RNA from the basal internodes of plants grown under inductive conditions for 1 week, 2 weeks, or 4 weeks. Hybridization analysis revealed no significant changes in levels of basal internode expression between the induced plants and their controls (data not shown).

Osmotin, but not FB7-4, Is Induced during TMV Infection

The expression of pathogenesis-related (PR) proteins can be induced by the infection of *N. tabacum* cv samsun *NN* with tobacco mosaic virus (Van Loon, 1983). Because both basic and acidic β-1,3-glucanases and chitinases as well as extensin are induced by TMV infection, the possibility that the levels of osmotin and FB7-4 transcripts are also influenced by viral infection was tested.

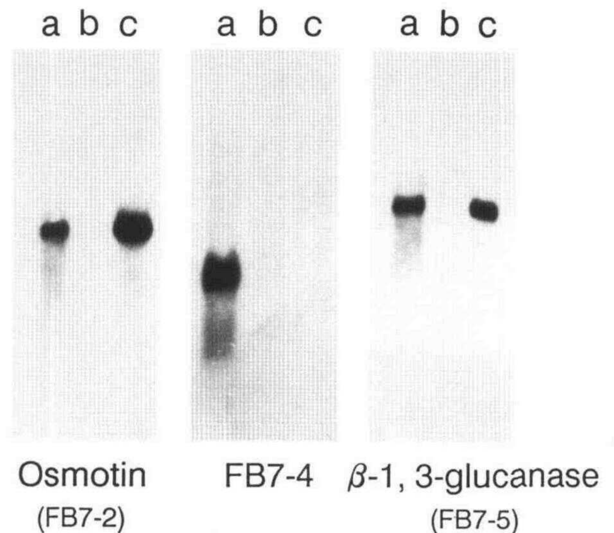

Figure 9. RNA Gel Blot Analysis To Determine Whether Expression of either Osmotin or FB7-4 Is Induced by Tobacco Mosaic Virus Infection.

Lanes (a) contain 10 μg (blot 1 and 3) or 50 μg (blot 2) of total RNA from the roots of a flowering samsun *nn* plant; lanes (b) contain 10 μg (blots 1 and 3) or 50 μg (blot 2) of total leaf RNA from healthy leaves of a control samsun *NN* plant; lanes (c) 10 μg (blot 1 and 3) or 50 μg (blot 2) of total leaf RNA from lesion-covered leaves of a samsun *NN* plant 4 to 5 days after TMV infection. The blots were hybridized with single-stranded ^{32}P-labeled RNA transcribed from FB7-2, FB7-4, and FB7-5.

As expected, hybridization analysis with chitinase (data not shown) and β-1,3-glucanase probes demonstrated that TMV-infection induced transcription of these genes. β-1,3-Glucanase transcript levels found in infected leaves were equal to or greater than the levels occurring in root tissues of uninfected plants, as shown in Figure 9, blot 3.

Figure 9 (blot 1) shows that osmotin is transcriptionally induced in TMV-infected leaves to levels equivalent to the high level found in healthy roots. Hybridization with FB7-4 probe shows a strong signal in the root lane, but hybridization could not be detected in healthy or TMV-infected leaves (Figure 9, blot 2), indicating that FB7-4 is not induced by TMV.

Wounding also Induces Osmotin but not FB7-4

Given the similarities in DNA sequence between most of the FB7 clones and genes known to be stress responsive, the induction of FB7 gene transcripts by wounding was examined. Figure 10 displays slot blots of RNA from wounded leaf samples hybridized separately with chitinase, osmotin, FB7-4, and β-1,3-glucanase DNA probes. The patterns of transcript accumulation for chitinase, os-

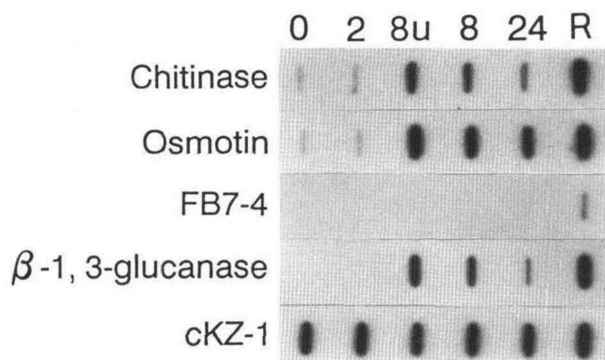

Figure 10. Slot Blots of RNA from Wounded Leaves.

Leaves were treated as described in Methods. Wounded leaves were removed after 2 hr, 8 hr, and 24 hr (columns 2, 8, 24) after the initial damage. In addition an unwounded leaf was removed from the wounded plant at 8 hr (column 8u). Three micrograms of total RNA from each sample was blotted through a slotted manifold onto nitrocellulose. For comparison an identical amount of total RNA from the roots of healthy plants (age 9 to 11) was included on the blots (column R). Filters were hybridized to random-primed DNA probes of FB7-1, FB7-2, FB7-4, FB7-5, and cKZ-1 (a ribosomal RNA) as indicated. The FB7-4 filter was overexposed to obtain a signal of similar intensity in the root sample to that obtained with the other filters.

motin, and β-1,3-glucanase are very similar. As demonstrated previously (Meeks-Wagner et al., 1989), the levels of these transcripts in leaves (Figure 10, column 0) are very low in comparison with the levels found in roots (column R). No increase in abundance of the specific mRNA is seen 2 hr after wounding, but the levels increase dramatically (14-fold for the osmotin transcript, as determined by scanning densitometry) 6 hr later (column 8). The pattern of transcript accumulation was confirmed for osmotin by RNA gel blot analysis, which showed that a single species was detected by the probe.

There appears to be a systemic response in the shoot because the unwounded leaf samples at this time point (column 8u) show the same level of transcript accumulation. By 24 hr after wounding, the signals decrease slightly. In contrast, the level of FB7-4 transcripts is not enhanced by wounding.

DISCUSSION

We have demonstrated that the expression of chitinase, osmotin, and β-1,3-glucanase is associated with the flowering process both during de novo flower formation of cultured tissue explants and, in various parts of the intact plant, during and after the transition to flowering. Another of the gene families isolated from TCL explants initiating floral development has been identified as extensin. Although significantly higher levels of extensin mRNA levels are found in tobacco flowers than in leaves (Memelink, 1988), the induction of this gene, unlike those previously mentioned, was not confined to explant tissue undergoing floral development (see FB7-3 of Meeks-Wagner et al., 1989).

These same genes, or related members of these gene families, which have been reported to be induced by stress conditions (see Moore and Stone, 1972; Sachs and Ho, 1986; Vogeli-Lange et al., 1988; Lamb et al., 1989; Varner and Lin, 1989) including pathogen infections, were observed also to be induced by wounding.

Both basic and acidic chitinases and glucanases are induced in the pathogen response (Legrand et al., 1987; Bol, 1988; Shinshi et al., 1988). Osmotin, which we show to be both TMV induced and wound induced, is likely to be the basic isoform of the acidic TMV-induced protein PR-S (Cornelissen et al., 1986). Whereas Lotan et al. (1989) have demonstrated that the acidic isozymes of glucanase and chitinase are expressed during growth and maturation of various flower parts, the chitinase and β-1,3-glucanase genes detected during in vitro flower formation encode only the basic isoforms. No acidic isoforms were detected among more than 50 cDNA clones isolated independently from the floral induction system (Meeks-Wagner et al., 1989). It is not known whether this is indicative of differential expression of the acidic *vs.* basic isoforms during the early stages of in vitro flower formation, or merely reflects the relative levels of expression of the different isoforms. The physiological significance of the association between the induction of these basic proteins and flower production is unclear. In the pathogen response the acidic isoforms of these proteins are secreted into the extracellular space, whereas the basic isoforms may be confined intracellularly (Bol and Van Kan, 1988). Each of the gene sequences we identified encodes a hydrophobic signal peptide at the N terminus, and previous reports (Boller and Vogeli, 1984; Singh et al., 1987a; Van den Bulcke et al., 1989; Varner and Lin, 1989) indicated that these gene products are targeted either to the vacuole or to the cell wall. The two β-1,3-glucanase cDNA clones that we studied may actually encode products that are delivered into two different intracellular locations. Further analysis with the use of genomic clones will be needed to verify that this is the case.

Is Expression of the FB7 Genes Stress Related?

TCL explant culture requires that the explant tissue adapt not only to allow uptake of water and nutrients but also to the radically altered availability of endogenous and exogenous hormones. It could be argued that the expression of the FB7 genes during in vitro flower formation is a response to one or more stress conditions that are im-

posed on explant tissue during culture. Even though these genes are only expressed in the one tissue culture system that leads to flower development, and not in the similar regime which leads to vegetative bud development, the presence of kinetin or absence of zeatin in the floral-specific medium could be the trigger for gene induction. Meeks-Wagner et al. (1989) argued against this possibility on the basis that the kinetin-containing medium did not induce these genes in explants taken from the lower stem. The extensin gene was the one exception to this and may be kinetin induced.

Alterations, resulting from in vitro culture, to the preexisting endogenous levels of other hormones in the explant tissue should also be considered as several hormones have been implicated in the regulation of these genes. Ethylene appears to be a common inducer of the acidic PR proteins (Van Loon, 1983) and of a number of the basic isoforms, as well as tobacco extensin (Memelink et al., 1988). Both chitinase and β-1,3-glucanase, including the basic isoforms, have been reported to be influenced by auxin and kinetin (Felix and Meins, 1987), gibberellic acid, and abscisic acid (see Mohnen et al., 1985). Abscisic acid also stimulates synthesis of osmotin in cultured tobacco cells (Singh et al., 1987b).

The strongest argument against the expression of FB7 genes in explant tissue undergoing floral development being solely related to stress induction is that the pattern of expression in the tissue culture system, as outlined below, is paralleled by the patterns observed for some of these genes during flower formation in the intact plant.

Expression of the FB7 Genes Correlates with Morphogenetic Changes in Plant Development

Meeks-Wagner et al. (1989) noted that the expression of FB7 genes was directly correlated with the timing of the floral meristem development. The temporal correlation was also observed when the adult vegetative meristems on the small plantlets, which had grown vegetatively under the zeatin regime, underwent the transition to floral meristems. In intact plants both chitinase and osmotin were shown to be highly expressed in prefloral and floral apices but not in vegetative apices (Meeks-Wagner et al., 1989). During in vitro flower development under the kinetin regime, Meeks-Wagner et al. (1989) observed that there was another period of increased induction of transcription of the genes corresponding to the period of floral organ development and bud growth. In intact plants, expression of both chitinase and osmotin occurred in floral organs at various stages during their development (data not shown). These observations suggest that elevated expression of these genes is correlated with periods of morphogenetic change. However, the relationship between FB7 gene induction and these morphogenetic changes may be indirect because expression is not confined spatially to the developing meristem.

Our experiments with different *Nicotianas*, those with either short-day or long-day photoperiod control of flowering induction, as well as the day-neutral cultivar we used for most of the analyses, showed that whatever controls the floral development-associated induction of the genes is not related to the photoperiod controls. However, there are other specific periods and places of elevated gene expression during vegetative development of the plant; these patterns are gene specific.

A puzzling observation was that the highest expression of all the genes, with the exception of FB7-4, was found at specific periods in the root system. The photoperiod *Nicotianas* exhibited the same complex developmental pattern of expression of the genes with major expression in the roots. In addition, the short-day Maryland Mammoth cultivar had a dramatic decrease in the root expression of β-1,3-glucanase, osmotin, and chitinase in the period immediately after photoperiod induction treatment. This difference was even more pronounced with the other gene FB7-4, for which we have not identified a product.

The photoperiod induction of flowering is postulated to be associated with an inducer transmitted from the leaf to the meristem (Knott, 1934). In the short-day Maryland Mammoth cultivar there may also be a signal after photoperiod induction that is translocated to the roots and downmodulates the expression of the FB7 genes. On the other hand, the level of root expression could be indirectly related to floral induction and may, for example, be mediated by a reduced level of ethylene in the roots. In this context it is interesting to note that Burley 21 tobacco, which exhibits a similar response to Maryland Mammoth (Kasperbauer, 1973), can be rendered unresponsive to flower-inducing conditions by treatment with exogenous ethylene (Kasperbauer and Hamilton, 1978).

Among the array of stress-related genes there are subsets that are differentially expressed in normal development. The basic isoforms of chitinase, β-1,3-glucanase, along with osmotin (Felix and Meins, 1986; Shinshi et al., 1987; this paper) and a basic form of the PR-1 protein (Memelink, 1988), are expressed at high levels in the roots of healthy tobacco plants, whereas the acidic chitinase, PR-1b, and PR-S genes are not (Memelink, 1988). Besides this report, which shows that the basic proteins are highly expressed in flower formation, there have been a number of reports of developmental regulation of stress-related genes (Felix and Meins, 1986; Hooft van Huijsduijnen et al., 1986; Shinshi et al., 1987; Memelink, 1988). Fraser (1981) and Lotan et al. (1989) reported PR protein accumulation during flower development and senescence. β-1,3-Glucanases have been found to be operative in seed germination (see Fincher, 1989).

Some morphogenetic changes occurring during plant development necessitate disruption to existing plant tissues. Such situations may occur during pollen tube

growth, the formation of various organs such as lateral or adventitious roots (Varner and Lin, 1989), seed germination, and the vegetative to floral meristem transition. It is possible that in these developmental changes in patterns of cell division, which disrupt existing tissue to give rise to new organs, there could be an increased susceptibility to foreign organisms. The PR genes may then perform an ancillary function as part of a general defense mechanism during these events in normal plant development.

Alternatively, it is possible that a number of hydrolytic enzymes, including those that are highly induced during pathogen attack, are directly involved in the vegetative to floral transition and the subsequent sequential development of floral organs, inasmuch as both of these processes involve novel cell divisions and growth patterns. Albersheim and Darvill (1985) suggested that the actions of phytohormones may be mediated by oligosaccharins released from plant cell walls by hydrolytic enzymes. It is conceivable that as a result of the hydrolytic action of some of the FB7 gene products, other genes whose products are needed in the cell divisions and differential cell growths may be induced.

Because we have not been able to identify the FB7-4 gene product, it is not possible to say whether it would fall into the hydrolytic class or cell division and expansion class; however, FB7-4 does have a unique expression pattern. Furthermore, the FB7-4 gene product is not induced by viral infection or wounding and has no signal sequence for transport across membranes.

We plan to experiment with both this gene and the other identified genes and use reduction of expression by antisense or ribozyme activity or use overexpression to help clarify the roles of these genes in flower induction and other critical plant developmental stages.

METHODS

The growth conditions of *Nicotiana tabacum* cv samsun plants, TCL culture, and isolation of the FB7 cDNA clones have been described earlier (Meeks-Wagner et al., 1989).

Plant Material and RNA Extraction from DN Tobacco

RNA was extracted from different tissues of healthy *N. tabacum* cv samsun plants representing several developmental ages. Three greenhouse-grown plants were harvested at each of the following ages (age defined as the total number of leaves $>$3 cm in width, counting from the base of the plant): age 2 to 4, age 5 to 7, age 10 to 12, and age 16 to 17 (small floral buds). For each plant, tissue samples were separately harvested from roots, consecutively paired leaves (i.e., leaves 1 to 2 and leaves 3 to 4), consecutively paired internodes, apical leaves less than 3 cm in length, as well as flower buds, if present. Plant tissue was frozen in liquid nitrogen immediately after harvesting and stored at −80°C. Samples were ground in a mortar and pestle and then covered with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and NTES (0.1 M NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.1% SDS). The supernatant from the phenol extraction was precipitated twice with 2.5 volumes of ethanol and 0.3 M sodium acetate. RNA concentrations were determined by OD measurement at 260 nm and gel electrophoresis comparisons. Total RNA prepared from each sample was dotted onto each of four nylon membranes.

Plant Material and RNA Extraction from Photoperiodic Plants

Twenty-four *N. sylvestris* and 24 Maryland Mammoth plants were initially grown under noninductive conditions. In these plants floral initiation is controlled by their requirement for a specific period of darkness within a 24-hr period as the signal to trigger flowering. Maryland Mammoth is a short-day plant that requires 10 to 14 days of $>$16 hr continuous dark before flowering. *N. sylvestris*, a long-day plant, needs 10 to 14 days of $<$8 hr dark for floral induction. These plants do not respond to photoperiodic induction until they have passed the juvenile phase and have 15 leaves $>$10 cm in length (after 3 to 4 months of growth). Twelve adult plants of each species were then transferred to inductive conditions. At intervals of 1 week, 2 weeks, 3 weeks, and 4 weeks of further growth, three induced and three noninduced plants were harvested. Flower buds had begun to form on those plants subjected to inductive conditions for 4 weeks. For each set of growth conditions, RNA from roots, one set of basal leaves, a set of middle leaves, the top pair of leaves, the top of the plant (leaves $<$3 cm), and floral buds, if present, was separately spotted onto a nylon membrane. Tissue samples were harvested in the same pattern as was used for the samsun plants. Tissue samples were also harvested from juvenile *N. sylvestris* and Maryland Mammoth plants. RNA was extracted from these tissues as described for DN plants.

Recombinant DNA Techniques

Subcloning and sequencing of the FB7 cDNA gene families were performed in the bacterial plasmids pUC118, pUC119, and pGEM3Z$^+$ (Promega Biotec). DNA sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977). The nucleotide sequences and deduced amino acid sequences were compared with sequences in the Genbank Genetic Sequence Data Bank, in the EMBL Data Library, and the NBRF protein identification resource.

DNA Gel Blot Hybridizations

DNA was isolated from tobacco plants according to the procedure described by Taylor and Powell (1982). This DNA was digested with the appropriate restriction enzymes, and DNA gel blot transfers (Southern, 1975) were performed after electrophoresis through 0.75% TAE-agarose gels (Maniatis et al., 1982). Probes were prepared by random-primed labeling (Bresatec Ltd.), and hybridizations were carried out according to Davis et al. (1986). For lower stringency the filters were hybridized and washed at 42°C. The final wash buffer was 0.1 × SSC and 0.1% SDS. At higher stringency the same procedures were carried out at 48°C.

RNA Gel Blot Hybridizations

RNA was electrophoresed in formaldehyde/formamide 1.5% agarose gels as described in Maniatis et al. (1982). Transfer to nylon membranes (GeneScreen, Du Pont) and hybridizations were performed according to the suppliers' instructions. The blots were hybridized with single-stranded ^{32}P-labeled RNA transcribed in the plasmid pGEM3Z^{+} (Promega Biotec).

TMV Infection and Wounding

Three fully expanded leaves at the top of healthy mature vegetative samsun *NN* plants were inoculated with TMV by rubbing the upper surface with an abrasive pad soaked in virus suspension diluted to produce 100 to 200 local lesions per leaf. Control plants were similarly treated with dilution buffer lacking virus. Five days after inoculation, total RNA was harvested separately from lesion-covered leaves of infected plants and undamaged leaves from the uninfected plants and was electrophoresed, blotted, and probed as described for RNA gel blot analysis.

The induction of FB7 genes in response to wounding was analyzed with mature vegetative *N. tabacum* cv samsun *nn* plants (six plants, each with 12 leaves greater than 3 cm in length). Leaves 4, 6, and 8 of each plant were punctured approximately 100 times with a dissecting needle; before wounding, leaf 7 was removed as a "time 0" control. Wounded leaves were removed at 2 hr, 8 hr, and 24 hr after wounding, and unwounded leaves from the same plant were removed at 8 hr. Once excised from the plant, all leaves were immediately frozen in liquid nitrogen and then stored at −80°C until processed for extraction of total RNA. During the experiment the plants were kept in ambient laboratory light. Samples 0 and 8 hr were from plants exposed to continuous light; the plants received one night period before collection of the 24-hr sample. Slot blots of total RNA from these leaf samples were hybridized separately with FB7 cDNA probes.

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