Rapid and Systemic Accumulation of Chloroplast mRNA-Binding Protein Transcripts after Flame Stimulus in Tomato[1]

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It has been shown that tomato (*Lycopersicon esculentum*) plants respond to flame wounding and electrical stimulation by a rapid (15 min) and systemic up-regulation of proteinase inhibitor (*pin*) genes. To find other genes having a similar expression pattern, we used subtractive cDNA screening between flamed and control plants to select clones up-regulated by flame wounding. We report the characterization of one of them, a chloroplast mRNA-binding protein encoded by a single gene and expressed preferentially in the leaves. Systemic gene expression in response to flaming in the youngest terminal leaf exhibited three distinct phases: a rapid and transient increase (5–15 min) in transcript accumulation, a decline to basal levels (15–45 min), and then a second, more prolonged increase (60–90 min). In contrast, after a mechanical wound the rapid, transient increase (5 min) was followed by a rapid decline to basal levels but no later, prolonged accumulation. In the petiole, the initial flame-wound-evoked transient increase (15 min) was followed by a continuous decline for 3 h. The nature of the wound signal(s) causing such rapid changes in transcript abundance is discussed in relation to electrical signaling, which has recently been implicated in plant responses to wounding.

Plants respond to local, damaging stimuli by changes in gene expression (Braam and Davis, 1990; Christensen et al., 1992; Henry-Vian et al., 1995; Schaller and Ryan, 1996). These changes are observed not only at the site of the stimulus, but also in distant, intact organs (Peña-Cortés et al., 1995; Bergey et al., 1996; Pastuglia et al., 1997; Vian et al., 1997; Bergey et al., 1999). In tomato (*Lycopersicon esculentum*), such stimuli have included insect chewing or mechanical wounding (Graham et al., 1986) and flaming or electrical stimulation (Stankovic and Davies, 1996), all of which evoke the expression of the proteinase inhibitor (*pin*) genes *pin-1* and *pin-2*. These *pin* genes have been used as very convenient molecular markers of stress perception to study the transmission of wound signals throughout the plant. Major candidates for the wound signal(s) include various chemicals (hormones) that can evoke *pin* gene expression (Pearce et al., 1991; Peña-Cortés et al., 1991; Ryan

and Farmer, 1991) and may be transmitted through the xylem (Malone et al., 1994; Malone, 1996). However, since *pin* mRNA accumulation can be detected in a distant leaf within 15 min after stimulation (Stankovic and Davies, 1996), it seems unlikely that these compounds produced at or near the wounded region could be the systemic signal evoking such rapid changes in gene expression. This premise was our starting point to explore alternative signaling pathways such as electrical signals as potential candidates.

Wildon et al. (1992) used a flame stimulus to provide the first evidence suggesting a link between *pin* expression and electrical signals. However, more convincing data unequivocally linking *pin* gene expression and electrical signals were provided by Stankovic and Davies (1996). These authors considered the two major kinds of electrical events in plants to be the variation potential (VP) and the action potential (AP). The genesis and general characteristics of these electrical waves are different: an injurious stimulus such as flaming evokes a VP, which results from a transmitted hydraulic signal evoking local membrane depolarization (Malone and Stankovic, 1991), while a noninjurious stimulus (DC pulse) triggers an AP, which is a genuine (i.e. self-propagating) electrical signal (Stankovic and Davies, 1996). Using stimuli selectively evoking an AP (electrical stimulus) or a VP (flame), they observed systemic *pin* transcript accumulation only when the recipient tissue exhibited a membrane depolarization exceeding 40 mV (Stankovic and Davies, 1996, 1997). To date, fully convincing links between gene expression and electrical events have been described only for *pin* genes, although a strong correlation was found for calmodulin (Vian et al., 1996, 1997).

The main goal of this research was to isolate, identify, and characterize additional genes whose expression is rapidly up-regulated after a distant flame-wound stimulus, thus implying the transmission of a wound signal. We used subtractive screening between intact (control) and flame-stimulated plants to isolate clones in distant tissue accumulating shortly after treatment to furnish potential candidates for electric signal-responsive genes. We used a flame wound, since we showed previously that it evokes hydraulic signals and their accompanying variation potentials in a highly reproducible manner (Stankovic and Davies, 1996). Others have also used flaming as the wound treatment of choice (Wildon et al., 1992). Here we report the cloning of a cDNA corresponding to a systemically activated, rapidly up-regulated, tissue-specific gene for chloroplast mRNA-binding protein (CMBP), which should be added to the category of systemic wound response

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proteins (SWRPs) (Bergey et al., 1996, 1999). The array of SWRPs that we are identifying will prove invaluable for our long-term efforts to determine which local signals (e.g. ABA, systemin, proteinase inhibitor-inducing factor) evoke the accumulation of different transcripts.

MATERIALS AND METHODS

Plant Culture and Treatment

Tomato (*Lycopersicon esculentum* cv Heinz 1439 VF) seeds were obtained from Stokes Seeds (Buffalo, New York) and grown in a standard substrate composed of gravel and Peat-Lite (developed at Cornell University, Ithaca, NY) under controlled conditions (16 h/8 h light/dark; 26°C/ 21°C, and 300 μmol s^{-1} m^{-2} PPFD) in the phytotron of North Carolina State University. Three-week-old plants (about 12 cm in height with the fourth leaf not fully expanded) were flamed for 2 s about 1 cm from the third leaf using a butane lighter (the flame was set to about 1.5 cm in height). Various tissues, including the fourth leaf, the petiole of the third leaf, the mature second leaf, and the internode, were then harvested at different times after treatment and immediately frozen in liquid nitrogen.

Nucleic Acid Isolation

Total RNA was isolated using Trizol reagent (Gibco-BRL, Cleveland), separated on a formaldehyde denaturing gel, and transferred onto a nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). Poly(A$^+$) RNA was isolated from 3 mg of total RNA using the PolyA-Tract system (Promega, Madison, WI).

Subtractive Screening

The subtraction was performed using a cDNA subtraction kit (PCR-Select, CLONTECH Laboratories, Palo Alto, CA) as described by the manufacturer, with minor modifications. Two micrograms of poly(A$^+$) RNA was isolated from the fourth leaf of untreated control (driver) and 60 min-flamed (tester) plants and used to make double-stranded, blunt-ended cDNA, which was then separately digested (2.5 h at 37°C) with the 4-bp restriction enzyme *Rsa*I. One-half of the digested tester cDNA was ligated with adaptor 1 and the other half with adaptor 2, while driver cDNA was not subjected to adaptor ligation. Denatured adaptor 1- or 2-ligated tester cDNA was then hybridized separately with an excess of denatured driver cDNA (10 μL final volume) for 8 h at 68°C in a hybridization oven. Then, while maintaining the incubation temperature at 68°C, the two hybridization reactions were mixed together, along with freshly denatured driver cDNA, and incubated at 68°C for 24 h. During this incubation, the flame-specific sequences formed new hybrid molecules with different adapters (1 and 2) on each end, while common sequences formed hybrids having only adaptor 1, adaptor 2, or no adaptor at their ends.

The hybridization product was then diluted to 200 μL with dilution buffer (20 mM HEPES/HCl [pH 8.3], 50 mM NaCl, and 0.2 mM EDTA), and 1 μL was subjected to PCR amplification in a mini-cycler (model PTC-150, MJ Research, Waltham, MA) using a PCR mix (Advantage Klentaq, CLONTECH Laboratories). This method ensures an automatic hot-start PCR and a low error rate during amplification. Thus, only the flame-specific sequences were amplified exponentially, while others were subjected to linear or no amplification. The first PCR amplification (30 cycles) was performed using the P1 and P2 primers complementary to adapters A1 and A2, the PCR reaction was diluted 30-fold, and an aliquot (1 μL) of this reaction was subjected to another amplification using nested PCR primers. The PCR products were then purified on a PCR-cleaning column (Qiagen, Valencia, CA) and quantified. Twenty nanograms were ligated into the T/A vector pT-7 Blue (Novagen, Madison, WI) for 2 h at room temperature using T4 DNA ligase (Gibco-BRL). After enzyme denaturation (10 min at 65°C), 2 μL of the reaction was used to transform the competent bacteria ElectroMax DH10B using a cell porator (Gibco-BRL).

Northern and Southern Blots

Fifty nanograms of cDNA probe were labeled using a kit (Ready-to-Go DNA, Pharmacia Biotech, Piscataway, NJ) with 50 μCi of [α-^{32}P]dCTP (ICN, Costa Mesa, CA), purified on a mini column (Tip-5, Qiagen), and heat denatured. Hybridization was performed in a hybridization oven as described by Sambrook et al. (1989) at 42°C in 12 mL of hybridization buffer (6× SSPE, 50% [v/v] formamide, 5× Denhardt's solution, 0.5% [w/v] SDS, and 100 μg mL^{-1} denatured herring-sperm DNA). The washes (stringencies as indicated in the text) were performed with SSPE buffer containing 0.1% (w/v) SDS.

Sequencing and RACE-PCR Amplifications

The B1-20 cDNA clone was sequenced using the University of Nebraska sequencing facilities and homology searched in GenBank using BLASTX. The coding region (521 bp) was incomplete at both the 3′ and 5′ ends. The primers B1-20S (CGGTTGGCGTAATTGGGGCTGGTCG) and B1-20AS (CCTCCAGGAGGTTCTCCGACTACA) were designed for both 3′ and 5′ RACE-PCR, and the amplifications were performed with a cDNA amplification kit (Marathon, CLONTECH Laboratories). All of the procedures were carried out as described by the manufacturer, except the PCR programs, which were adapted to our PCR mini-cycler. The cDNA was made from 1 μg of poly(A$^+$) RNA isolated from plants flamed for 60 min. The amplified products were then cloned in pGEM-T and p-GEM-T Easy (Promega) and sequenced. Three sets of clones were separately made and sequenced to avoid possible sequence errors due to PCR amplifications.

RESULTS

Plant Model Description

Figure 1 shows the tomato plant model used for these experiments. The plants were stimulated on the third leaf
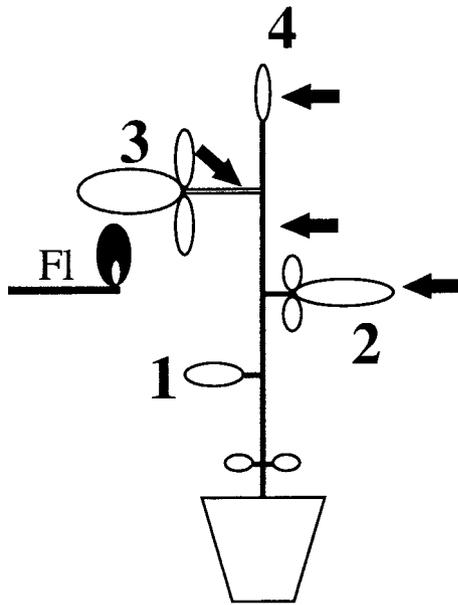
Figure 1. Schematic drawing showing the stimulation treatment. Three-week-old plants (12 cm in height) were used for all experiments. Stimulations were made on leaf 3 by flaming for 2 s with a gas lighter. The effects of this treatment were analyzed in intact, distant tissues (black arrows), including the petiole of the third leaf, the underlying internode, the terminal leaf (leaf 4), and the mature leaf (leaf 2).

(Fig. 1, leaf 3), and responses were studied in distant tissues (Fig. 1, black arrows). To determine the array of genes up-regulated by flame wounding, we harvested the fourth leaf (Fig. 1, leaf 4) from plants stimulated 60 min earlier on the third leaf to yield the tester cDNA, and this was used to generate a subtractive library against the cDNA (driver) from the fourth leaf of control (non-flamed) plants. We first evaluated the efficiency of the subtraction by monitoring its enrichment in calmodulin cDNA, which is rapidly induced to a high level by flame wound treatment in tomato (Stankovic and Davies, 1996). We found this cDNA to be much more abundant (about 6.5-fold) in the subtracted cDNA (data not shown). Assuming that calmodulin cDNA is representative, this suggests that the subtractive library is a useful source for up-regulated clones.

B1-20 Cloning

We then prepared the cDNA from subtracted clones and made two sets of identical dot-blot membranes to perform a differential screen. The clone B1-20 was selected after this test because it displayed an intense hybridization signal only on the membrane hybridized with the cDNA flamed for 60 min (data not shown). Sequence analysis of the B1-20 insert showed high homology with the spinach CMBP (Yang et al., 1996) and also revealed that the cDNA was incomplete: sequences were missing from both the 3′ and 5′ ends. To obtain the full-length clone, RACE-PCR amplifications were performed and a single band was generated by each PCR amplification. Sequencing confirmed that the four gene-specific primers designed for the RACE-PCR

amplified the expected fragments. Three sets of clones were independently produced and sequenced to avoid possible errors due to PCR amplification.

Gene Sequence Analysis

The 3′-, 5′-, and full-length RACE products were sequenced on both strands and the corresponding cDNA was identified as a CMBP. Figure 2 shows the complete deduced amino acid sequence for the tomato CMBP and its comparison with a previously cloned CMBP from spinach (Yang et al., 1996). The complete cDNA sequence, including a poly(A$^+$) tail, is available in GenBank under the accession no. AF106660. The encoded protein has a calculated molecular mass of 36.2 kD and a pI of 5.88. The sequence also displayed a putative chloroplast transit peptide (data not shown) upstream of the coding region.

The homology of the tomato CMBP to the spinach clone (Yang et al., 1996) is high (84% identity), while the tomato protein has an additional Ala residue at the end (Fig. 2). The corresponding gene was found to be encoded by a unique gene in tomato (data not shown), as was also found for spinach (Yang et al., 1996). This result is consistent with the fact that the RACE-PCR amplifications always generated a single band (data not shown).

Accumulation of CMBP mRNA after Flaming

RNA was isolated from various tissues of control plants or plants flamed for 60 min (Fig. 1), blotted, and the membranes probed with the B1-20 cDNA; results are shown in Figure 3. The initial level of the CMBP transcript was always low in all of the control tissues, but particularly in

Figure 2. Sequence of clone B1-20. The predicted amino acid sequence of tomato is compared with spinach CMBP (Yang et al., 1996). The tomato CMBP shows an additional terminal amino acid (Ala). An incomplete chloroplast transit signal showing analogies with the spinach counterpart was also identified (data not shown). The complete cDNA sequence is available in GenBank under the accession no. AF106660.
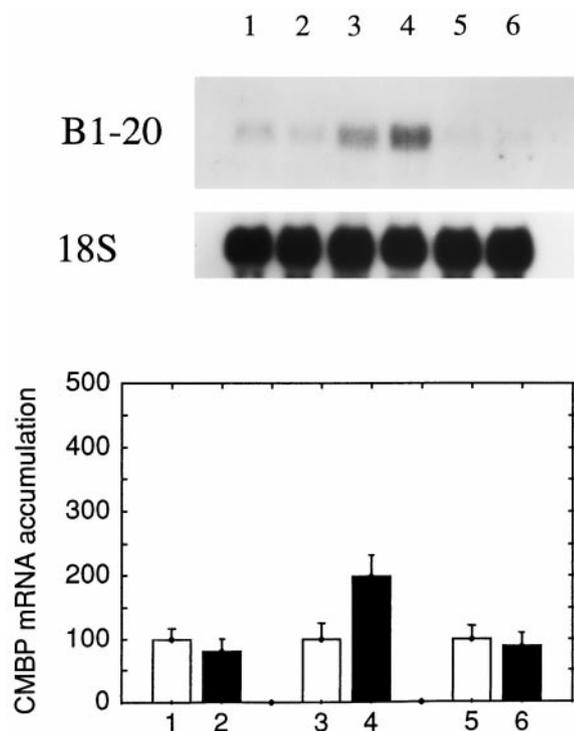
Figure 3. Accumulation of CMBP mRNA in various tissues. RNA was isolated from the petiole of the third (stimulated) leaf (lanes 1 and 2), the mature second leaf (lanes 3 and 4) or the internode (lanes 5 and 6). The plants were either non-stimulated controls (lanes 1, 3, and 5) or plants flamed on the third leaf for 60 min (lanes 2, 4, and 6), which corresponds to the time after flaming chosen for the tester condition in the subtraction procedure. B1-20, Hybridization using B1-20 cDNA as a probe. 18S, Control for equal loading of RNA (5 μg) in each lane using an 18S probe. Treatment values (black bars) are expressed relative to their respective controls (100%, white bars). Each point is the average of five replicates ± SE.

the petiole (Fig. 3, lane 1) and internode (Fig. 3, lane 5), and was somewhat higher in the mature leaf (Fig. 3, lane 3). Flame treatment, however, led to a consistent 2-fold accumulation of CMBP only in the mature leaf (Fig. 3, lane 4). In the other tissues tested, flame treatment had no significant effects (Fig. 3, lanes 1 and 2 and 5 and 6).

We then analyzed changes in CMBP transcript in a young, actively growing terminal leaf (leaf 4), the tissue used for previous work on *pin-1* and *pin-2* gene expression (Stankovic and Davies, 1996, 1997). Changes in the abundance of CMBP transcripts in the terminal leaf were complex. The initial level was extremely low (Fig. 4, lane 1), lower than that observed in the mature leaf (Fig. 3, lane 3), but flame wounding caused an extremely rapid accumulation of CMBP transcript. Within 5 min after flaming the third leaf, the transcript level increased 2.8-fold (Fig. 4, lane 2) and reached a maximum of about 4-fold after 15 min (Fig. 4, lane 3). This initial period of accumulation (phase I) was followed immediately by a period of decline (phase II), such that by 30 min (Fig. 4, lane 4) the transcript level had returned to that observed in control plants. A second, limited accumulation (phase III) occurred between 60 and 90 min (Fig. 4, lanes 5 and 6), while after 3 h (Fig. 4, lane 7)

the level had once more returned to that observed in control plants.

Since these plants were grown in a phytotron at a relatively high light flux (300 μmol s^{-1} m^{-2}) and since CMBP is a chloroplast protein, we wanted to find out if transcript accumulation also occurred when plants were stimulated in darkness. Plants were transferred from the phytotron to the dark for 24 h before flame wounding of leaf 3, and the RNA in leaf 4 was analyzed. The initial level was similar between plants grown in high light (Fig. 5, lane 1) and those grown in darkness for 24 h (Fig. 5, lane 2). However, in plants kept for 24 h in the dark, flame treatment did not cause any significant change in CMBP transcript level 15, 30, or 60 min after flaming (Fig. 5, lanes 3–5).

Since the wound signal must pass through the petiole of the treated leaf (leaf 3), we decided to analyze the kinetics of CMBP transcript accumulation in this petiole (Fig. 6). The treatment caused a slight (not significant) increase in CMBP transcript 15 min after treatment (Fig. 6, lane 2), followed by an immediate decline in transcript abundance at 30 and 90 min (Fig. 6, lanes 3–5), which continued until 3 h (Fig. 6, lane 5), by which time it was only about one-third of that observed in the control plants.

We were interested in finding out if this response was specific to flame wounding or if a mechanical wound would evoke the same response. Therefore, plants were crushed with metal forceps on leaf 3 and mRNA was isolated from leaf 4. The pattern observed was quite different from that obtained with the flame stimulus (Fig. 4). After mechanical wounding, a sharp and extremely tran-
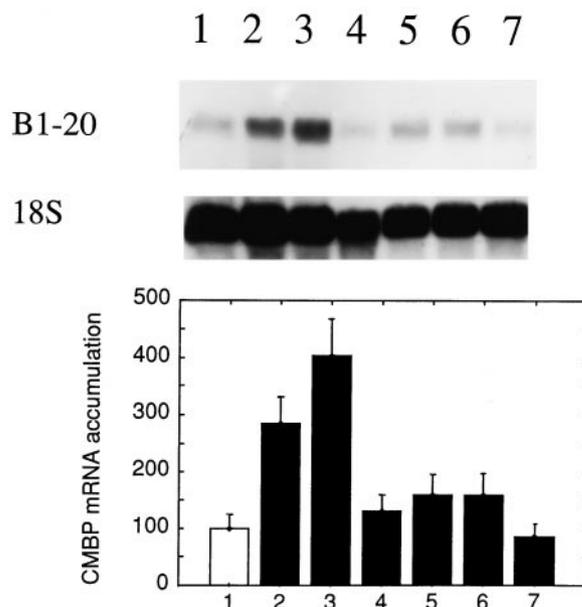

Figure 4. Kinetics of accumulation of CMBP mRNA in the fourth (terminal) leaf after flaming the third leaf. RNA was isolated from the fourth terminal leaf of control plants (lane 1), or 5, 15, 30, 60, 90, and 180 min after flaming the third leaf (lanes 2–7). B1-20, Hybridization using B1-20 cDNA as a probe. 18S, Control for equal loading of RNA (5 μg) in each lane using an 18S probe. Treatment values (black bars) are expressed relative to the control (100%, white bar). Each point is the average of six replicates ± SE.
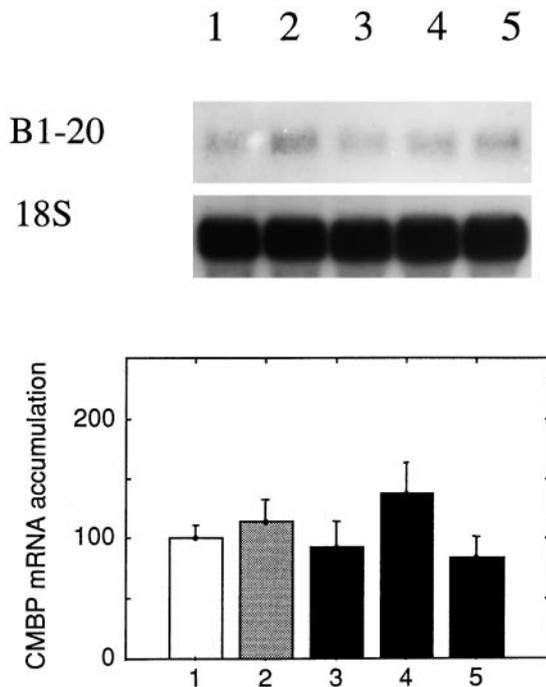
Figure 5. Accumulation of CMBP mRNA after flaming plants in the dark. RNA was isolated from the fourth terminal leaf of unflamed plants subjected to normal light culture conditions (lane 1), from plants placed in the dark for 24 h and harvested without prior treatment (lane 2), or 15, 30, and 60 min after flaming the third leaf (lanes 3–5). B1-20, Hybridization using B1-20 cDNA as a probe. 18S, Control for equal loading of RNA (5 μg) in each lane using an 18S probe. Treatment values (black bars) and the dark control (gray bar) are expressed relative to the light control (100%, white bar). Each point is the average of four replicates $\pm$ SE.

sient accumulation of CMBP transcript occurred after 5 min (Fig. 7, lane 2), but thereafter (15 min–6 h: Fig. 7, lanes 2–7) dropped to the level in control plants (Fig. 7, lane 1).

DISCUSSION

Since the pioneering work of Ryan and co-workers (Ryan, 1974; Lee et al., 1986), the tomato plant has been a model system for studying wound-regulated systemic gene expression. However, much of this work has focused on a limited number of transcripts, primarily pin, which are involved in plant responses to insect feeding (Johnson et al., 1989) and calmodulin (Stankovic and Davies, 1997), although work has been extended recently into SWRPs as well (Bergey et al., 1996, 1999). Several different systemic signals have been advocated, including hormones such as proteinase inhibitor-inducing factor (Ryan, 1974), systemin (Pearce et al., 1991), ABA (Peña-Cortés et al., 1991), and oligosaccharides (Ryan and Farmer, 1991), the genuine electrical signal or AP, and the hydraulic signal with its electrical aftermath, the VP. It is therefore quite possible that different systemic signals regulate the expression of different genes. Accordingly, the main goal of this work was to begin characterizing the array of genes up-regulated

by wounding in order to determine which systemic and which local signals regulate which gene(s).

To identify novel up-regulated genes, we chose to use a subtractive cloning strategy (Bonaldo et al., 1996), which has become a relatively easy technique since a commercial kit became available (CLONTECH Laboratories). We also used this technique to avoid possible artificial selection of particular transcripts, such as those introduced by PCR cloning with primers designed from existing genes or the screening of a library with a previously cloned cDNA probes. We found the new two-step PCR-based protocol to be a major simplification over the existing subtraction methods. We effectively selected stress-related sequences, and additional screening steps (i.e. differential screening of subtraction-selected clones) were unnecessary since virtually all of the randomly selected subtractive clones displayed positive differential expression (data not shown).

The current study focused on one of these clones, B1-20, which was identified as a CMBP based on results from the clone recently isolated from spinach (Yang et al., 1996). As a consequence of the *Rsa*I digestion step in the subtraction procedure, B1-20 lacks some of both the 5' and 3' ends. RACE-PCR successfully amplified these ends, and analyzed the complete sequence using a multiple set of clones obtained from independent cloning/sequencing events (Fig. 2). The tomato CMBP displayed a high degree of homology (Fig. 2) with its spinach counterpart (84%). The CMBP is present in only one copy in the tomato genome
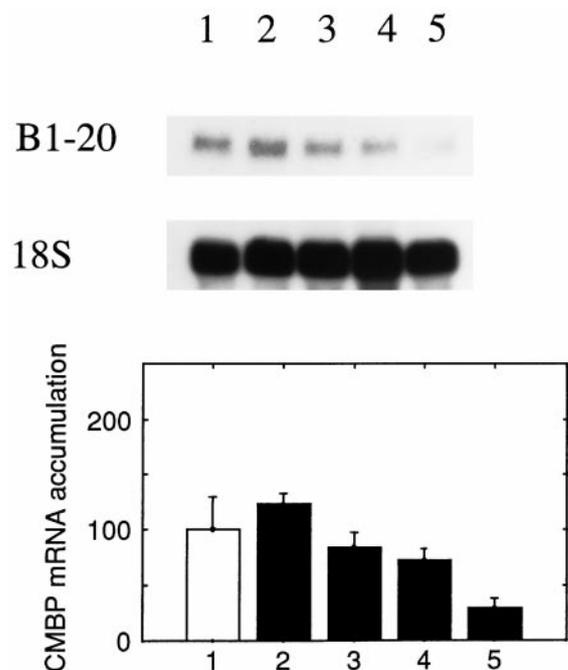

Figure 6. Kinetics of accumulation of CMBP mRNA in the petiole of the third leaf after flaming the third leaf. RNA was isolated from the petiole of the third leaf of control plants (lane 1) 15, 30, 60, and 180 min after flaming (lanes 2–5). B1-20, Hybridization using B1-20 cDNA as a probe. 18S, Control for equal loading of RNA (5 μg) in each lane using an 18S probe. Treatment values (black bars) are expressed relative to the control (100%, white bar). Each point is the average of five replicates $\pm$ SE.
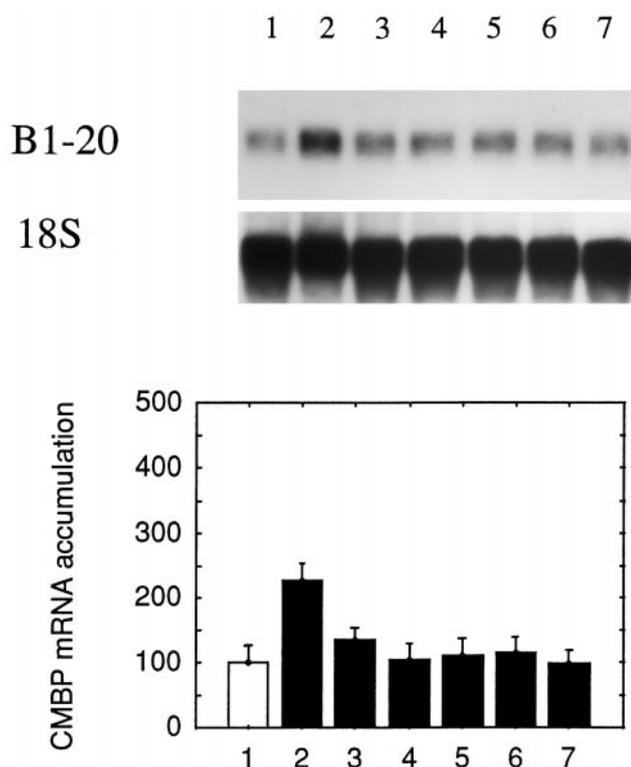
Figure 7. Kinetics of accumulation of CMBP mRNA in the fourth terminal leaf after mechanical wounding of the third leaf. RNA was isolated from the fourth terminal leaf of intact, non-stimulated plants (lane 1) or after mechanical wounding (crushing the third leaf with metal forceps). Tissue was harvested 5, 15, 30, and 60 min and 2 and 6 h after the treatment (lanes 2–7, respectively). B1-20, Hybridization using B1-20 cDNA as a probe. 18S, Control for equal loading of RNA (5 μg) in each lane using an 18S probe. Treatment values (black bars) are expressed relative to the control (100%, white bar). Each point is the average of five replicates ± SE.

(data not shown), as was found previously in spinach (Yang et al., 1996). This result is consistent with the RACE-PCR amplifications that always gave a single band (data not shown).

CMBP transcripts were low in all non-stimulated tissues, but they increased in all tissue after flame wounding, with a larger accumulation in the mature leaf than in the petiole or internode (Fig. 3). It seems reasonable that a chloroplast-related transcript would accumulate more in photosynthetically active tissues such as the leaves and less in tissues low in chloroplasts (e.g. the petiole or internode). It was surprising that the steady-state level was so low in an actively growing, photosynthetically active tissue such as the terminal leaf (Fig. 4). This may be because the protein has either a high catalytic activity or a limited substrate specificity, since its major or sole function is in maturation of the petD pre-mRNA (Yang et al., 1996), and it may only be needed at a low level.

After flaming the third leaf, the kinetics of CMBP transcript accumulation in the terminal leaf (leaf 4) were quite complex and occurred in three distinct phases (Fig. 4). Phase I involved extremely rapid and major transcript accumulation. Within just 5 min, the CMBP transcript ac-

cumulated over 2-fold above control levels and had reached 4.5-fold by 15 min. This demonstrates that the wound signal must have been transmitted from the third leaf to the terminal leaf within 5 min. It also suggests (but does not prove) that enhanced transcription, rather than lessened degradation, was responsible for this rapid accumulation. Phase II involved a decline of CMBP transcript levels back to control levels between 15 and 30 min. Considering that almost all of the newly accumulated transcript disappeared as quickly as it had been made, an active degradation process is likely to be involved.

Phase III involved another period of transcript accumulation between 60 and 90 min after flaming, but this was much smaller than the initial accumulation. This triphasic pattern has already been observed (with much slower kinetics) for the mRNA-binding protein rbpA3 in the cyanobacterium *Anabaena variabilis* (Sato and Maruyama, 1997). These authors showed that after a temperature shift from 38°C to 22°C, the amount of rbpA3 transcript peaked after 30 min, returned to control levels after 2 h, and then exhibited a second and weaker peak after 5 h. Despite the lack of sequence homology between the tomato CMBP and rbpA3, the similarity between these patterns is quite striking, although the response was far more rapid in tomato. The faster response may have been due to the treatment, which was much more drastic in tomato (flame) than the temperature shift of *A. variabilis*.

Since xylem tension is lost after plants are kept for a long time in the dark, and plants therefore lose their ability to generate hydraulic signals and the accompanying variation potentials (Stankovic and Davies, 1997), we wondered whether transcript accumulation would occur in plants that had been placed in the dark 24 h before the flame treatment. Accumulation of CMBP transcript did not take place (Fig. 5), implying (but not proving) that the hydraulic signal is involved. However, since expression of some wound-regulated genes such as *pin* is dependent on Suc (Johnson and Ryan, 1990), the signal may be generated/transmitted, but no response can ensue in the absence of sugar, which is likely to be low after 24 h in the dark.

Since transcript accumulation begins within 5 min in tissue 4 to 5 cm away from the wounded region, the wound signal must be generated and transmitted extremely rapidly. Although we have not totally ruled out this possibility, it seems unlikely that a chemical (hormone) could move from the damaged tissues to the terminal leaf in the phloem sufficiently rapidly to turn on the gene. It is possible, however, that hormones could be transported in the xylem and somehow liberated into the adjacent living cells (Malone et al., 1994; Malone, 1996). Alternative signaling pathways involving electrical signals have recently been proposed (Wildon et al., 1992; Stankovic and Davies, 1996, 1997, 1998a, 1998b; Vian et al., 1996). Stankovic and Davies (1996, 1997) demonstrated that the systemic signal evoking a rapid (30 min) and massive (7- to 10-fold) accumulation of pin mRNA is an electrical wave of depolarization, an AP induced by electrical stimulation, or a VP induced by flame stimulus.

These signals would be good candidates for rapid transmission of the wound information from damaged tissues to intact, distant tissues, since their velocity ranges from about 0.8 to 4 cm s^{-1} for the AP (Sibaoka, 1950) and 0.6 to 1 mm s^{-1} for the VP (Van Sambeeck and Pickard, 1976; Julien et al., 1991; Zawadzki et al., 1991). These velocities are far higher than those generally proposed for the diffusion of a chemical (i.e. hormonal) signal. We do not yet have data unequivocally linking membrane depolarization with evocation of CMBP mRNA accumulation. However, since such correlations have been demonstrated for ethylene production (Inaba et al., 1995) and for other genes such as *pin* (Wildon et al., 1992; Stankovic and Davies, 1996, 1997) and calmodulin (Vian et al., 1996), such a signaling system may in reality play an important role in the expression of several genes.

Mechanical wounding (crushing with forceps) leads to a different response from that induced by flame wounding. As with flame wounding (Fig. 4), both the rapid increase (phase I) and the rapid return to control levels (phase II) are seen after crushing (Fig. 7), but no subsequent accumulation (phase III) occurs. This suggests that the two periods of accumulation are under the control of different signals— and we suspect that the later increase is more likely controlled by hormones than the initial, rapid increase.

We have shown that the subtractive screen furnishes at least one novel clone rapidly up-regulated in distant, intact tissues after flaming or mechanical wounding. Since there are major questions concerning the nature of the systemic wound signal(s), we are in the process of isolating additional wound-up-regulated clones. We intend to use these to find out whether the same systemic signal is involved in their regulation, or if different signals regulate different genes. In this context, experiments are in progress to determine if CMBP gene expression can be coupled to membrane potential, thus demonstrating that a large array of wound-related genes are evoked by a signal in which changes in membrane potential constitute a major role.

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