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Sequence-specific interactions of wound-inducible nuclear factors with mannopine synthase 2′ promoter wound-responsive elements

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Abstract

A 318 bp mannopine synthase 2′ (*mas2′*) promoter element from the T-DNA of *Agrobacterium tumefaciens* can direct wound-inducible and root-preferential expression of a linked *uidA* gene in transgenic tobacco plants. Wound inducibility is further enhanced by sucrose in the medium. Promoter deletion analysis indicated that the sucrose enhancement is conferred by a region extending from −318 to −213. DNase I footprinting indicated that an A/T-rich DNA sequence in this region is protected by tobacco nuclear factors. Regions extending from −103 to +66 and from −213 to −138 directed wound-inducibile expression of a linked *uidA* gene when placed downstream of a CaMV 35S enhancer or upstream of a truncated (−209) CaMV 35S promoter, respectively. DNase I footprinting analyses indicated that proteins from wounded tobacco leaves specifically bound to three contiguous motifs downstream of the *mas2′* TATA box. In addition to a common retarded band formed by the upstream wound-responsive element complexed with proteins from either wounded or unwounded tobacco leaves, two unique retarded bands were observed when this element was incubated with protein from wounded leaves. Methylation interference analysis additionally identified an unique motif composed of promoter elements and nuclear factors derived specifically from wounded tobacco leaves. We propose a model to describe the involvement of nuclear factors with *mas2′* promoter elements in wound-inducible gene expression.

Introduction

Crown gall tumor formation by *Agrobacterium tumefaciens* results from the transfer of T-DNA from the bacterium to wounded plant tissues. In addition to the production of high levels of auxins and cytokinins directed by the *tms* and *tmr* genes, T-DNA also encodes genes that direct the synthesis of low-molecular-weight tumor-specific compounds termed opines (for recent reviews, see [15, 16, 50]). The *mas1′* and *mas2′* genes encode enzymes of a two-step pathway for the synthesis of the opine mannopine [12, 26]. These genes share a dual bidirectional promoter [47]. Promoters of both the *mas1′* and *mas2′* genes direct similar patterns of developmental and root-

preferential expression, as well as wound and auxin inducibility, in transgenic tobacco plants [28, 44]. Deletion analyses have implicated various *cis* elements in the developmental, tissue-specific, and environmental regulation of the *mas* genes [9, 14, 31, 36]. However, most of these studies were based on transient assays in protoplasts or stably transformed callus tissue, and no detailed analyses have yet been carried out to define these functional *cis* elements.

A number of plant genes are inducible by mechanical wounding of plant tissue. These genes include those encoding proteinase inhibitor II [29, 45], soybean storage proteins [35], nopaline synthase (*nos* [1]), and *win* [48]. Some genes, such as *mas*, *nos*, and one encoding a tobacco proteinase inhibitor I [33], are wound-inducible only in cells found locally around the wound site, while other genes respond systemically because they are induced in non-wounded as well as in wounded leaves [45]. The wound inducibility of gene expression implies the existence of a signal transduction pathway in response to the environmental stimulus. At the terminus of this signal transduction pathway, a wound-specific factor(s) would bind to wound-responsive promoter elements and activate gene expression. For the proteinase inhibitor II gene, deletion analysis identified a 421 bp sequence necessary for wound-inducible gene expression in transgenic plants [37]. Utilizing a DNA mobility shift assay, these authors identified a 10 bp sequence (AAGCGTAAGT) within the 421 bp region that bound a nuclear factor from both non-wounded and wounded leaves. A 10 bp putative Z-DNA sequence (GCACATACGT) between −123 and −114 or an element overlapping this sequence in the *nos* promoter is essential for the wound-inducible response [1]. Recently, Guevara-Garcia *et al.* [19] reported the effects of deletion mutations upon the wound-inducible gene expression directed by the *mas1'* promoter. However, the *cis* element responsible for wound inducibility is redundant throughout the whole promoter region.

No data have yet been presented for the existence of a concensus wound-responsive *cis*-acting motif that interacts with cognate wound-inducible *trans*-acting factors. In this study, we demonstrate that an upstream region extending from −213 to −138 and a downstream region extending from −103 to +66 (relative to the transcription start site) of the *mas2'* promoter confer wound inducibility upon an *uidA* gene linked to a heterologous promoter. DNase I footprinting and methylation interference analyses identified DNA sequence elements within these two regions that complex specifically and differentially to wound-inducible factors from tobacco leaves.

Materials and methods

Materials

Restriction endonucleases were from Bethesda Research Laboratories and used according to the manufacturer's specifications. We purchased DNA polymerase I Klenow fragment and T4 DNA ligase from Pharmacia LKB Biotechnology, and used them according to the manufacturer's specifications. [α-^{32}P]-dCTP was from Amersham. We labeled DNA fragments for the RNA blot assay using an Amersham multiprime DNA labeling system 1601Z kit. Reagents for the GUS assays and antibiotics were from Sigma.

Strains and culture conditions

We grew *Escherichia coli* strains in LB medium [34] and *Agrobacterium tumefaciens* LBA4404 [21] in AB minimal medium [32]. Antibiotic concentrations, when used, were: for *E. coli* kanamycin 50 μg/ml, ampicillin 100 μg/ml, for *A. tumefaciens* kanamycin 50 μg/ml, rifampicin, 10 μg/ml. These experiments were conducted under P1 containment conditions as specified by the National Institutes of Health Recombinant DNA Guidelines.

Plasmid constructions

The base plasmid for all binary vector constructions was the plasmid pBI101.2 from Clontech. We initially cloned an *Eco* RV-*Xba*I fragment cor-

responding to the *mas* promoter region −318 to + 66, relative to the transcription initiation site (nucleotides 20513–20128 [2]), from pKan2–318 [9] into the *Sma* I and *Xba* I sites of Cup31 (a pUC13 derivative with the pUC13 backbone but polylinker reading 5′ to 3′ as *Hin*d III, *Pst* I, *Sst* I, *Sma* I, *Bam* HI, and *Xba* I). In a similar manner, we also cloned the *mas* promoter regions −138 to + 66 (nucleotides 20343–20128 [2] from pKan2–138 [9] and the −103 to + 66 *Rsa* I-*Xba* I fragment (nucleotides 20296–20128 [2]) into Cup31. We then cloned *Hin*d III-*Xba* I restriction endonuclease fragments from Cup31 into the *Hin*d III-*Xba* I sites of pBI101.2 to generate the plasmids pNi1, pNi2, and pNi3 as *mas* −318, −138, and −103 deletions, respectively. We isolated a *Xho* I-*Hae* III fragment (from −318 to −213), filled in the ends using Klenow fragment of DNA polymerase I, added *Hin*d III linkers to the ends of the fragment, and cloned the fragment into the *Hin*d III site of pNi3 to construct the internal promoter deletion −213 to −103. We added *Xba* I linkers to the *Hae* III-*Xba* I fragment (from −213 to + 66) and cloned this fragment into the *Xba* I site of pBI101.2 to construct the plasmid pNi4 as the *mas* −213 promoter deletion plasmid.

We used the plasmid pBI121 (Clontech), containing a 800 bp *Hin*d III-*Bam* HI fragment, as the source of the CaMV 35S promoter. We added *Hin*d III linkers to a *Hin*d III-*Eco* RV fragment (−800 to −90) and cloned this fragment into the *Hin*d III site upstream of the −103, −138, and −213 deletions of pNi2, pNi3, and pNi4, respectively. We added a *Bam* HI linker to a *Hae* III-*Bam* HI fragment (−209 to 0) and cloned this fragment into the *Bam* HI site of pBI101.2 to construct the plasmid pNi5. pNi5 contains a CaMV 35S promoter truncated at position −209. We next converted a *Rsa* I-*Hae* III fragment (−103 to −213), a *Hae* III-*Xho* I fragment (−213 to −318), and a *Mnl* I-*Xho*I fragment (−111 to −318) to blunt-end fragments by filling in the ends using the Klenow fragment of DNA polymerase I. We added *Xba* I linkers to these fragments and cloned them into the *Xba* I site of pNi5. We amplified the *mas* promoter fragments −138 to −104 and −212 to −135 by the polymerase chain reaction using the upstream primers 5′-TACGCTGACACGCCAAGC-3′ or 5′-CTGAATTTCGCGGGTATTC-3′ and the downstream primers 5′-ACACTTTTGACTAGCGAG-3′ and 5′-CGTATCTATTCAAAAGTCG-3′, respectively. We added *Xba* I linkers to these fragments and cloned them into the *Xba* I site of pNi5.

We cloned a *Bam* HI-*Eco* RI fragment containing an *ocs* minimal promoter and part of the *uidA* structural gene (−116 to + 296 relative to the transcription initiation site, nucleotides 13774 to 13362 [2]), and a *Xba* I-*Eco* RI fragment containing the *ocs* activator plus promoter and part of the *uidA* structural gene (−333 to + 296 relative to the transcription initiation site, nucleotides 13991 to 13362 [2]) from the plasmid pEN1 [30] into the *Bam* HI-*Eco* RI and *Xba* I-*Eco* RI sites, respectively, of pBluescriptII SK(+) (Stratagene). We excised from these plasmids *Xba* I-*Eco* RV fragments and cloned them into the *Xba* I-*Sma* I sites of pBI101.2 as GUS translational fusions to give rise to plasmids pNi6 and pLH3, respectively. We cloned an *ocs* activating element (−333 to −116; nucleotides 13774 to 13991 [2]) containing *Hin*d III ends from the plasmid pENΔ1 [30] into the *Hin*d III site of pNi2. We added *Xba* I linkers to various *mas* promoter fragments (−213 to −103, −318 to −213, and −318 to −111) and cloned these fragments into the *Xba* I site of pNi6.

We transformed each of the constructions into *E. coli* DH5α and verified the orientations of each insert either by restriction endonuclease mapping or by DNA sequence analysis.

Plant materials and transformation

We mobilized plasmids into *A. tumefaciens* LBA4404 by a triparental mating procedure [10] using *E. coli* MM294 harboring pRK2013 [13]. *A. tumefaciens* transconjugants were selected on AB minimal medium containing 0.5% sucrose, 10 μg/ml rifampicin, and 50 μg/ml kanamycin. We verified the introduction of the plasmid into the recipient *A. tumefaciens* strain by DNA blotting.

We transformed six-week old sterile shoot tip cultures of *Nicotiana tabacum* cv. Wisconsin 38 by a leaf disk transformation-regeneration method [22]. After incubation of infected leaf disks for 3 days on MS3$^+$ medium, we transferred disks to shoot induction medium containing 1250 μg/ml carbenicillin and 200 μg/ml kanamycin. After 4 to 5 weeks, we transferred a single shoot per leaf disk to root induction medium containing 500 μg/ml carbenicillin and 50 μg/ml kanamycin. After two weeks, we established shoot tip cultures of each line by transferring the plant to BGS medium containing 50 μg/ml kanamycin.

Wound induction

We cut leaf discs with a cork borer and floated the disks in MS3$^-$ medium (or other media, as indicated) with the adaxial side up in Petri dishes sealed with parafilm. We incubated the disks in a growth chamber at 25 °C under continuous light for 24 h. For each series of experiments, we prepared discs from the same leaves to avoid the effects of different physiological conditions of different leaves. For preparation of cell extracts, we wounded the leaves with a cork borer and floated the wounded leaves (with the discs still attached) in MS3$^-$ medium for 24 h.

GUS activity assay

We harvested tissues from the leaves and roots of transgenic plants, ground them in 200 μl extraction buffer [23], and stored them at −70 °C. We assayed GUS activity according to Jefferson and Wilson [23] using 10 μl extract (about 20 to 30 μg protein) and MUG (4-methylumbelliferyl-β-*D*-glucuronide) as a substrate. We determined protein concentrations according to Patterson [38].

Nucleic acid manipulations

We isolated total RNA according to de Vries *et al.* [8] and fractionated 5 μg per lane by formaldehyde gel electrophoresis [36]. We transferred RNA to a nylon filter according to Maniatis *et al.* [34]. The integrity of the RNA was checked by agarose gel electrophoresis followed by ethidium bromide staining. The fluorescence of the nucleic acids also served to verify that equal amounts of RNA were loaded in each lane. We fixed the RNA onto the membrane by baking for 2 h at 80 °C under vacuum. We prehybridized the filters for 2–4 hr at 65 °C in 1.5 × SSC, 1.0% SDS, 0.5% Blotto, 500 μg/ml sheared salmon sperm DNA. We hybridized the filters overnight in fresh solution with probe containing the *uidA*-coding region (a *Xba* I-*Sst* I fragment from pBI101.2). We washed the membrane successively for 15 min at room temperature in the following solutions: 2 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS, 0.1 × SSC/0.1% SDS. The final wash was done for 30 min with 0.1 × SSC/1.0% SDS at 60 °C.

Preparation of extracts and probes for gel mobility shift assays

We prepared extracts from 100 g each of control leaves or wounded leaves according to Green *et al.* [17], and resuspended the protein in Buffer B (20 mM Hepes-KOH pH 7.5, 40 mM KCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, and 0.8 mM PMSF).

We 3′-end labeled restriction endonuclease fragments with the Klenow fragment of DNA polymerase I using α-labeled ^{32}P-dCTP according to Maniatis *et al.* [34]. Probes were isolated by agarose gel electrophoresis and electro-eluted. We determined probe DNA concentrations by comparison with standards on ethidium bromide-stained agarose gels.

Gel mobility shift assays

We conducted binding reactions in a 20 μl volume in a reaction containing 20 mM Hepes-KOH pH 6.7, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.8 mM PMSF, 0.5–1.0 ng DNA (10^5 cpm), 3.75 to 7.5 μg dI:dC, and 30 μg

protein [17]. After incubation at room temperature for 30 min, we analyzed the reaction products by electrophoresis through 8% polyacrylamide gels (29:1 acrylamide: bisacrylamide) containing 0.25 × TBE buffer at 20 mA.

We treated protein extracts with alkaline phophatase according to Sun *et al.* [43] with modifications. Briefly, we incubated 30 μg protein for 10 min in a 20 μl reaction containing EB buffer and 0.5 units alkaline phosphatase (Boehringer Mannheim). Where indicated, 60 μg protein were either boiled for 10 min or treated with 30 μg of proteinase K (Boehringer Mannheim) for 30 min at 37 °C before addition to the binding reaction. For RNase treatment, we included 5 μg RNase A (Pharmacia) in the binding reaction.

In vitro *footprinting and methylation interference assays*

Binding conditions for DNA footprinting were the same as those used for gel mobility shift assays except that we used 60 μg protein. We initiated the reactions by the addition of 3 ng DNase I (Pharmacia) for 1 min at room temperature in the presence of 5 mM $MgCl_2$ and 1 mM $CaCl_2$, and stopped the reactions by the addition of 1.6 μl 0.5 M EDTA. We performed preparative gel eletrophoresis as described above. We excised the retarded band and eluted the DNA according to Green *et al.* [17]. DNA samples were resuspended in 4 μl of formamide dye, boiled for 3 min, and loaded onto 8% acrylamide (19:1 acrylamide bisacrylamide) sequencing gels.

We methylated the DNA probe as described by Green *et al.* [17]. After the methylation reaction was stopped, we precipitated the DNA probe with ethanol and purified it by agarose gel electrophoresis. Gel retardation assays (50 μl) contained 2.5 ng methylated DNA probe, 19 μg poly-dI:dC, and 150 μg protein in EB buffer. Band excision, DNA elution, and probe cleavage with piperidine were done according to Green *et al.* [17].

Results

The −318 mas *promoter deletion is sufficient for wound-inducible and root-preferential expression*

The transcription of the *mas1'* and *mas2'* genes is divergent and a 479 bp intergenic region contains all the *cis*-acting elements necessary for the regulated transcription of both genes [9, 14, 31]. Figure 1 shows the nucleotide sequence of the *mas2'* promoter −318 deletion [9] that we have used in this study. The TATA box for the *mas1'* gene is located 2 bp upstream of the *mas2'* −318 deletion and reads in the opposite direction. Bouchez *et al.* [6] identified two regions with limited homology to *ocs*-(octopine synthase)-like elements at positions −289 and −64. According to Leung *et al.* [31], three complementary sequences labeled a'', a', and a can potentially hybridize to form alternative palindromic structures. However, the importance of these *cis*-acting elements remains largely unknown.

Our previous deletion analysis showed that sequences within 138 bp of the transcription initiation site are sufficient for accurate transcription

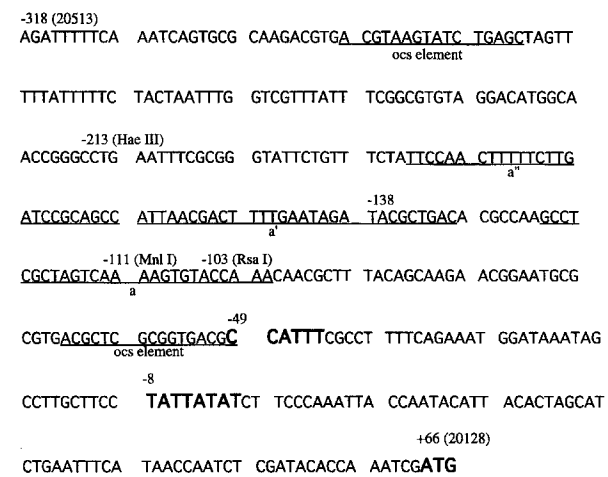

Fig. 1. Nucleotide sequence of the *mas2'* promoter region. The region extends from −318 to +66 (nucleotides 20128–20513 [2]) relative to the transcription initiation site. Proposed functional elements are indicated as *ocs* elements [6], and a'', a' and a [31]. Restriction endonuclease sites used for subcloning are indicated above the sequence. The CATT box, TATA box, and ATG translation initiation codon are indicated in bold face.

initiation of a *mas2'/nptII* fusion gene in sunflower crown gall tissue, while sequences between −138 and −318 may be involved in the quantitative regulation of the levels of *nptII* mRNA in sunflower tumors [9]. Based upon these data, we constructed a plasmid containing a −318 promoter element linked to an *uidA* reporter gene in the binary vector pBI101.2 and generated transgenic tobacco plants using an *Agrobacterium*-mediated transformation system.

To investigate the kinetics of wound inducibility, we floated leaf disks cut from these transgenic plants on MS3$^-$ medium (lacking hormones) and assayed GUS activity at various times. Figure 2 shows that there was a rapid increase in GUS activity within the first hour, followed by a relatively slow increase until 16 h. Subsequently, we observed another sharp increase in GUS activity that lasted until at least 24 h. This biphasic pattern of wound inducibility resembles that directed by the *win* gene promoter [48], but differs from that directed by the proteinase inhibitor II promoter, for which activity increases for 12 to 16 h and thereafter ceases or declines [45]. To investigate whether components of the incubation medium contribute to the induction, we incubated leaf discs from transgenic plants harboring the −318/*uidA* fusion gene in different media (Fig. 3). Plants wounded *in situ* by forceps (leaves remained on the plant during the induction period) showed only a 2 to 4-fold induction of GUS activity, whereas GUS activity in leaf discs floated

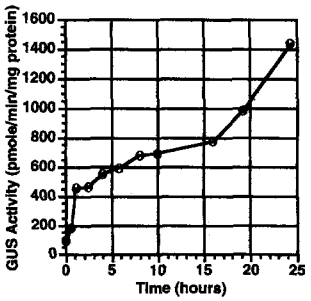

Fig. 2. Kinetics of wound inducibility of the *mas2'* promoter. GUS activity was measured at various times after leaf disks from a transgenic plant were floated in MS3$^-$ medium with sucrose.

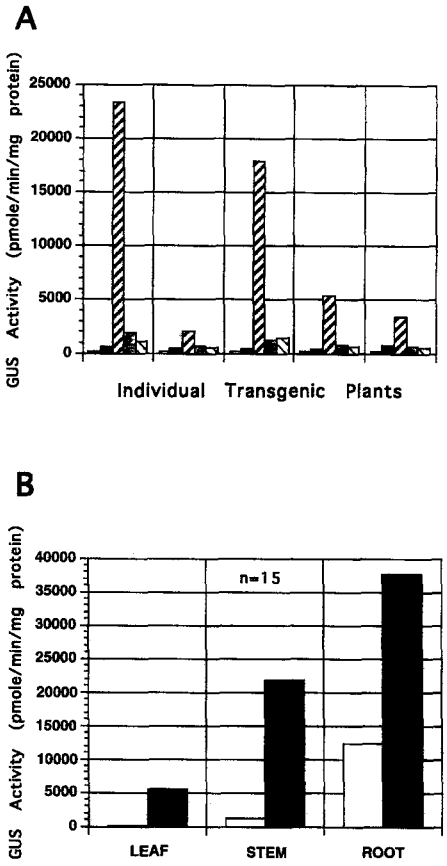

Fig. 3. Wound inducibility of the *mas2'* promoter in different media and in different plant tissues. A. The effects of different medium components upon wound induction were studied by wounding plant leaves *in situ* (2nd bar from the left), excising and floating leaf disks in MS3$^-$ medium with sucrose (3rd bar), without sucrose (4th bar), or in distilled water (5th bar) for 24 h. Protein was also extracted from disks of control unwounded leaves (1st bar). B. Wound induction in different tissues was conducted by incubating leaf disks, and stem and root fragments in MS3$^-$ medium with sucrose for 24 h. □, control unwounded tissues; ■, wounded tissues. n indicates the number of individual transgenic plants assayed. In this and subsequent figures, the standard deviation approximately equaled the mean values and is not shown because of the great variation resulting from the T-DNA integration site position effect upon gene expression.

in MS3$^-$ medium without sucrose or in distilled water increased 4- to 12-fold over a 24 h period (Fig. 3A). Inclusion of sucrose in MS3$^-$ medium strongly enhanced the wound inducibility: GUS activity increased 12- to 145-fold compared to

unwounded control plants. For plants wounded *in situ*, the increase in GUS activity closely related to the degree of wounding, and leaves of even moderately wounded plants wilted after a few hours. In contrast, excised leaf disks floated in MS3$^-$ medium remained turgid and yielded relatively reproducible results. The extent of wound inducibility differed in various plant tissues. Inducibility averaged 32-fold in leaves, 17-fold in stems, and 3-fold in roots (Fig. 3B). Under unstressed (unwounded) conditions, GUS activity in roots was 72-fold and 10-fold higher than in leaves and stems, respectively (Fig. 3B). These data closely resemble those previously reported [36]. In order to verify that wound-induced gene activation resulted from higher levels of *uidA* mRNA, we floated leaf disks from several different plants in MS3$^-$ medium and extracted RNA after 24 h. We also performed GUS assays on some of these disks. We assayed equal amounts of total RNA (5 μg) for the presence of *uidA* transcripts by RNA blot analysis. As shown in Fig. 4, *uidA* mRNA levels increased greatly in wounded leaves. The induced level of *uidA* transcripts generally remained less, however, than that found in non-wounded roots. In addition, *uidA* transcripts in roots were slightly larger than those in leaves. We do not currently know whether this size increase results from an altered transcription initiation site or from a longer poly(A) tail.

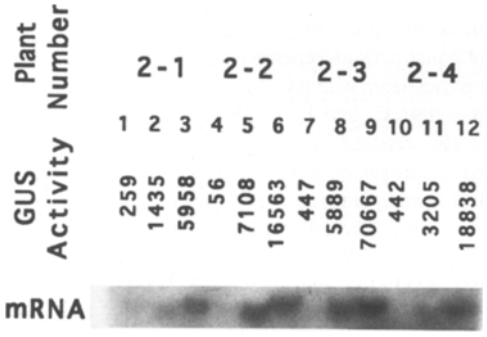

Fig. 4. Analysis of wound-inducible gene expression by RNA blotting. Total RNA and proteins were isolated from unwounded (lanes 1, 4, 7, and 10) or wounded (lanes 2, 5, 8, and 11) leaf tissues and unwounded root tissues (lanes 3, 6, 9, and 12) of four different transgenic plants as indicated. The blot contained 5 μg RNA per lane and was hybridized with radiolabeled *uidA* gene-coding sequences. GUS activity was assayed and expressed as pmol/min per mg protein.

Promoter deletion and swapping experiments reveal wound-responsive and sucrose-response regions

We constructed a series of 5′ external and internal deletions to define functional elements within the *mas2′* promoter (Fig. 5A). Compared to the −318 promoter, deletion of the promoter to −213 did not result in a significant alteration of GUS activity in non-wounded leaves (Fig. 5B). However, the magnitude of wound inducibility in leaves (leaf disks floated in MS3$^-$ medium containing sucrose) and the level of GUS activity in roots were greatly reduced, although the patterns of wound induced gene expression and root-preferential expression still remained (Fig. 5B).

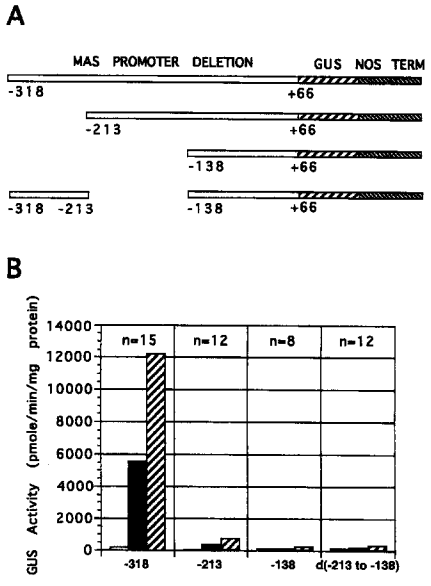

Fig. 5. Analysis of the effect of external and internal deletions upon *mas2′* promoter activity. A. Schematic diagram of the constructions. B. GUS activity was assayed in unwounded leaves (white bar), wounded leaves (black bar), and unwounded roots (hatched bar). Plants were wounded by floating leaf disks in MS3$^-$ medium containing sucrose for 24 h. Average GUS activity is from n individual transgenic plants assayed. The end points of each deletion are indicated relative to the transcription start site.

Because we conducted these experiments in the presence of sucrose, and because deletion of sequences between −213 and −138, but not −318 and −213, resulted in loss of wound inducibility (Fig. 5B, but also see Fig. 6D), we conclude that sequences from −318 to −213 act as a sucrose response element of wound inducibility in leaves. These sequences additionaly serve as a general activator of gene activity in root tissues. Under non-stressed conditions, the functioning of this activator is not easily detected in leaves because the overall activity is low. Further deletion to −138 rendered the promoter virtually inactive. DiRita and Gelvin [9] previously described this low activity directed by the −138 promoter fragment.

Our initial promoter mutations may have deleted regions that act as general enhancers of promoter activity as well as regions that specifically regulate promoter activity in response to wounding or developmental cues. We therefore may have missed identifying important regulatory regions because overall promoter activity was too low to detect. We therefore conducted 'promoter swapping' experiments using various regions of a heterologous cauliflower mosaic virus (CaMV) 35S promoter that, in our hands, is not wound-inducible (Fig. 6). However, linking various *mas* promoter fragments to the 35S enhancer resulted in wound inducibility (Figs. 6A and 6C). Analysis of transgenic tobacco plants containing a 35S enhancer (−800 to −90) upstream of a series of *mas* promoter deletions indicated that a wound-responsive element is located downstream of −103 (Figs. 6A and 6C). The possibility that wound-responsive elements also exist further upstream of −103 was tested by placing different *mas* promoter fragments upstream of a truncated 35S promoter (−209 to 0; Fig. 6B). We found that the activity of this 'short' 35S promoter (35S P′) is strongly enhanced by sucrose in the MS3⁻ medium (data not shown). We therefore used MS3⁻ medium lacking sucrose, or even distilled water, to avoid this interference in these experiments. Figure 6D demonstrates that a 77 bp region of the *mas* promoter from −212 to −135 contains a wound-responsive element that could confer wound inducibility upon a truncated 35S promoter.

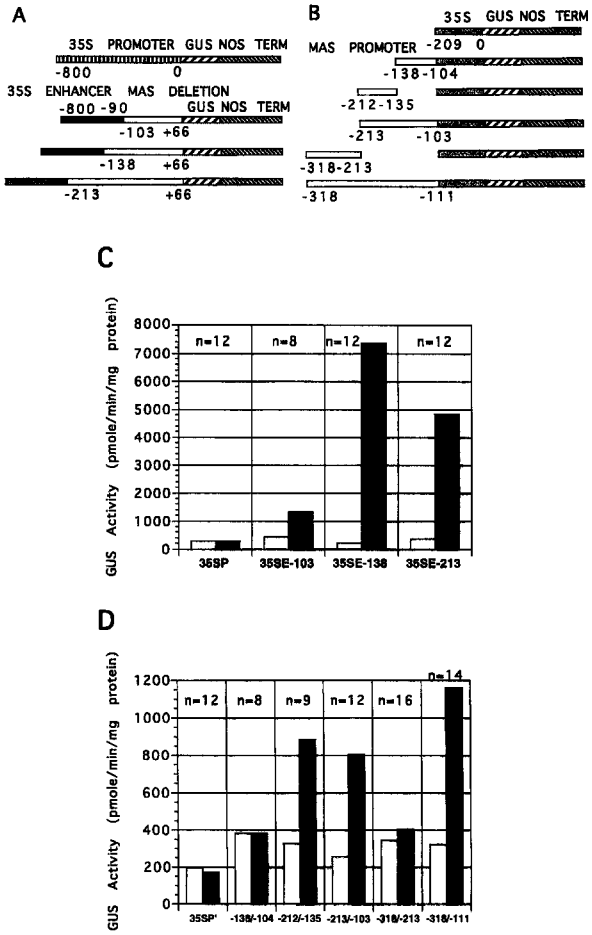

Fig. 6. Promoter swap analysis of *mas2′* wound-responsive elements. A. Schematic representation of constructions generated by combining the CaMV 35S enhancer (−800 to −90) with various *mas* promoter deletions. B. Schematic representation of constructions generated by combining different *mas* promoter fragments with a CaMV 35S promoter (truncated at position −209). C. and D. GUS activity was assayed using total protein prepared from unwounded leaves (white bar) or wounded leaves (black bar). n indicates the number of individual transgenic plants assayed. The end points of each construction are indicated relative to the transcription start site of the *mas2′* gene. 35SP, full CaMV 35S promoter (−800 to 0); 35SE-103, 35SE-138, and 35SE-213, CaMV 35S enhancer (−800 to −90) linked 5′ to the *mas* −103, −138, or −213 deletions, respectively; 35SP', CaMV 35S promoter truncated at position −209; −138/−104, −212/−135, −213/−103, −318/−213, and −318/−111, *mas* promoter fragments linked 5′ to the CaMV 35S promoter (truncated at position −209).

An element conferring root-preferential expression is located in a promoter-proximal region of the mas *promoter*

As discussed above, plants containing a deletion of the *mas* promoter to −213 still retained the root-preferential expression pattern, whereas further deletion to −138 rendered the activity of the mutated promoter below the limit of detection (Fig. 5). These results do not discount the possibility of several elements, such as a promoter-proximal element residing far downstream and a general enhancing element located far upstream, contributing synergistically to the root-preferential pattern of expression. We investigated this possibility by combining various portions of the octopine synthase (*ocs*) promoter with portions of the *mas* promoter (Fig. 7A). The *ocs* promoter region functions approximately equally well in the leaves and roots of transgenic tobacco plants ([36] and Fig. 7B). When the *ocs* activating element (−333 to −116) was added upstream of the deleted −138 *mas* promoter, we observed a strong pattern of root-preferential expression (Fig. 7B). This result indicates that a *cis*-activating element conferring a pattern of root preferential expression is present downstream of −138 within the *mas* promoter. Analysis of the DNA sequence within this region revealed the presence of an AS-1 tandem repeat motif [27] at position −66 of the *mas* promoter. In the CaMV 35S promoter, this element interacts with the *trans*-acting factor ASF-1 [27], which may be the same as the *trans*-acting factor TGA1a [24], and directs a tissue-specific expression pattern with high activity in the roots of transgenic tobacco plants [4]. Regions of the *mas* promoter from −213 to −103 or from −318 to −213 did not confer this root-preferential expression pattern when linked individually to a minimal (−116) *ocs* promoter (Fig. 7B). When both of these two *mas* promoter regions together were linked to a minimal *ocs* promoter, however, the pattern of root-preferential expression was restored (Fig. 7B). DNA sequence analysis identified an additional AS-1 tandem repeat motif at position −290 of the *mas* promoter, located near the *mas1′* TATA box. Although present in the −318 to −213 *mas* promoter fragment, the AS-1 element in this latter sequence context was not alone sufficient to direct the root-preferential pattern of expression (Fig. 7B).

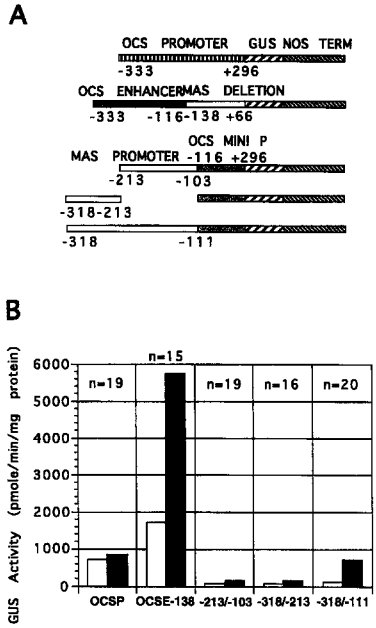

Fig. 7. Promoter swap analysis of *mas* promoter elements involved in root-preferential expression. A. Schematic diagram of constructions generated by combining elements from the octopine synthase (*ocs*) and *mas* promoter regions. B. Different *mas* fragments were added either 3′ downstream of an *ocs* enhancer or 5′ upstream of an *ocs* promoter truncated at position −116. GUS activity was assayed using total protein prepared from leaf □ and root ■ tissues. n indicates the number of individual transgenic plants assayed. The end points of each construction are given relative to the transcription start site of the *mas2′* gene. OCSP, full *ocs* promoter (−333 to +296); OCSE-138, *ocs* activator linked 5′ to the *mas* −138 promoter deletion; −213/−103, −318/−213, −318/−111, *mas* fragments linked to the minimal (−116 to +296) *ocs* promoter.

Sequences in the mas2′ *promoter region interact specifically with wound-inducible nuclear factors*

To investigate the interaction of DNA sequences within the *mas2′* promoter region with tobacco proteins, we end-labeled three DNA probes covering the region from −318 to +66 with ^{32}P and

incubated them with extracts from the leaves of either unwounded or wounded plants. Fig. 8 shows that we could detect DNA-protein complexes with all three probes. For probes 2 (−213 to −103; lanes 6–10) and 3 (−318 to −218, lanes 11–15), the complexes migrated with slightly lower mobility when proteins from wounded leaves were used for the binding assays. The DNA fragment from −103 to +66 (probe 1, lanes 1–5) interacted with tobacco proteins to form only a diffuse smear. When we treated the protein extracts with alkaline phophatase, however, the resulting complex was both more intense and distinct. In addition, the complex now migrated with a greater mobility. We distinguished two complexes using probe 2 (lanes 7 and 8). Treatment of whole cell extracts with alkaline phosphatase resulted in the formation of an additional complex (lanes 9 and 10). The region from −318 to −213 (probe 3) complexed with proteins to form two retarded bands, although these bands were often diffuse (lanes 12–15).

We next examined the specificity of each protein-DNA probe interaction. We observed distinct complexes with increasing polydI:dC concentrations up to 3 μg/reaction. A 50-fold molar excess of unlabeled probe, but not the pUC19 polylinker region, completely eliminated the formation of complexes. Boiling the protein extracts, or treatment of the protein extracts with proteinase K but not with RNase, also eliminated the formation of complexes (data not shown).

We next studied specific interactions of proteins with the *mas2′* promoter region by DNA footprinting analysis. We incubated DNA-protein complexes with DNase I, separated these complexes from the bulk of the free DNA probe by a preparative gel mobility shift assay, and subsequently performed DNA sequence analysis upon the retarded bands that were excised from the gel. As shown in Fig. 9A, DNase I footprinting of probe 1 (extending from −103 to +66) revealed three unique protected regions on the upper strand with protein extracts from wounded (lane 3) but not from unwounded (lane 2) tobacco leaves. We designated these regions, located downstream of the TATA box, a1, a2, and a3 (Fig. 9B).

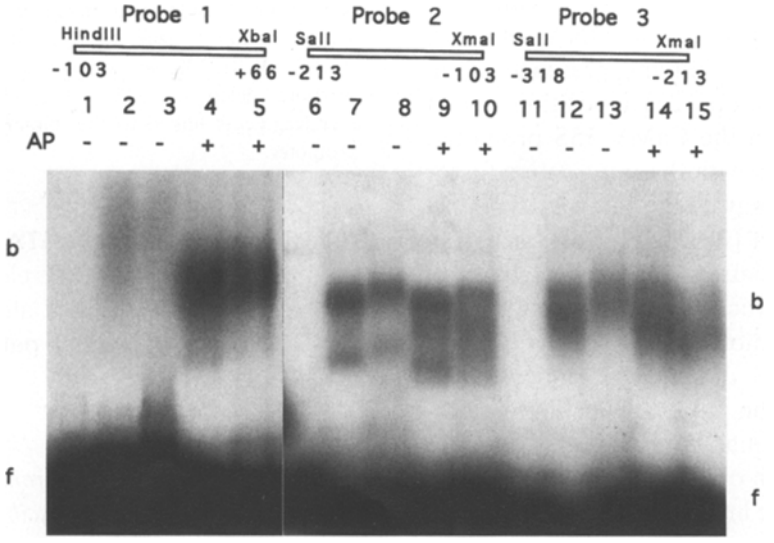

Fig. 8. Gel mobility shift assays of DNA-protein interactions. 1 ng of each end-labeled DNA probe was incubated with 60 μg of whole cell extracts isolated from either non-wounded leaves (lanes 2, 4, 7, 9, 12, and 14) or wounded leaves (lanes 3, 5, 8, 10, 13, and 15). 20 μl reactions were performed in EB buffer containing 7.5 μg polydI:dC. No protein extract was added in lanes 1, 6, and 11. Treatment of protein extracts with alkaline phosphatase (AP) was as described in Materials and methods. b, bound complex; f, free probe.

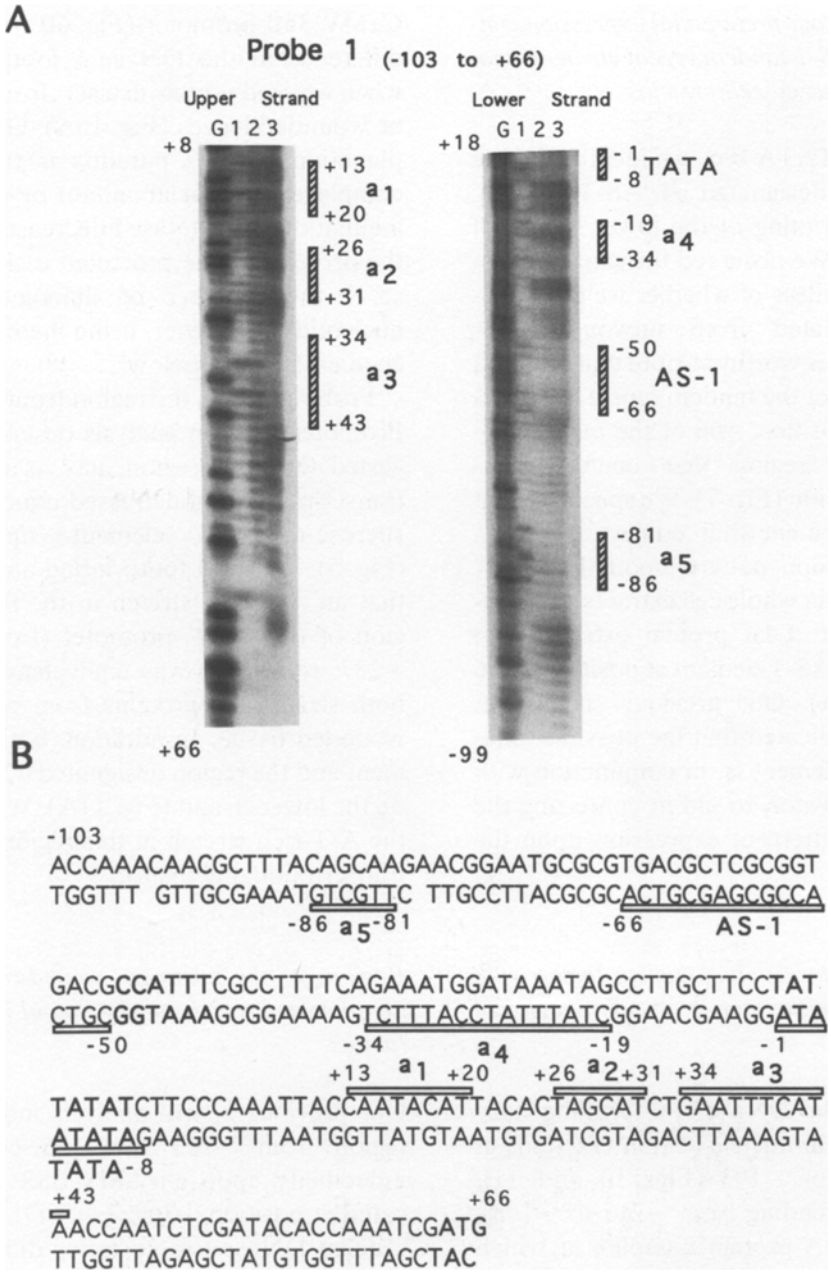

Fig. 9. DNase I footprinting analysis of probe 1 extending from −103 to +66. A. 1 ng DNA probe 1 end-labeled either in the upper strand (left) or lower strand (right) were incubated with 60 μg protein extracts isolated from non-wounded plants (lanes 2) or wounded plants (lanes 3) in the presence of 7.5 μg polydI:dC. Maxam-Gilbert G sequence ladder (lanes G) and free probe (lanes 1) are indicated. Subsequent DNase I digestion and preparative gel electrophoresis were performed as described in Materials and methods. The protected regions are indicated on the right by boxes and denoted by the corresponding labels. All numbers indicate the nucleotide position relative to the transcription initiation site. B. DNA sequence of probe 1 indicating the regions protected by protein extracts on either upper (bars above the sequence) or lower (bars below the sequence) strands. Regions a1, a2, and a3 are specifically protected by protein extracts only from wounded leaves. CATT box, TATA box, and ATG translation initiation codon are indicated in bold face.

Regions conferring root-preferential expression contain two identical AS-1 tandem repeat elements that are protected by tobacco cell proteins

In addition to the TATA box, we identified three protected regions, designated a4, AS-1, and a5, by DNase I footprinting of the lower strand of probe 1 (Fig. 9A). We observed the same pattern of protection regardless of whether we used protein extracts isolated from unwounded or wounded leaves. It is worthy to note that an AS-1 element consisting of the tandem repeat TGACG [27] is found at position −66 of the *mas2'* promoter within the region that confers root-preferential expression (Fig. 7). We speculate that it is this AS-1 element that confers the root-preferential expression pattern upon the *mas2'* promoter. Proteins in whole cell extracts also protected this element. Leaf protein extracts also protected a similar AS-1 element at position −296 (probe 3, Fig. 11A). Our previous *cis*-element analysis (Fig. 7) indicated that the possible function of this latter element is, in conjunction with a downstream activator, to aid in conferring the root-preferential pattern of expression upon the *mas* promoter.

Sequence-specific interaction of nuclear factors with an A/T-rich stretch in the sucrose-response region of the mas *promoter*

We performed similar DNase I footprinting studies to analyze protein-nucleic acid interactions in regions upstream of −103 (Figs. 10 and 11). Using probe 2 (extending from −213 to −103), we detected a DNA-protein complex in which nucleotides in the regions designated b1 and b2 were protected on both strands (Fig. 10). These regions may bind two factors (bF1 and bF2), or may be protected by a single protein. Protection additionally extended to cover regions designated b3 and b4. We determined in our previous *cis*-element analysis that this region functions as a general activator of transcription (Fig. 5). Although this region from −213 to −138 confers wound inducibility upon a heterologous truncated CaMV 35S promoter (Fig. 6B), we observed no difference in the DNase I footprinting pattern when we used protein extracts from non-wounded or wounded leaves (Fig. 10A). One possibile explanation for this paradox is that the binding complexes are not abundant or stable, and that incubation with DNase I decreases the stability of the proteins in the protected region. We did observe the presence of differentially protected nucleotides, however, using methylation interference assays (see below).

Probe 3 covers the region from −318 to −213. Promoter deletion analysis described above suggested that this region acts as an enhancer of transcription in nonstressed conditions and as a sucrose-response element upon wounding (Fig. 5). DNase I footprinting analysis indicated that an A/T-rich stretch in the far upstream region of the *mas2'* promoter (from ca. −272 to −237; region c1) was equivalently protected on both strands by proteins from wounded or unwounded tissue. In addition, both the AS-1 element and the region designated c2 were protected on the lower strand (Fig. 11A). We speculate that the A/T-rich stretch in this region functions as a transcriptional activator.

Regions of the upstream wound-responsive region are protected specifically by wound-inducible nuclear factors

Our previous *in vivo* analysis indicated that the region from −212 to −135 confers wound-inducibility upon a CaMV 35S promoter truncated at position −209 (Fig. 6D). However, subsequent DNase I footprinting did not reveal any difference in the protection pattern when using extracts from wounded or from unwounded leaves (Fig. 9). It is possible that the DNase I digestion conditions used could destroy any weakly formed binding complexes. We therefore analyzed in more detail the interaction of leaf proteins with two subfragments derived from this region (Fig. 12). We observed a major complex formed when either of these probes was incubated with leaf protein extracts. This complex

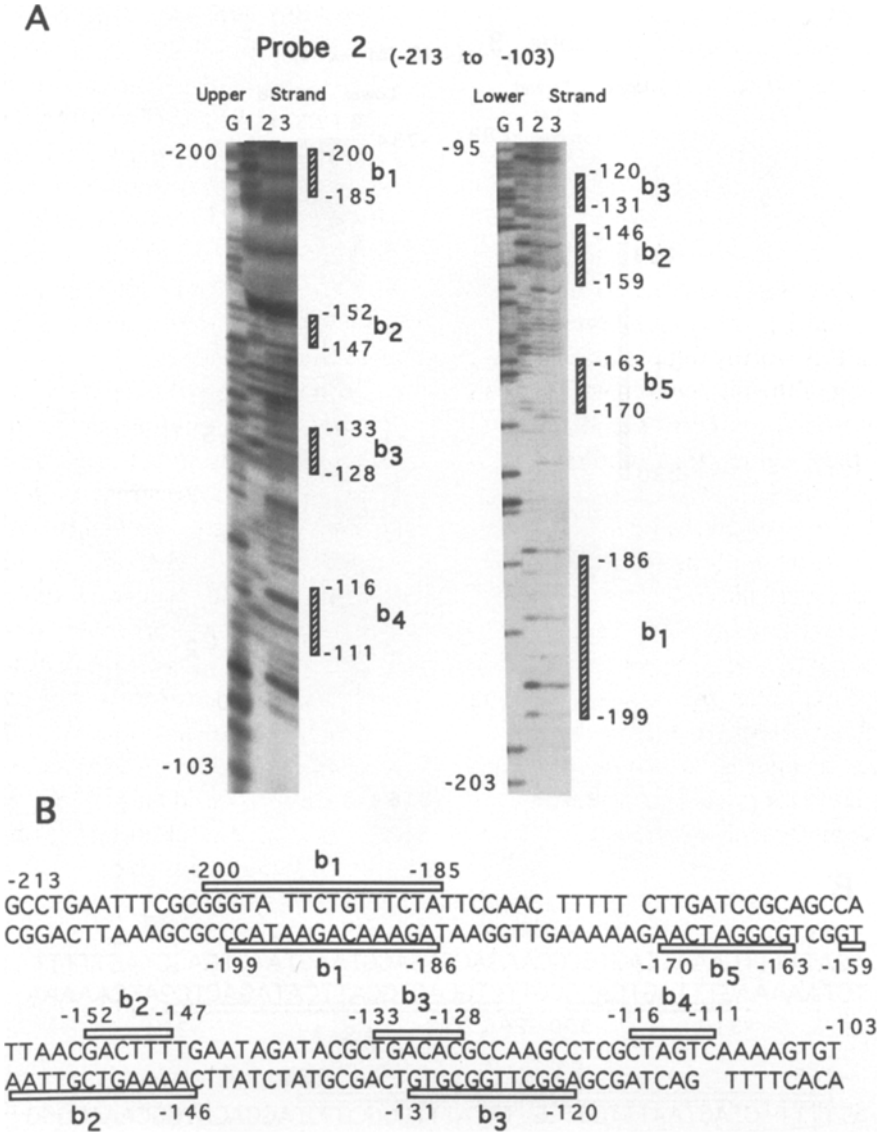

Fig. 10. DNase I footprinting analysis of probe 2 extending from −213 to −103. A. 1 ng DNA probe 2 end-labeled either in the upper strand (left) or lower strand (right) were incubated with 60 μg protein extracts isolated from unwounded leaves (lanes 2) or wounded leaves (lanes 3) in the presence of 7.5 μg polydI:dC. Maxam-Gilbert G sequence (lanes G) and free probe (lanes 1) are indicated. Subsequent DNase I digestion and preparative gel electrophoresis were performed as described in Materials and methods. The protected regions are indicated on the right by boxes and denoted by the corresponding labels. All numbers indicate the nucleotide position relative to the transcription initiation site. B. DNA sequence of probe 2 indicating regions protected by protein extracts on either upper (bars above the sequence) or lower (bars below the sequence) strands.

appeared when we used extracts from either wounded or non-wounded leaves. In addition to the complexes formed commonly by both extracts, we observed two unique DNA-protein complexes when the probe from −212 to −135 (probe 5) was incubated with extracts from wounded but not from non-wounded leaves (Fig. 12, probe 5, lane 3; complexes specific to extracts from wounded leaves are denoted by arrows). Incubation of probe 5 in the presence of either a 10- or 50-fold molar excess of unlabeled probe 5 (lanes 4 and 5, respectively) or a 50-fold

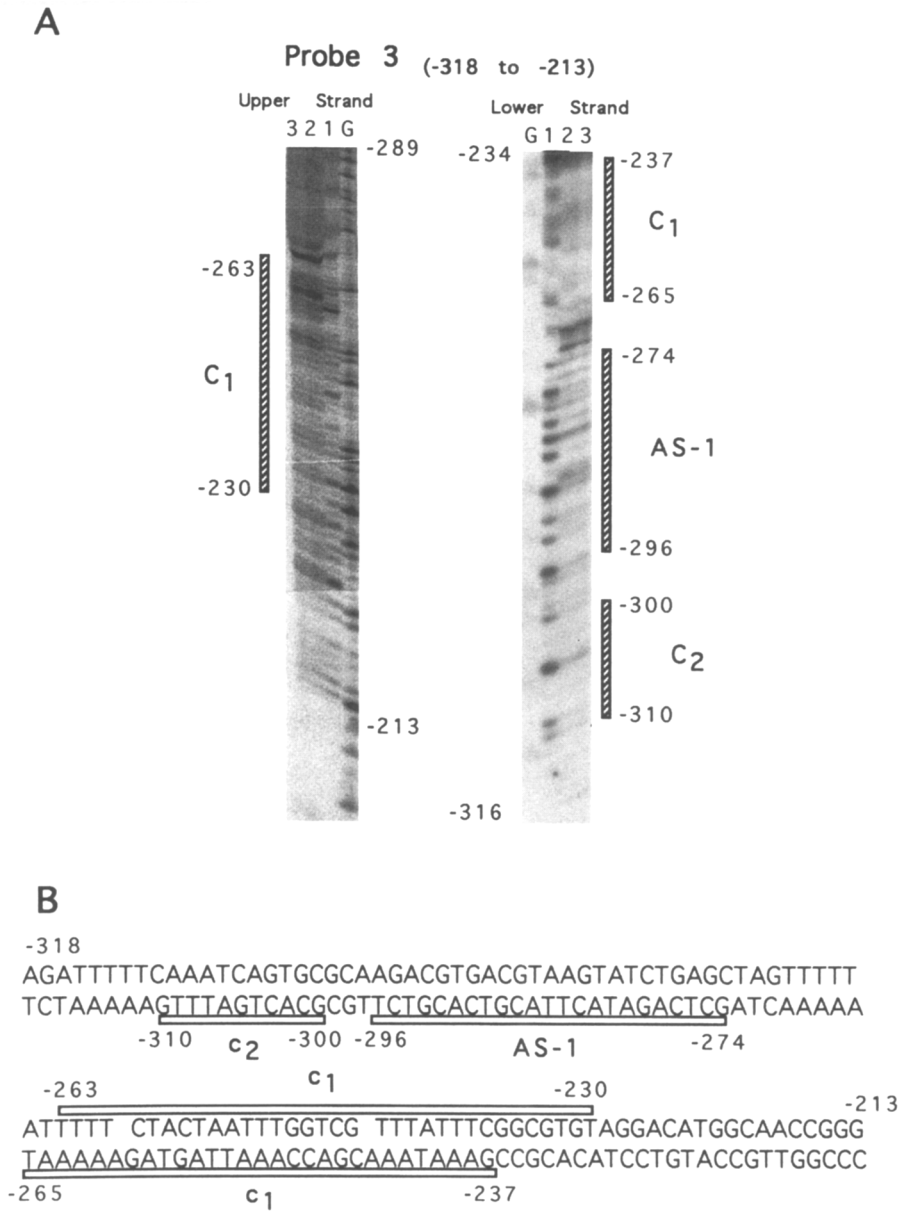

Fig. 11. DNase I footprinting analysis of probe 3 extending from −318 to −213. A. 1 ng DNA probe 3 end-labeled either in the upper strand (left) or lower strand (right) were incubated with 60 μg protein extracts isolated from unwounded leaves (lanes 2) or wounded leaves (lanes 3) in the presence of 7.5 μg polydI:dC. Maxam-Gilbert G sequence (lanes G) and free probe (lanes 1) are indicated. Subsequent DNase I digestion and preparative gel electrophoresis were performed as described in Materials and methods. The protected regions are indicated on the right by boxes and denoted by the corresponding labels. All numbers indicate the nucleotide position relative to the transcription initiation site. B. DNA sequence of probe 3 indicating regions protected by protein extracts on either upper (bars above the sequence) or lower (bars below the sequence) strands.

molar excess of unlabeled probe 2 (covering the entire region from −213 to −103; lane 7), but not a 50-fold excess of probe 4 resulted in the inhibition of complex formation.

We investigated the interactions of specific nucleotides within this wound-responsive region with proteins from wounded and non-wounded leaves using methylation interference analysis. We

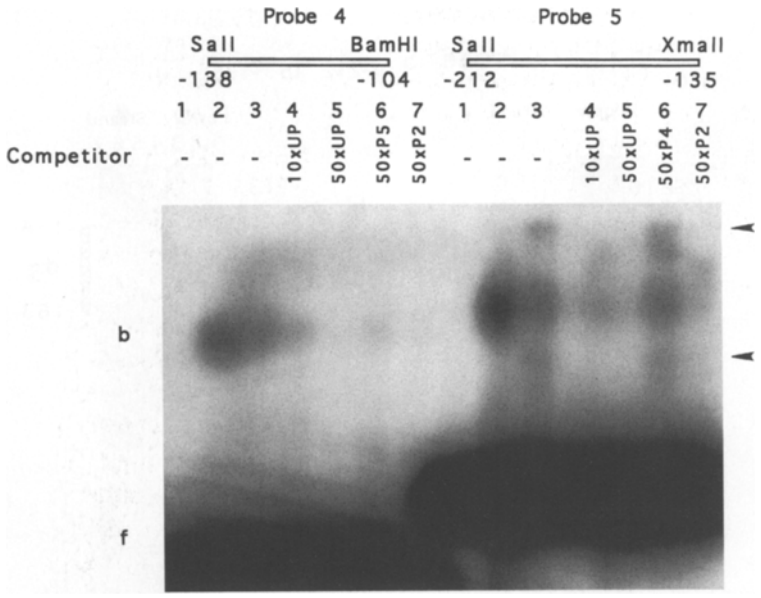

Fig. 12. Gel mobility shift analysis of the upstream wound-responsive region of the *mas2'* promoter. 0.5 ng end-labeled DNA probes 4 and 5 were incubated with 30 μg protein extracts from non-wounded leaves (lanes 2) or wounded leaves (lanes 3 to 7) in the presence of 3.75 μg polydI:dC. No protein extracts were added in lanes 1. For competition assays, molar excesses of unlabeled probes as indicated were included in the binding reactions. UP, unlabeled homologous probe; P2, unlabeled probe 2; P4, unlabeled probe 4; P5, unlabeled probe 5; b, bound complex; f, free probe.

performed this study on the unique lower band of fast-mobility formed by the interaction of probe 5 with extracts from wounded leaves, and with the major 'common' band formed by the interaction of probe 5 with extracts from both wounded or unwounded leaves (Fig. 13). We did not investigate those interactions of probe 5 with extracts from wounded leaves that resulted in the formation of the upper band in Fig. 12 (lane 3) because this band often comigrated with a non-specific band. We identified in the major 'common' band regions in which methylated G residues interfered with DNA binding by extracts from both wounded and non-wounded leaves (d1 in the upper strand and d4 in the lower strand; Fig. 13A, lanes 3 and 5). In contrast, we identified a short region, termed d3, on both strands (including part of the d4 region) and a region, termed d2, in the upper strand that also contained methylated G residues that abolished DNA-protein interactions in the unique lower band of fast mobility. Apparently, wound-inducible nuclear factors complex with only a small proportion of probe 5 fragments to result in this unique binding complex. The binding of these wound-induced factors to probe 5 displaces some of the non-wound-induced factors. We hypothesize that the displacement of these factors in this DNA region, along with the binding of other wound-inducible factors to regions downstream of the TATA box (Fig. 9A), strongly activate gene expression from the *mas2'* promoter.

Discussion

The activity of the *mas2'* promoter is highly regulated in plants. We and others have shown that this promoter shows different levels of activity in various types of plant tissues and cells [31, 36, 39, 44, 46]. In addition, the activity of the *mas2'* promoter is induced by wounding [39, 44] and is affected by phytohormones [28, 31]. Thus, this promoter may serve as a model to study gene regulation in plants. From this and other analyses, it is clear that the combinatorial properties of

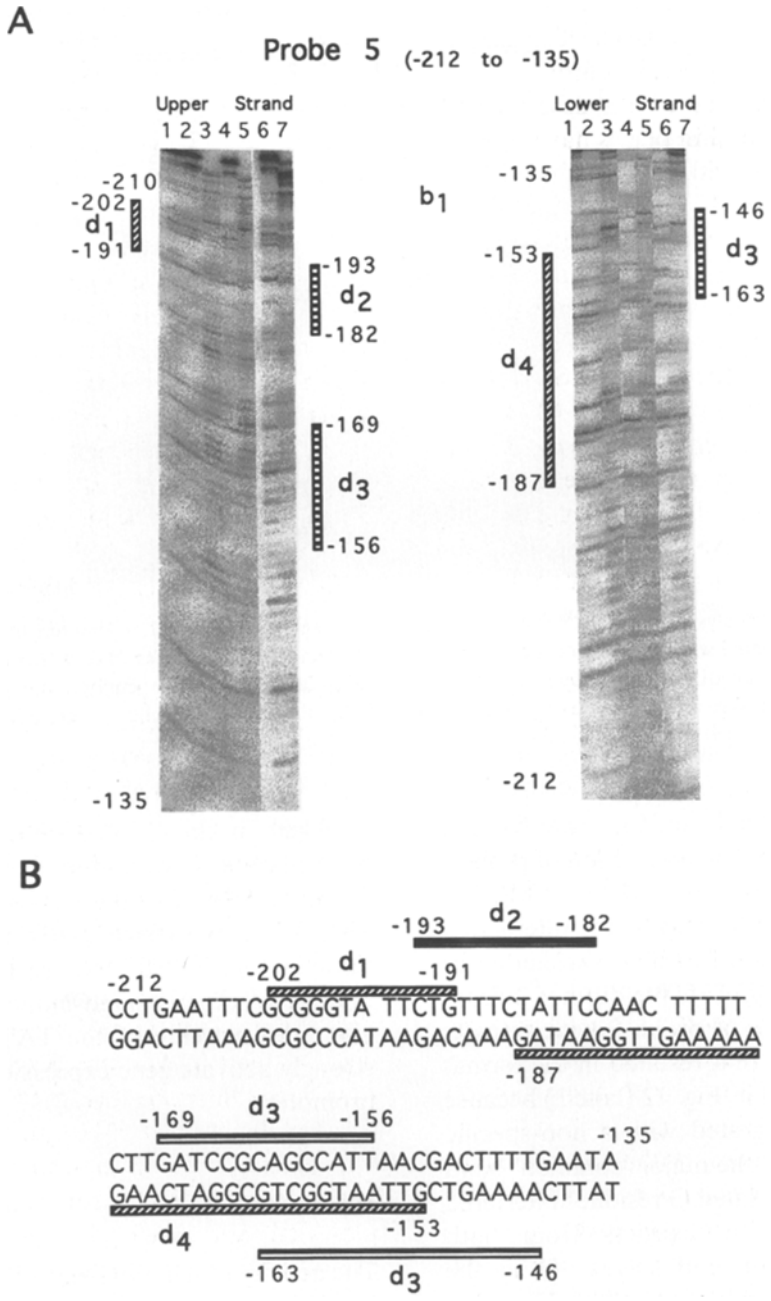

Fig. 13. Methylation interference analysis of probe 5 (−212 to −135). A. 2.5 ng methylated DNA probe 5 end-labeled on either the upper strand (left) or the lower strand (right) were incubated with 150 μg protein extracts from either unwounded leaves (lanes 2 and 3) or wounded leaves (lanes 4 to 7) in the presence of 19 μg polydI:dC. No protein extracts were added in lanes 1. The free probes (lanes 1, 2, 4, and 6), 'middle retarded bands' common to both unwounded and wounded extracts (lanes 3 and 5), and the wound-induced unique 'lower retarded' bands (lanes 7) were excised and eluted following a preparative gel mobility shift assay as described in Materials and methods. The protected regions (bars on the left) are derived from the common middle bands and the bars on the right from the wound-induced lower bands. (B) DNA sequence of probe 5 indicating the protected regions d1 (upper strand) and d4 (lower strand) derived from the common middle bands and d2 (upper strand) and d3 (both strands) from the wound-induced lower bands.

several *cis*-regulatory elements are required to define the overall regulatory program of the *mas2'* promoter [6, 7, 9, 14, 31]. Such combinations of promoter 'subdomains' have been described for numerous genes [11] and in plants have perhaps best been documented with the CaMV 35S promoter. The CaMV 35S promoter contains a TATA-proximal subdomain that directs expression in the roots of transgenic plants, whereas a more distal subdomain directs expression in leaves [4]. Interestingly, an element within the proximal subdomain of the CaMV 35S promoter that directs expression in the root, AS-1, also occurs within a TATA-proximal region of the *mas2'* promoter that is responsible for root-preferential expression of this promoter. We speculate that it is this AS-1 element within the *mas2'* promoter that is responsible for the root-preferential expression. We note, however, that there is another AS-1 element in a more distal location of the *mas2'* promoter (position −296), and that this element may also contribute to the pattern of root-preferential expression. Both of these elements are protected by proteins from tobacco cells (Figs. 9 and 11).

Our *in vivo* analyses indicated that two regions of the *mas2'* promoter, from −213 to −138 and from −103 to +66, are responsible for wound inducibility. In addition, a region from −318 to −213 conferred sucrose enhancement of wound inducibility upon the promoter. DNase I and methylation interference studies indicated that tobacco leaf proteins protected oligonucleotide stretches from each of these regions. In addition, specific DNA sequences within the wound-responsive promoter regions were protected differently by proteins from wounded and unwounded leaves. To our knowledge, this is the first report of such differential interactions between a wound-responsive DNA element and protein factors.

We mapped a sucrose-response element to an A/T-rich sequence in the far upstream region of the *mas2'* promoter. Kim *et al.* [25] showed that promoter activity of a potato proteinase inhibitor II promoter in transgenic tobacco was strongly induced by the addition of sucrose to medium in which leaf disks were incubated or to the site of wounding on leaves. However, sucrose did not increase promoter activity when added to unwounded leaves. A/T-rich sequences were also reported responsible for the sucrose enhancement of promoter activity for genes encoding patatin-1 [5] and sporanin-A1 [20].

It is interesting to note that the promoter-proximal wound-responsive *cis* elements of the *mas2'* promoter are located in a position downstream of the TATA box. Such downstream regulatory elements have, however, been identified in other genes. For example, the maize shrunken feedback control element spans the transcription start site and binds to a homeodomain in the Zmhoxla protein at three sites flanking the TATA box [3].

Our data demonstrated that dephosphorylation of protein extracts can alter the affinity and mobility of protein-DNA complexes when probes 1 and 3 were used (Fig. 8). Apparently, these factors can bind to *mas2'* promoter elements *in vitro* in both their phosphorylated and dephosphorylated forms, although their affinity for the *mas2'* promoter was lower when they were phosphorylated. Changes in mobility of DNA-protein complexes by dephosphorylation have also been observed by others [40, 43]. The possible functional significance of protein dephosphorylation in DNA-protein interactions remains unknown. Treatment of extracts with alkaline phosphatase also resulted in the formation of an additional complex with probe 2 (Fig. 8). Phosphorylation-induced binding of DNA to proteins, and the subsequent increase in transcriptional efficacy, have been reported for some nuclear factors such as CREB and RcaA [42, 49]. In contrast, it is possible that some tobacco transcription factors become active and bind to DNA only in their dephosphorylated forms.

Based upon our *in vivo* and *in vitro* analyses, we present in Fig. 14 a model for the involvement of *mas2'* *cis* elements and wound-inducible *trans*-acting factors in wound-inducible gene expression. Under nonstressed conditions, an A/T-rich stretch in the far upstream region acts as a generalized transcriptional activator of the *mas2'*

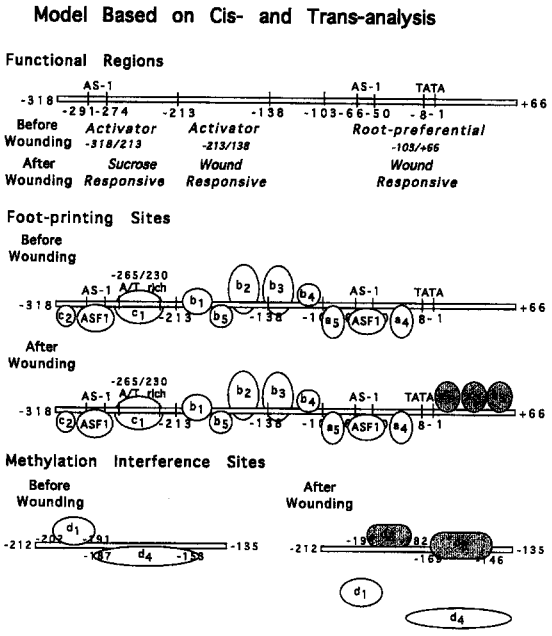

Fig. 14. Model of DNA-protein interactions within the *mas2'* promoter region based upon *cis* and *trans* analysis. Functional regions were defined by promoter deletion and swap analyses. The regions involved in interaction with nuclear factors were defined by either DNase I footprinting or methylation interference analysis. For example, C1 indicates a nuclear factor that binds to the c1 region as revealed by either DNase I footprinting or methylation interference analysis. TATA, TATA box; ATG, translation initiation codon. All numbers indicate the nucleotide position relative to the transcription initiation site.

promoter and binds to a nuclear factor(s) c. Full promoter strength is observed when c1 binds to this A/T-rich sequence and two other factors, b1 and b2, bind to a downstream activation sequence. How the A/T-rich sequence acts either as a general activator or a sucrose-response element after wounding remains unknown. AS-1 elements in the promoter-distal and promoter-proximal regions determine the pattern of root-preferential expression when interacting with ASF-1 in root tissues. Upon wounding of leaf tissue, several factors (a1, a2, and a3) are induced and bind to regions downstream of the TATA box, as revealed by DNase I footprinting analysis. Methylation interference studies revealed the displacement of the factors d1 and d4 by the wound-inducible factors d2 and d3 in the region from −212 to −103. *In vivo* analyses also indicated the involvement of this promoter region in wound-inducible gene expression.

Intensive studies have been performed to define a protein factor (OCSBF-1) that binds to the palindromic element of the *ocs* promoter of the Ti-plasmid [41]. Bouchez *et al.* [6] have implied that this factor can complex with two *ocs*-like elements in the *mas2'* promoter (Fig. 2). Our *in vivo* and *in vitro* analyses suggest that these two elements may be involved in the root-preferential expression of the *mas2'* promoter, and therefore function in a manner more similar to the AS-1 element of the CaMV 35S promoter. In our present studies, we have identified a number of additional elements involved in regulating *mas2'* promoter activity in unstressed and stressed conditions. In particular, the promoter-distal A/T-rich region and the downstream b1 and b2 elements function as general enhancers of full promoter activity, whereas another set of DNA motifs function as wound-responsive elements. DNA footprinting and methylation interference studies revealed the existence of cognate factors that bind to these elements. Further studies will be focused on identifying these enhancer-binding and wound-induced factors by biochemical as well as genetic approaches.

Acknowledgments

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