

Identification of G-Box Sequence as an Essential Element for Methyl Jasmonate Response of Potato Proteinase Inhibitor II Promoter¹

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ABSTRACT

The potato proteinase inhibitor II promoter was studied to identify *cis*-acting regulatory sequences involved in methyl jasmonate (MJ) response using transgenic tobacco plants carrying various lengths of the promoter fused to a chloramphenicol acetyltransferase reporter gene. An internal fragment between –625 and –520 was sufficient to confer a response to MJ, wounding, or sucrose when it was placed upstream of the *nos* promoter –101, which contains the CAAT-TATA region. Deletion of the proteinase inhibitor II promoter sequence upstream of –611 did not affect the MJ response, but a further deletion to –573 eliminated the response. The 3′-deletion study showed that the DNA sequence downstream from –520 is dispensable. However, 3′-deletion mutant –574 did not respond to the MJ treatment. These results indicated that an element essential for the MJ response is located at the –574/–573 region where the G-box sequence (CACGTGG) is located. The G-box sequence was not required for the sucrose enhancer effect, suggesting that the MJ response mechanism is different from that of sucrose.

JA² and MJ occur commonly in the plant kingdom (4, 32, 34). These chemicals are synthesized in plants from linolenic acid by an oxidative pathway (4, 28, 33). It has been proposed that these compounds function as phytohormones because JA and its methyl ester alter the growth of plant tissues (4) and induce senescence (32). Recently, JA and MJ were shown to influence expression of several plant genes. For example, the application of MJ selectively induced the accumulation of vegetative storage proteins in soybean (12, 30) and PIs in tomato (11).

The PIs also accumulate in many plant species in response to various environmental or developmental factors (24). For example, the level of the proteinase inhibitor transcripts is increased drastically by either insect attack or mechanical wounding (13). In addition to the wound stress response,

several other factors also influence the expression of PI genes. Pectic polysaccharides derived from the cell wall have been shown to be inducers of the PIs (25). The phytohormone ABA has been suggested as a wound-affiliated hormone because ABA-deficient mutants do not accumulate the PIs upon wounding (23). It also was recently reported that the addition of sucrose to wounded leaves further enhanced expression of the potato PI-II gene (16, 19). It is unknown whether these various factors induce the PI-II promoter by independent regulatory sequences or whether some of the regulatory elements are shared by different environmental factors. In this study, we have shown that the induction mechanism of MJ is independent from that of wounding or sucrose.

MATERIALS AND METHODS

Bacterial Strains and Plant Materials

Escherichia coli strain MC1000 (5) was used as a host for the routine cloning experiments. *Agrobacterium tumefaciens* strain LBA4404 (15), carrying avirulent helper tumor-inducing plasmid pAL4404, was used for the transformation of tobacco (*Nicotiana tabacum* L. cv Xanthi or cv Petit Havana SR1) plants grown aseptically on MS agar medium supplemented with 3% sucrose (21).

Constructions and Plant Transformation

The generation of 5′- and 3′-deletion mutants was reported previously (19). Internal fragments were generated by digesting the 3′-deletion mutants at the *Ssp*I site that cuts between –625 and –626. The 3′-deletion mutants and internal fragments were then connected to the truncated nopaline synthase (*nos*) promoter –101, which carries the CAAT box and the TATA box region (1). Both strands of the 23-base pair nucleotide sequence between –522 and –500 of the PI-II promoter were synthesized and inserted at the *Xho*I site located at the junction between the PI-II promoter 3′-deletion mutant –574 and the *nos* –101 fragment to generate a chimeric promoter, 3′-574–23-mer.

These mutant promoter fragments were linked to the CAT gene (*cat*) and subcloned into a binary tumor-inducing vector (2). The plasmids were transferred into *A. tumefaciens* by the direct DNA transformation method (2). Tobacco leaf slices were transformed stably by the cocultivation method as de-

¹ Supported in part by a grant from U.S. Department of Agriculture, CSRS 89–37262–4892, and National Science Foundation grant DCG-8919154.

² Abbreviations: JA, jasmonic acid; MJ, methyl jasmonate; PI, proteinase inhibitor; MS, Murashige-Skoog; CAT, chloramphenicol acetyltransferase.

scribed earlier (2). Transgenic R1 plants were selected from selfed seeds on MS agar medium supplemented with 3% sucrose and 50 $\mu\text{g}/\text{mL}$ kanamycin.

Induction Assay

Leaves were wounded by cutting into pieces, which were floated on MS liquid medium for 20 h at room temperature. Sucrose effect was studied by addition of 3% sucrose during the incubation period. MJ (Bedoukian Research Inc., Danbury, CT) effect was studied by either the isopiestic movement of the volatile ester from an adjacent cotton-tipped dowel placed in the same chamber as the plants (11) or by addition of MJ into the MS liquid medium at the final concentration of 0.1 mM. The CAT assay was conducted with 2 to 100 μg of total soluble protein at 37°C for 20 to 60 min by the TLC method using [^{14}C]chloramphenicol as described previously (2).

RESULTS

MJ Effects on the PI-II Promoter

It was previously reported that transgenic tobacco plants carrying about 1 kilobase of the potato PI-II promoter fragment that was fused to the *cat* reporter gene did not express the chimeric gene. The gene was, however, activated by mechanical wounding or by incubating the leaf pieces in water (3, 18). Results in Figure 1 demonstrate that expression of the transgene was induced by MJ as well as by wounding. We observed that the PI-II promoter activity was inducible either by treating an intact plant with MJ gas or by incubating leaf pieces in water containing MJ. Such effects were not observed in control transgenic tobacco plants carrying the cauliflower mosaic virus 35S promoter fusion to the *cat* reporter gene (data not shown).

Analysis of 5'-Deletion Mutants

It was previously reported that the wound response of the PI-II promoter was further enhanced by addition of sucrose to wounded tissues and that the sucrose-responsive and wound-responsive sequences are located at separate distinctive regions. Because both sucrose and MJ induce the PI-II promoter by a mechanism that is different from that of wound response, we investigated whether there is any connection between the MJ effect and sucrose effect.

It was shown earlier that the 5'-deletion mutant lacking the DNA sequence upstream of -573 of the PI-II promoter was unable to respond to the mechanical wounding but was able to respond to sucrose (19). If MJ is involved in the sucrose effect, transgenic plants carrying this mutant should also respond to MJ. Figure 1 shows that the mutant -573 was unable to respond to MJ, although it responded to sucrose. Longer promoter fragments (-892 or -611) responded to wound, MJ, or sucrose, whereas the 5'-deletion mutant -453 did not respond to any of these stimuli. These results indicate that the DNA sequence between -611 and -573 is necessary for the MJ response and that this upstream sequence is not required for the sucrose effect.

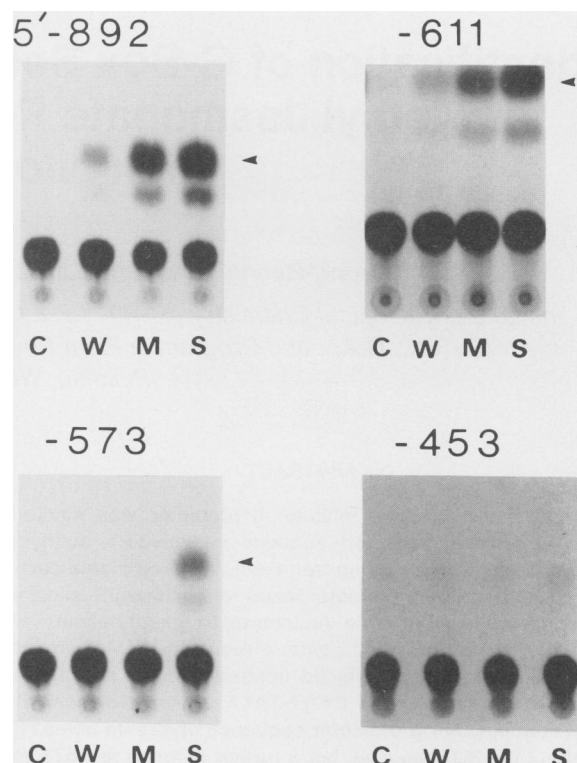


Figure 1. Analysis of the 5'-deletion mutants. Leaf pieces of transgenic R1 seedlings carrying either the parental molecule (5'-892) or three 5'-deletion mutants (-611, -573, and -453) were assayed for the CAT activity before wounding (lane C) or after floating wounded pieces on MS medium (lane W), MS medium with 0.1 mM MJ (lane M), or MS medium with 3% sucrose (lane S). The results shown are a representative sample from at least four independently transformed transgenic plants tested in this study. Assay conditions: 4 μg protein and 20-min incubation period for the promoter -892; 40 μg and 20 min for -611 and -573; 100 μg and 60 min for -453. Arrowheads indicate the position of 3-acetylchloramphenicol.

Analysis of 3'-Deletion Mutants

To further locate the MJ-responsive element on the PI-II promoter, transgenic plants carrying various 3'-deletion mutants were studied for MJ responsiveness. We demonstrated earlier that deletion of the sequence downstream of -500 did not significantly alter the promoter strength or the responsiveness to sucrose or wound induction (19). Deletion of an additional 20 nucleotides between -500 and -520, however, reduced the promoter activity by a factor of 10 without altering the sucrose or wound responsiveness. Further deletion to -574 abolished the sucrose-enhancing effect (Fig. 2), which was also observed previously (19). Examination of these transgenic plants showed that treatment with MJ gas induced the 3'-deletion mutants -500 and -520 to the level equivalent to that of the wound-induced level. However, plants carrying the mutant -574 or -624 were no longer inducible by the MJ treatment (Figs. 2 and 3).

Similar results were observed with the 3'-deletion mutants lacking the far upstream region that was deleted by digestion of the mutants with *Ssp*I site at -625 (Fig. 4, A-C). These

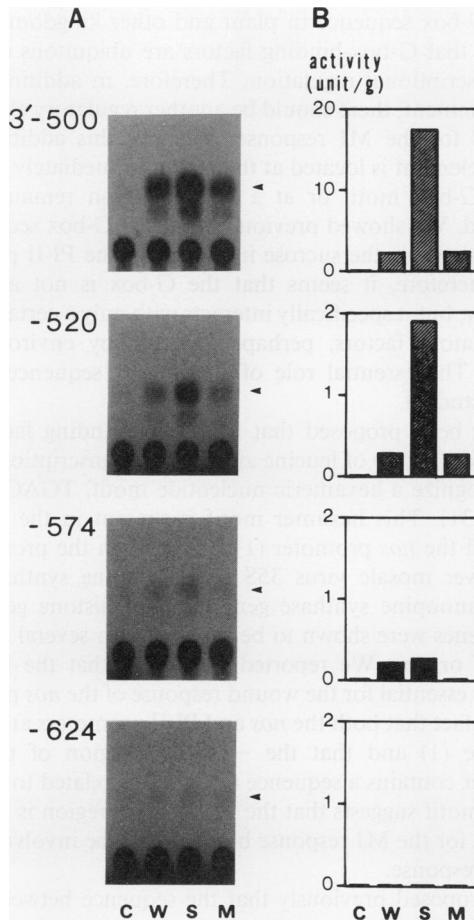


Figure 2. Analysis of the 3'-deletion mutants. Leaves of transgenic R1 plants were sampled before wounding (lane C), after floating leaf pieces on sucrose-free MS medium (lane W) or on MS medium with 3% sucrose (lane S), or treated for 20 h with MJ gas (lane M). A, Analysis of CAT activity from a representative sample. The assay conditions were 40 μ g of protein for a 60-min incubation period. B, Average values from five to nine experiments with at least three independently transformed plants are shown as a specific CAT activity (units per gram). One unit of CAT converts 1 ng of [14 C]chloramphenicol to acetylchloramphenicol per min. Arrowheads indicate 3-acetylchloramphenicol.

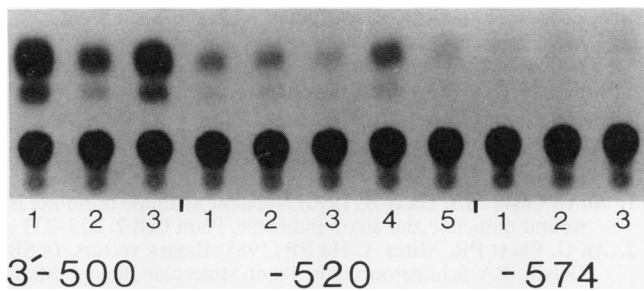


Figure 3. MJ response of the 3'-deletion mutants. Transgenic R1 plants that were grown on MS agar medium with 3% sucrose in a Magenta box were treated with MJ gas for 20 h. CAT assay conditions were described in Figure 2. The numbers indicate independently transformed plants.

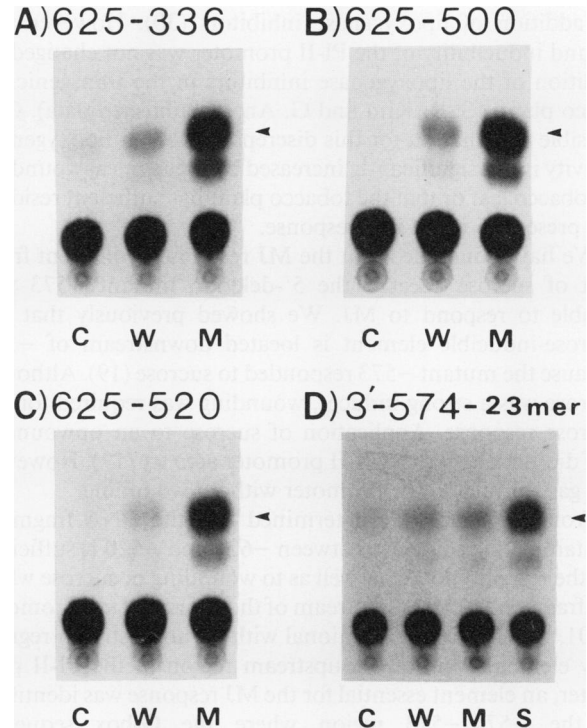


Figure 4. Analysis of the internal fragments. The samples were treated as described in Figure 1. Assay conditions: Samples A-C, 40 μ g protein and 20 min; sample D, 50 μ g and 60 min. C, Control; W, floated on sucrose-free liquid MS medium; M, 0.1 mM MJ treatment; S, floated on MS medium containing 3% sucrose.

results and those of the 5'-deletion mutants indicate that the DNA sequence between -625 and -520 is sufficient for the MJ response and that an element at the -574/573 region is essential for the response.

The 3'-deletion analysis indicated that the sequence between -520 and -500 was not necessary for the MJ response or sucrose response, but deletion of this region resulted in about 10-fold reduction of the responses. To investigate the role of this region, a synthetic oligonucleotide containing the 23-nucleotide sequence between -522 and -500 was inserted into the 3'-deletion mutant -574, which alone was able to respond weakly to wounding but not to MJ or sucrose. The result in Figure 4D shows that addition of the 23-mer at this region restored the sucrose response, but the MJ response was not recovered.

DISCUSSION

We studied the MJ response of the potato PI-II promoter using the transgenic tobacco system. Because the promoter is inducible by several factors such as mechanical wounding, MJ, and sucrose, we investigated whether there were any relationships among the responses invoked by these factors. Mechanical wounding may stimulate accumulation of MJ or sucrose at the wound site, thereby further amplifying the response. It was reported previously that three vegetative storage protein genes of soybean were also inducible by wounding or MJ and that the wound response was blocked

by addition of lipoxygenase inhibitors (30). However, the wound inducibility of the PI-II promoter was not changed by addition of the lipoxygenase inhibitors in the transgenic tobacco plants (S.-R. Kim and G. An, unpublished data). One possible explanation for this discrepancy is that lipoxygenase activity is not significantly increased by mechanical wounding in tobacco leaf or that the tobacco plant has sufficient residual MJ present to result in a response.

We have concluded that the MJ response is different from that of sucrose because the 5'-deletion mutant -573 was unable to respond to MJ. We showed previously that the sucrose-inducible element is located downstream of -573 because the mutant -573 responded to sucrose (19). Although sucrose was a strong inducer, wounding was required for the sucrose response. Application of sucrose to an unwounded leaf did not change the PI-II promoter activity (19). However, MJ gas stimulated the promoter without wounding.

From this study, we determined that the DNA fragment containing the sequence between -625 and -520 is sufficient for the response to MJ as well as to wounding or sucrose when the fragment is placed upstream of the truncated *nos* promoter -101, which was not functional without an upstream regulatory element. Within the upstream region of the PI-II promoter, an element essential for the MJ response was identified at the -574/-573 region where the G-box sequence (CACGTGG) is located (Fig. 5). The G-box sequence has been found to be important for activity of several promoters regulating genes involved in photosynthesis (9, 10, 26), chalcone synthase (27), ABA responsiveness (14, 22), and *Arabidopsis* alcohol dehydrogenase (8). The common occurrence

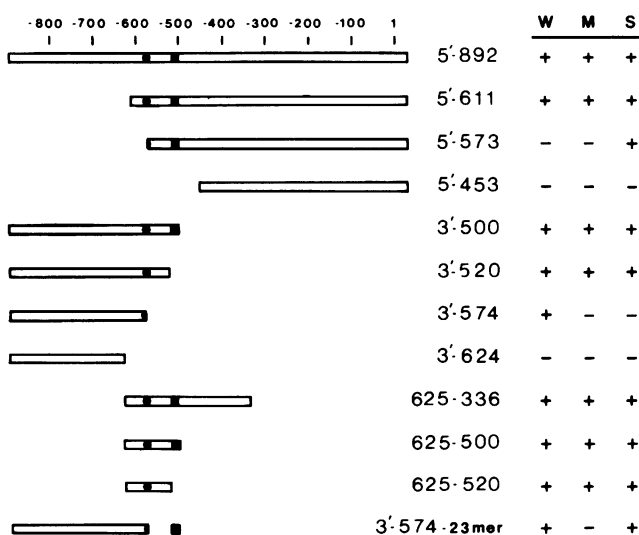


Figure 5. Summary diagram of the PI-II promoter mutants. The DNA fragment containing the 892-base pair untranscribed region and 32-base pair untranslated sequence of the potato PI-II promoter is shown as open bars at the left. ●, G-box element; ■, 23-nucleotide region between -522 and -500. The numbers above the open bars indicate the distance in the number of bases from the transcription initiation site at the position 1. The promoter deletion mutant constructs are identified at the right of each bar. The positive response and negative response by mechanical wounding (W), MJ (M), or sucrose (S) are indicated by + and -, respectively.

of the G-box sequence in plant and other kingdoms (6, 10) suggests that G-box-binding factors are ubiquitous elements for transcriptional regulation. Therefore, in addition to the G-box element, there should be another regulatory element(s) required for the MJ response. Whether this additional sequence element is located at the region immediately adjacent to the G-box motif or at a distant region remains to be answered. We showed previously that the G-box sequence is not required for the sucrose induction of the PI-II promoter (19). Therefore, it seems that the G-box is not a general enhancer, but it specifically interacts with only a certain group of regulatory factors, perhaps triggered by environmental stimuli. The essential role of this G-box sequence will be further studied.

It has been proposed that the G-box-binding factors are related to a group of leucine zipper-type transcription factors that recognize a hexameric nucleotide motif, TGACGT (17, 22, 29, 31). This hexamer motif is present in the essential region of the *nos* promoter (1) as well as in the promoter of cauliflower mosaic virus 35S (20), octopine synthase gene (29), mannopine synthase gene (7), and histone gene (31). These genes were shown to be functional in several different types of organs. We reported previously that the hexamer region is essential for the wound response of the *nos* promoter (1). The fact that both the *nos* and PI-II promoters are wound inducible (1) and that the -574/573 region of the PI-II promoter contains a sequence (TCACGT) related to the hexameric motif suggests that the PI-II G-box region is not only essential for the MJ response but also may be involved in the wound response.

We proposed previously that the sequence between -574 and -520 contains a sucrose-response element because the 3'-deletion mutant -520 is inducible by sucrose, and further deletion to -574 removed the sucrose response (19). However, the results shown in Figure 4D indicate that addition of the 23-nucleotide sequence (between -522 and -500) to the 3'-deletion mutant -574 restored the sucrose response as well as the wound response but not the MJ response. This experiment further supports that the G-box region is essential for the MJ response but not for wound response or sucrose response. The use of heterologous promoters and other approaches for studying plant promoters would facilitate further analysis of these complex promoter elements.

ACKNOWLEDGMENTS

We thank Dr. C.A. Ryan and E.E. Farmer for helpful suggestions and Jonathan Todd for technical assistance.

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