Plant Molecular Biology **26**: 403–414, 1994.

Jasmonate-inducible expression of a potato cathepsin D inhibitor-GUS gene fusion in tobacco cells

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Received 7 April 1994; accepted in revised form 29 June 1994

Key words: cathepsin D inhibitor, methyl jasmonate, promoter analysis, transformed tobacco cells, tuberonic acid

Abstract

A potato gene encoding cathepsin D inhibitor (CDI) is expressed constitutively in tubers and flower buds and it is inducible in leaves upon wounding of the tissue or by treatment with methyl jasmonate (MJA). A fusion gene (CDI:GUS) in which the 2.4 kb long promoter of the CDI gene was translationaly fused with the coding sequence for β-glucuronidase (GUS) showed MJA-inducible expression in transformed tobacco cells in suspension. The maximum level of induction by MJA was obtained in the absence of auxin and repression of MJA-inducible expression of the fusion gene by auxin was released by aphidicolin, the results suggesting that MJA-inducible expression is repressed during active cell division. JA and MJA showed similar activities in inducing the expression of the fusion gene, while other JA-related compounds such as cucurbic acid, tuberonic acid and dihydrojasmonic acid neither induced expression of the fusion gene nor inhibited the MJA-inducible expression of the fusion gene. Methyl dihydrojasmonate specifically stimulated the MJA-inducible expression of the fusion gene. The MJA-inducible expression of the fusion gene was observed even with a 100 bp long promoter of the CDI gene albeit with significantly decreased level of expression compared to the 2.4 kb long promoter. The 100 bp long CDI promoter did not contain a G-box or hexamer motif that had been implicated in the MJA-responsive expression of several other plant genes. Further mutagenesis of the 100 bp long promoter by deletion or oligonucleotide insertion suggested that although a sequence between −100 and −82 is required for the MJA-responsive expression, the presence of this sequence alone does not confer the MJA-responsive expression.

Abbreviations: BA, N^6-benzyladenine; CA, cucurbic acid; CaMV, cauliflower mosaic virus; CDI, cathepsin D inhibitor; DMSO, dimethyl sulfoxide; GUS, β-glucuronidase; HJA, dihydrojasmonic acid; JA, jasmonic acid; MCA, methyl cucurbate; MJA, methyl jasmonate; MHJA, methyl dihydrojasmonate; MTA, methyl tuberonate; PI-II, proteinase inhibitor II; TA, tuberonic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Introduction

Jasmonic acid (JA, 3-oxo-2-[(z)-2-pentenyl]-1-cyclopentaneacetic acid), methyl jasmonate (MJA), and other compounds that are structurally related to JA occur naturally in plants and they are called jasmonates [28]. Exogenous application of jasmonate to plants causes diverse physiological effects such as inhibition of growth, promotion of leaf senescence, promotion of stomata closure and induction of potato tuber formation [19, 28]. JA and MJA have also been shown to induce the synthesis of specific proteins, which include storage proteins, stress- or wound-inducible enzymes and proteinase inhibitors [3, 5, 9, 32]. Since a rapid increase in the endogenous levels of JA and MJA occurs after wounding of leaves [4] and after elicitor treatment of cells [8, 24], JA and MJA probably act as natural mediators of these stress signals in the signal transduction of defense-related genes.

Similar to the wound-inducible gene for proteinase inhibitor II (PI-II) of potato [5, 7, 30], expression of the potato genes encoding cathepsin D inhibitor (CDI) was induced in leaves upon treatment with MJA [10, 12, 13]. In addition to MJA, various other jasmonates induced both genes for PI-II and CDI with different strength, and activities of various jasmonates in the induction of expression of genes encoding CDI and PI-II were similar, suggesting that two genes are regulated by similar mechanisms [12]. In order to understand the molecular mechanisms involved in the signal-induced gene expression, use of the suspension-cultured plant cells has various advantages over using whole plants or explants.

In the present study, we examined the inducibility of expression of a fusion gene, CDI:GUS, composed of the 2.4 kb long promoter of the potato gene for CDI with the coding sequence of β-glucuronidase (GUS [15]) in transformed and suspension-cultured tobacco cells. In contrast to experiments using whole plants or leaf explants of potato, induction of expression of the fusion gene by various JA-related compounds was highly specific for JA and MJA in tobacco cells. Auxin in the culture medium strongly repressed the MJA-inducible expression of the fusion gene, and experiments using aphidicolin suggested that induction by JA is repressed in cells undergoing active cell division. The MJA-inducible expression of the fusion gene was observed even with a 100 bp long promoter of the CDI gene albeit with significantly decreased level of expression compared to the 2.4 kb long promoter. It is suggested that synergistic action of at least two *cis*-regulatory elements in the 100 bp long promoter is required for the MJA-inducible expression.

Materials and methods

Chemicals

JA, MJA and other JA-related compounds used in this paper were described previously [12, 20]. They were racemic and mixture of *cis* and *trans* with respect to the plane of the cyclopentane ring. MJA and its related compounds were dissolved in 1% Tween 20 or dimethyl sulfoxide (DMSO) and diluted with water to a final concentration of 50 μM unless otherwise indicated.

Construction of fusion genes in binary plasmid

The 2.4 kb long 5′-upstream sequence of a potato gene for CDI (gCDI-A1 [13]) was subcloned into *Hin*d III-*Bam* HI sites of pBI101 (Clonetech) to give a plasmid pBI-CGN harboring the fusion gene CDI:GUS^{CGN} with terminator from the nopaline synthase gene (*nos*). Substitution of the *nos*-terminator fragment in CDI:GUS^{CGN} with the 3′-downstream sequence of the CDI gene gave a plasmid construct pBI-CGC harboring the fusion gene CDI:GUS^{CGC}. As a control, the plasmid pIG121-Hm harboring the cauliflower mosaic virus (CaMV) 35S promoter: intron-GUS fusion gene [26] in a double selection vector for kanamycin and hygromycin (S. Ohta *et al.*, manuscript in preparation; referred to in [2]) was used. Serial deletion of the 5′ terminus of the CDI promoter were obtained by exonuclease III/exonuclease VII. The deletion end-points were

determined by sequencing. The binary Ti plasmids were transferred to *Agrobacterium tumefaciens* LBA4404 by triparental mating.

Culture and transformation of tobacco cell line BY-2

Suspension-cultured cell-line BY-2 derived from tobacco (*Nicotiana tabacum* cv. Bright Yellow [11]) was grown and maintained by subculturing every 7 days in the BY-2 medium containing 2,4-D (0.2 mg/l) and transformed with *Agrobacterium* as described previously [23]. Transformants were selected on solid medium containing 200 mg/l kanamycin and 500 mg/l carbenicillin, and either the mixture of several hundred transformants or the individual transformants selected randomly were brought into suspension.

Treatment of transformed tobacco cells with methyl jasmonate and assays of GUS activity

Seven-day-old cells of transformed tobacco cells were collected by centrifugation and diluted with the fresh medium containing 2,4-D (0.2 mg/l) or benzylaminopurine (BA; 0.5 mg/l). JA and its related compounds were added to the culture medium at a final concentration of 50 μM and cultured for 24 h unless otherwise indicated. Cells were collected by centrifugation and resuspended in GUS extraction buffer [15], and they were broken by sonication at 50 W for 20 s with a sonicator (Ohtake Works, Tokyo). The cell extracts obtained by centrifugation were assayed for GUS activity by the fluorimetric methods described by Jefferson *et al.* [15]. Proteins in the extracts were determined by the method of Lowry *et al.* [21].

Treatment of cells with aphidicolin

After a 7-day subculture, the suspension of cells at stationary phase was diluted with culture medium which contained 2,4-D and 5 μg/ml of aphidicolin (Wako Chemical Industry, Osaka, Japan), and cultured for 24 h. Cells were washed and resuspended with culture medium which either contained or did not contain aphidicolin. After 12 h, when cells in the culture medium without aphidicolin resumed cell division [1], MJA was added to give the final concentration of 50 μM and cultured for an additional 24 h.

Results

MJA-inducible expression of the CDI:GUS fusion gene in suspension cultured cells of tobacco

A potato gene encoding CDI, gCDI-A1, isolated previously [13] does not contain any introns. A DNA fragment that covers the 2.4 kb long promoter fragment of the gCDI-A1 gene was fused to the coding region of the *uidA* gene in such a way that translation of the GUS protein is initiated by the initiator codon for the CDI gene. The fusion gene thus constructed (CDI:GUS^{CGN}; Fig. 1) contained a terminator from the nopaline synthase gene (*nos*). The *nos*-terminator fragment in CDI:GUS^{CGN} was replaced with the 3′-downstream sequence of the CDI gene (+ 688 to + 1057, where + 1 indicating the transcription start site [13]) to yield a CDI:GUS^{CGC} fusion gusion construct (Fig. 1). These two CDI:GUS fusion genes were used to transform tobacco cells. In a preliminary experiment, we assayed GUS activities in more than twenty independent kanamycin-resistant cells, for each construct, that had been cultured in the presence or absence of 50 μM MJA. About 15% of the kanamycin-resistant cells did not express GUS activity at all (data not shown). The CDI:GUS fusion genes in all of the other transformants examined responded to MJA although the levels of GUS activities varied significantly among individual transformants (data not shown). Three independent transformants for each construct with different levels of GUS expression were brought into suspension in the medium containing 2,4-D and maintained by subculturing every 7 days for further analyses.

MJA dissolved in DMSO was added to the suspension culture of cells transformed with the

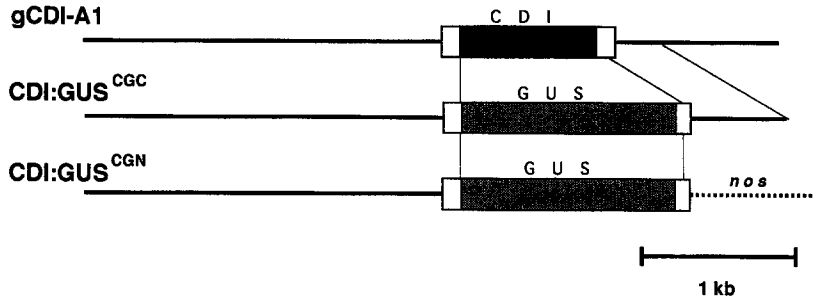

Fig. 1. Structures of the CDI:GUS fusion genes. The coding sequence of CDI and GUS were indicated by black box and dotted box, respectively. The open boxes indicate the 5′- and 3′-untranslated regions of the gene. The terminator fragment of the *nos* gene was indicated by a dotted line.

CDI:GUS^{CGC} fusion gene to give the final concentrations of 50 μM for MJA and 0.1% for DMSO. At various time after the addition of MJA, extracts were prepared from cells and GUS activities were assayed. Three transformants showed similar time course of increase in GUS activity and representative results with one of them are shown in Fig. 2A and 2B. Treatment of cells with MJA resulted in increase of the specific activity of GUS after about 12 h of lag-time (Fig. 2A). The specific activity of GUS in cell extracts slightly decreased when cells were treated with 0.1% DMSO alone. Under these conditions, MJA did not affect growth of cells and increase in the amount of proteins (data not shown). Similar results were obtained with cells transformed with the CDI:GUS^{CGN} fusion gene (data not shown). In control cells harboring the Intron-GUS fusion gene under the control of the CaMV 35S promoter [26], no such increase in the level of GUS activity by MJA was observed (Fig. 2C). These results indicated that the promoter of the CDI gene confers MJA-inducible expression of the GUS reporter gene in tobacco cells and the 3′-terminator fragment did not affect the MJA-inducible expression.

MJA-inducible expression of the CDI:GUS fusion gene in tobacco cells is repressed by auxin

When cells transformed with CDI:GUS^{CGC} cultured in the medium containing 2,4-D were diluted 10-fold with the medium containing benzyladenine (BA), the level of MJA-inducible expression of the fusion gene was significantly higher than those obtained with cells cultured in the 2,4-D medium (Fig. 2B). After treatment with 50 μM MJA in the BA medium, the specific activity of GUS in transformants with high levels of GUS expression like that shown in Fig. 2B, was significantly higher than that in cells transformed with CaMV 35S:Intron-GUS fusion gene (Fig. 2C). The CaMV 35S:intron-GUS fusion gene has been shown to give higher level of expression of GUS activity than the CaMV 35S:GUS fusion gene in tobacco [26].

Transformed cells were treated with various concentrations of MJA in the 2,4-D medium for 24 h and the levels of GUS activities were determined. As shown in Fig. 3A, stimulation of expression of the fusion gene by MJA was observed with concentrations higher than 5 μM and it did not show saturation for up to 500 μM. Similar results were obtained in another independent experiment with the same transformant as well as in an experiment with another transformant. Induction of expression of the CDI gene by MJA in excised leaves of potato also occurs at concentrations higher than 5 μM [12].

When effects of various concentrations of MJA on expression of the CDI:GUS fusion gene were examined in tobacco cells cultured under various growth conditions, the level of expression of the fusion gene was significantly higher in cells cultured in BA medium compared to cells cultured in 2,4-D medium at all of the concentrations of MJA (Fig. 3B). High-level of MJA-inducible

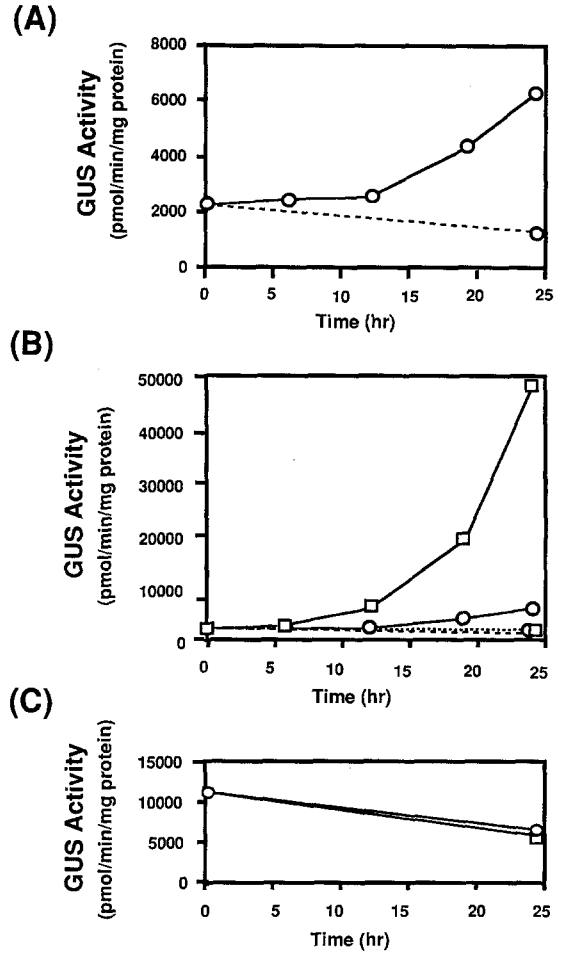

Fig. 2. Time course of increase in specific activities of GUS in transformed tobacco cells after the addition of MJA to the culture. Tobacco cells transformed with the CDI:GUS^{CGC} fusion gene were maintained by subculturing every 7 days in the 2,4-D medium. Experiments were carried out with three independent transformants, and the representative results with one of them is shown. A. After a 7-day subculture, cells were diluted 10-fold with the 2,4-D medium in the presence (—○) or absence (- - -○- - -) of 50 μM MJA. After various time periods indicated in the figure, cell extracts were prepared and specific activities of GUS were determined. B. Cells were diluted with the 2,4-D medium [○; same as in A] or with the BA medium (□) in the presence (———) or absence (- - - - -) of MJA. C. The 7-day-old cells of tobacco cells transformed with the CaMV 35S:intron-GUS fusion gene were diluted 10-fold with the 2,4-D medium (○) or with the BA medium (□) in the presence of 50 μM MJA. Two independent transformants showed similar results.

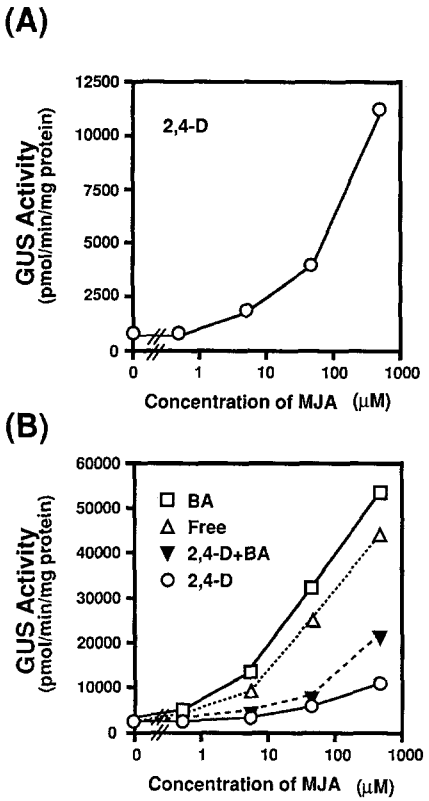

Fig. 3. Effects of various concentrations of MJA on expression of the CDI:GUS fusion gene in transformed tobacco cells. The 7-day-old culture of tobacco cells transformed with the CDI:GUS^{CGC} fusion gene was diluted 10-fold with the 2,4-D, BA, 2,4-D plus BA, or hormone-free medium in the presence of various concentrations of MJA. After incubation for 24 h, specific activities of GUS in cell extracts were determined. The data shown in A are the same as those shown in B and presented on a different scale.

expression of the fusion gene was also observed with cells transferred to the hormone-free medium. Furthermore, addition of 2,4-D to the medium containing BA reduced the MJA-inducible expression of the fusion gene to the level that was obtained with cells cultured in the 2,4-D medium. These results suggest that 2,4-D represses the MJA-inducible expression of the CDI:GUS fusion gene in tobacco cells.

To examine whether the effects of 2,4-D in the medium are due to active division of cells, we treated cells cultured in 2,4-D medium with aphidicolin, an inhibitor of DNA synthesis, to arrest the cell division. Treatment of tobacco BY-2 cells with 5 μg/ml aphidicolin inhibits DNA synthesis and decreases the mitotic index of cells

to zero [1]. When cells that had been pretreated with 5 μg/ml aphidicolin for 36 h were treated with 50 μM MJA in the presence of aphidicolin for 24 h, a high level of MJA-induced expression of the fusion gene, i.e. specific activity of GUS around 40000 pmol/min per mg protein, was obtained (Fig. 4). This value was similar to those obtained with cells cultured in the BA or hormone-free medium (see Fig. 3B). By contrast, when cells that had been arrested for cell division by treatment with aphidicolin for 24 h were resumed for cell division by transferring them to aphidicolin-free medium for 12 h [1] before treatment with 50 μM MJA for 24 h, the level of GUS activity obtained was significantly lower than that obtained in cells cultured and treated with MJA in the presence of aphidicolin (Fig. 4).

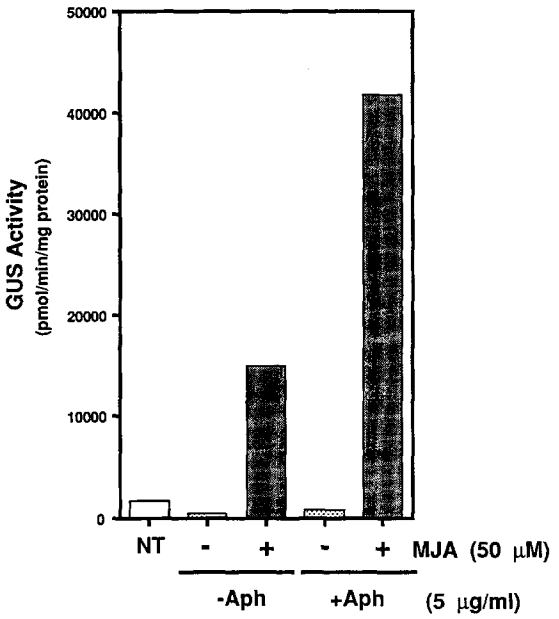

Fig. 4. Effect of treatment of cells with aphidicolin on MJA-inducible expression of the CDI:GUS fusion gene in transformed tobacco cells. The culture of tobacco cells transformed with the CDI:GUS^{CGC} fusion gene in the 2,4-D medium was treated with aphidicolin for 24 h to arrest the cell division. Cells were collected by centrifugation, and half of the cells was resuspended in the 2,4-D medium containing aphidicolin (+Aph), while the other half was resuspended in the fresh medium without aphidicolin (−Aph) to resume the cell division [1]. After 12 h of incubation, cells were treated with 50 μM MJA in Tween 20 (+) or with Tween 20 alone (−) for 24 h. NT, non-treated cells. Similar results were obtained with additional two independent experiments.

Induction of expression of the CDI:GUS fusion gene by various MJA-related compounds

Various compounds structurally related to MJA (Fig. 5A) were examined for their activities to induce the expression of the CDI:GUS fusion gene. These compounds were previously examined for their activities to induce tuber formation in potato shoots [20] and to induce expression of genes for CDI and PI-II in potato leaves [12]. Transformed tobacco cells in the BA medium were treated with 50 μM solutions of various JA-related compounds for 24 h and analyzed for the level of GUS activity. Each compound was examined in more than three separate experiments which contained treatment of cells with JA and Tween 20 as controls. Figure 5B shows representative results with one of the transformant and similar results were obtained with another independent transformant. The highest levels of GUS activities were obtained with JA and MJA (Fig. 5B). None of the other JA-related compounds examined showed inducing activity. JA-related compounds which showed weak activities to induce the genes for CDI and PI-II in leaves of potato such as cucurbic acid (CA), tuberonic acid (TA) and dihydrojasmonic acid (HJA) [12] had almost no activity in inducing the CDI:GUS fusion gene in tobacco cells (Fig. 5B).

In order to examine whether JA-related compound would interfere with the binding of JA to a potential receptor for JA in tobacco cells, we preincubated transformed cells with various JA-related compounds at 50 μM for 30 min, and then MJA was added to the culture to give a final concentration of 5 μM. After incubation for 24 h, we assayed for GUS activities in cell extracts. As shown in Fig. 5C, none of the JA-related compounds blocked the MJA-inducible expression of the fusion gene. Interestingly, MHJA significantly and specifically enhanced the level of MJA-induced expression of the fusion gene. Similar results were obtained with two independent transformants.

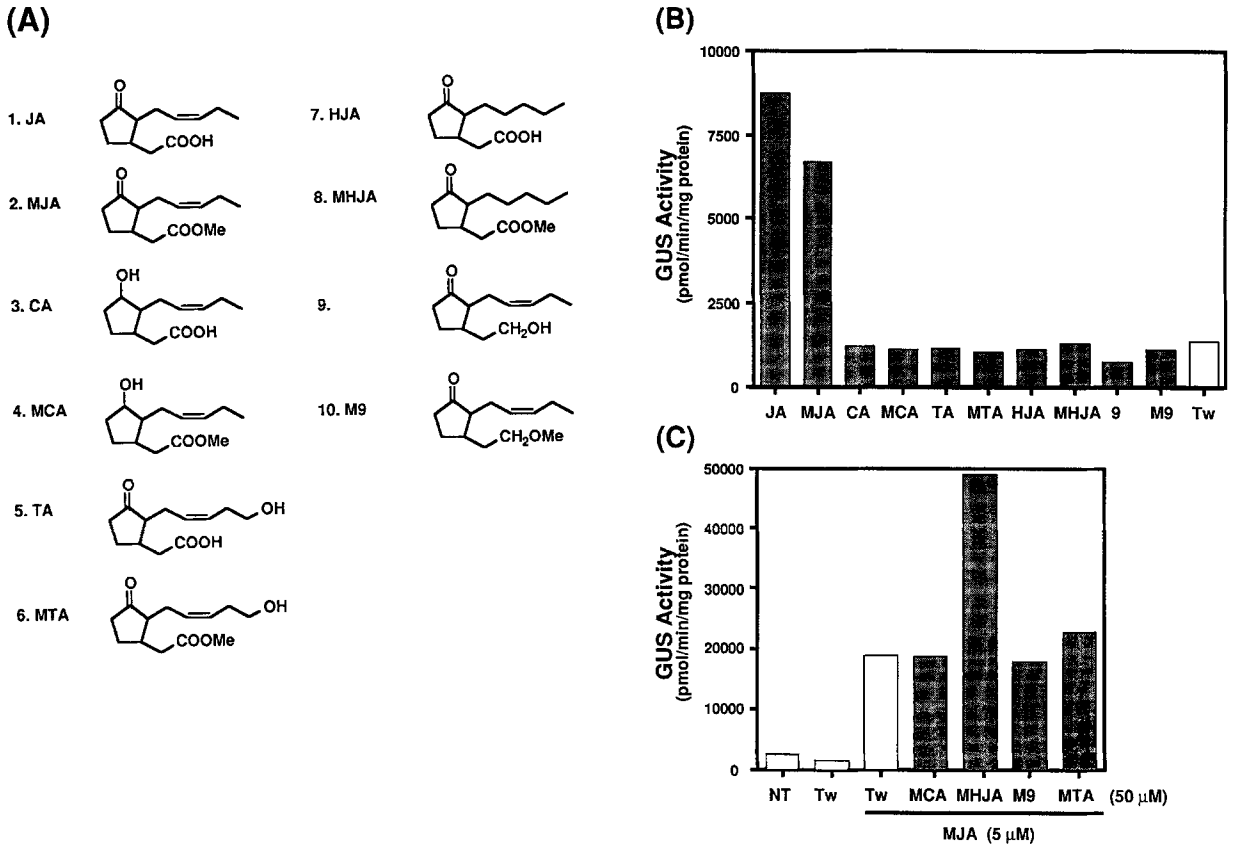

Fig. 5. Effects of various JA-related compounds on expression of the CDI:GUS fusion gene in transformed tobacco cells. A. Structure of various JA-related compounds. 1, JA, jasmonic acid; 2, MJA, methyl jasmonic acid; 3, CA, cucurbic acid; 4, MCA, methyl cucurbic acid; 5, TA, tuberonic acid; 6, MTA, methyl tuberonic acid; 7, HJA, dihydrojasmonic acid; 8, MHJA, methyl dihydrojasmonic acid; 9, compound 9, i.e. 3-(2-hydroxyethyl)-2-[(z)-2-pentenyl]-1-cyclopentanone; 10, M9, methyl ester of compound 9. B. The 7-day-old culture of tobacco cells transformed with the CDI:GUS^{CGC} fusion gene was diluted 10-fold with the BA medium in the presence of various compounds at the final concentrations of 50 μM. After incubation for 24 h, GUS activities were assayed. TW, Tween 20 alone. C. Transformed cells transferred to the BA medium were incubated with methyl esters of various JA-related compounds at 50 μM for 30 min before the addition of MJA to a final concentration of 5 μM. After incubation for 24 h, GUS activities were assayed. NT, untreated cells; Tw, cells treated with Tween 20 alone.

Analysis of MJA-responsible elements in the CDI promoter

The 5′-upstream region of the CDI promoter in the CDI:GUS^{CGC} fusion gene was deleted from its upstream side to various lengths and analyzed for their MJA-inducible expression in transformed tobacco cells cultured in the BA medium. In these experiments, mixtures of several hundred kanamycin-resistant transformants rather than individual transformants for each construct were brought into suspension in order to eliminate the variabilities in the level of expression of the fusion gene among individual transformants.

As shown in Fig. 6A, the level of MJA-induced expression of the fusion gene decreased with decreasing length of the 5′-upstream region of the CDI promoter. The level of expression increased when the deletion extended from −230 to −210, suggesting that there exists some negative element in this region. However, the basal level of expression in the absence of MJA did not change in these 5′-deletion mutants. Deletion of the 5′-upstream region to position −100 still showed

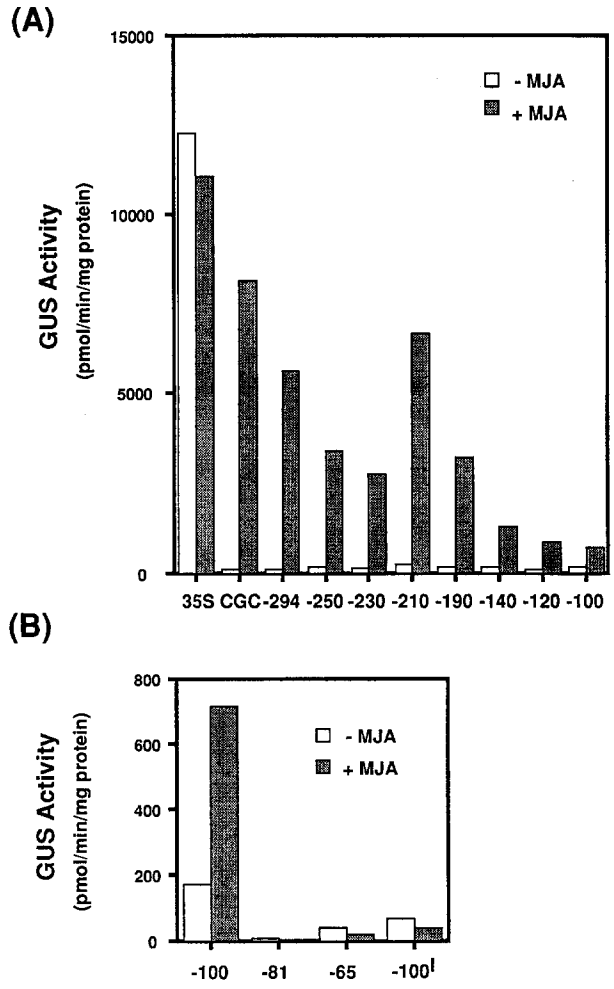

Fig. 6. Effects of mutation in the CDI promoter on MJA-inducible expression in transformed tobacco cells. Tobacco cells transformed with various GUS fusion constructs were assayed for their MJA-responsible expression by incubating in the BA medium with or without 50 μM MJA for 24 h. 35S, CaMV35S:Intron-GUS; CGC, CDI:GUS^{CGC}. Numbers in abscissa indicate position of the deletion endpoints of the 5′-deletion mutants of the CDI promoter with respect to the transcription start site.

MJA responsiveness, although the level of GUS activity after the induction by MJA was reduced significantly compared to the construct with the 2.4 kb long promoter (Fig. 6, A and B).

In contrast to the 5′-deletion mutants described above, the 5′-deletion mutants −81 and −65 showed only the basal levels of GUS activities which could not be enhanced by treatment of cells with MJA (Fig. 6B), the results suggesting that the 19 bp sequence between −100 and −82 plays an important role in the MJA-responsive expression of the 100 bp CDI promoter. This result is consistent with the fact that the 5′-upstream regions of the CDI gene and another structurally related potato gene, gCDI-B1, are highly homologous for up to the position −72 from the transcription start site even though the latter gene is not inducible by MJA [13].

During the course of our studies, reports on the analysis of MJA-responsible domains in the 5′-upstream regions of genes coding for potato PI-II [17], *nos* [18] and soybean vegetative storage protein (Vsp-B; [22]) appeared. The sequence required for the MJA-responsible expression in these promoters contained a G-box motif (CACGTG [17, 22]) or a hexamer motif (TGACGT [18]), both of which containing a core sequence of ACGT. The 5′-upstream region of the CDI promoter for up to −100 from the site of transcription start does not contain the ACGT core motif (Fig. 7A). However, a sequence between −68 and −58 is similar to the sequences encompassing the MJA-responsive regions of other plant genes with the G box or hexamer motif (Fig. 7B). Since the 5′-deletion mutant −81 that contains this homologous region did not show MJA-responsive expression (Fig. 6B) and this sequence is highly conserved in the corresponding region of the gCDI-B1 promoter [13], it is highly unlikely that this homologous region alone is sufficient to confer MJA-responsive expression.

A mutant promoter −100^{I} in which a 17 bp oligonucleotide was inserted between position −66 and −65, or between position −61 and −62, of the 5′-deletion mutant −100 (Fig. 7A) did not show MJA-responsive expression (Fig. 6B) indicating that the sequence between −100 and −61 is not sufficient to confer the MJA responsiveness to the basal level of expression observed with the 5′-deletion mutant −65 (Fig. 6B). It is possible that the homologous sequence between −58 and −68, in addition to the sequence between −100 and −82, is also required for the MJA-responsive expression. Alternatively, changes in the distance of the upstream element from the TATA box due

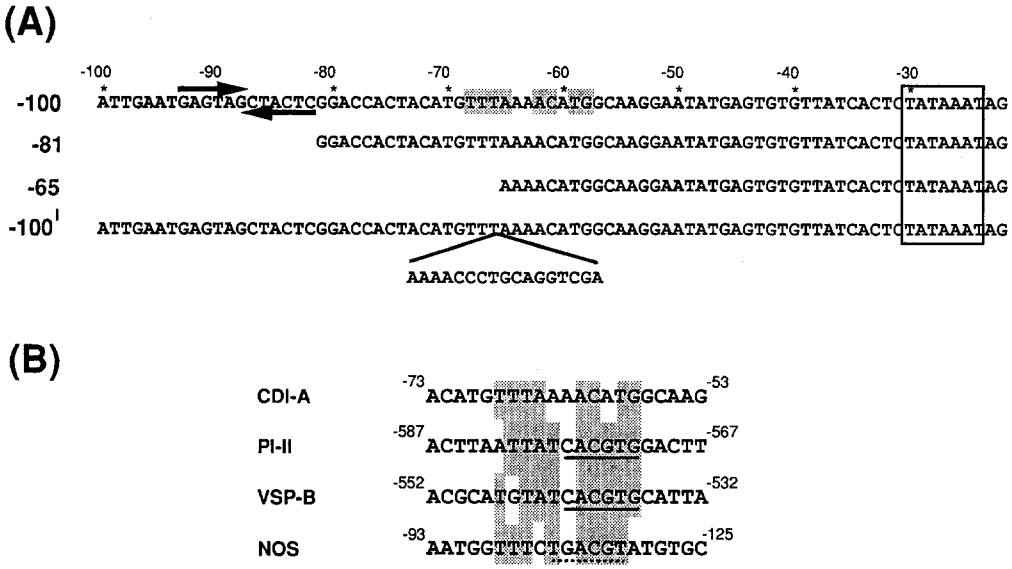

Fig. 7. Nucleotide sequence of the promoter mutants of the CDI promoter. Nucleotide sequence of the 5′-deletion mutants −100, −81 and −65 are shown. The mutant -100^{I} contains an insertion of 17 bp oligonucleotide between −66/−65, or −62/−61, of the mutant −100. An inverted repeat sequence is indicated by an arrow, and the TATA box is boxed. B. Comparison of nucleotide sequence of the −60 region of the CDI gene promoter [13] with the MJA-responsive region of potato proteinase inhibitor II (PI-II [17], soybean VspB [22], and *nos* [18]. Numbers indicate position of nucleotide relative to the site of transcription start. The G-box motif and the hexamer motif are indicated by the underline and by the dotted underline, respectively.

to insertion of the oligonucleotide may have eliminated the MJA-responsive expression.

Discussion

The potato gene encoding cathepsin D inhibitor (CDI) is expressed constitutively in tubers and flower buds [10, 13, 29]. However, its expression is inducible in leaves upon wounding of the tissue or by treatment of the tissue with MJA [10, 12, 13]. In the present study, we demonstrated that expression of the CDI:GUS fusion gene, which is composed of the promoter fragment of the gene for CDI and the GUS-coding sequence, was also inducible in transformed tobacco cells upon treatment with MJA. It is suggested that tobacco BY-2 cells in suspension harbor mechanisms of regulating gene expression in response to MJA. Indeed, we observed that changes in population of translatable mRNA in tobacco BY-2 cells occur within a few hours after treatment with 50 μM MJA, and we isolated several MJA-inducible cDNAs (K. Hashizume, A. Ishikawa and K. Nakamura, manuscript in preparation).

The MJA-inducible expression of the CDI-:GUS fusion gene was severely repressed by the presence of 2,4-D in the culture medium. Previously, it has been reported that expression of a fusion gene, *pin-2*-CAT, composed of a promoter of the PI-II gene and the coding sequence for chloramphenicol acetyltransferase (CAT) in calli and leaves of tobacco is down-regulated by auxin [16]. In contrast to the expression of the CDI-:GUS fusion gene in suspension-cultured tobacco cells described in this paper, expression of the *pin-2*-CAT fusion gene in tobacco calli cultured on auxin-free solid medium occurs in the absence of exogenous supply of MJA. Down regulation by auxin of several other plant defense genes has also been reported [25, 27]. The reduced level of MJA-inducible expression of the CDI:GUS fusion gene in tobacco cells cultured in the 2,4-D medium was released by treatment of cells with aphidicolin (Fig. 4). These results suggest that the effect of auxin on the MJA-inducible expression

of the CDI:GUS fusion gene in tobacco cells is an indirect one, and that MJA-inducible expression of the fusion gene is repressed in cells under active cell division. In contrast to the MJA-inducible gene expression, disruption of cortical microtubles of tobacco BY-2 cells by MJA occurs only in cells at the S phase of the cell cycle, and microtubles in cells kept in aphidicolin were insensitive to MJA [1]. These results suggest that MJA-induced gene expression and MJA-induced disruption of microtubles in tobacco BY-2 cells occur by different mechanisms.

Among various JA-related compounds examined (Fig. 5A), only JA and MJA, were effective to induce expression of the CDI:GUS fusion gene in tobacco cells and compounds other than JA and MJA did not show such an activity (Fig. 5B). In leaves of potato, CA, TA and HJA and their methyl esters in addition to JA and MJA induced the expression of the CDI gene albeit with efficiencies significantly lower than those of JA and MJA [12]. In both cases, whether or not the compound is methylated did not severely affect the activity. Compounds other than JA and MJA were not only unable to induce the expression of the fusion gene, but also did not repress the MJA-induced expression of the fusion gene in tobacco cells (Fig. 5C). It seems that the receptor in tobacco cells would have high structural-specificity for JA and the binding of the receptor with JA is not competed by other JA-related compounds. Previously, Koda *et al.* [20] have reported that JA-induced tuberization from single-node segments of potato is partially inhibited by analogues of JA that lack a carboxyl group at the C-1 position of JA. The structure-activity relationships of various JA-related compounds in inducing tuber formation [20] are not identical with those in inducing the expression of genes encoding CDI and PI-II in leaves of potato [12]. The receptor for JA in tobacco cells involved in the regulation of gene expression could be different from those in shoots and leaves of potato in the specificity of binding with JA-related compounds. Alternatively, JA is the natural signalling molecule recognized by the receptor and activities expressed by compounds other than JA could be the secondary effects. Wounding of leaves results in increase in endogenous levels of JA and MJA [4]. In experiments using whole plants or excised tissues and in experiments which require longer periods of time for assays, the transport and metabolism of JA-related compounds suplied exogenously must be taken into account.

The significance of stimulation of the MJA-inducible expression of the fusion gene by MHJA (Fig. 5C) is not known at present. MHJA alone does not induce expression of the CDI:GUS fusion gene (Fig. 5B). HJA occurs naturally in plants, and it is active in promoting senescence of oat leaves [31] but not in promoting tuber formation [20]. It is tempting to speculate that HJA is also a natural signalling molecule and JA and HJA signals interact each other to regulate expression of defence genes in plant cells.

The results presented in Fig. 6A indicate that the 100 bp sequence upstream from the transcription start site of the CDI gene can exhibit MJA-responsive expression in tobacco cells. Neither the 5′-deletion mutants −81 and −65 nor the insertion mutant -100^{I} showed MJA-responsible expression (Fig. 6B). The sequence between −100 and −82 that is required for the MJA-responsible expression contains a palindromic sequence with a center of symmetry at position −87/−86 (Fig. 7A). However, this sequence is not sufficient to confer the MJA-responsiblility of the expression. In the mutant -100^{I}, interaction of the protein that binds to this upstream element with other proteins may have affected by the insertion of the oligonucleotide. Alternatively, insertion of the oligonucleotide may have disrupted the functional element which functions synergistically with the upstream element. The sequence between −68 and −58 of the CDI promoter is similar to the sequences encompassing the MJA-responsive domains of promoters of other plant genes (Fig. 7B) and the oligonucleotide was inserted in the middle of this sequence. The MJA-responsive domains of PI-II [17], nos [18] and VspB [22] contain the G-box or hexamer motif to which a family of plant bZIP proteins bind [14]. Mason *et al.* [22] also noticed that both of the G-box motifs in the MJA-responsive domains of VspB

and PI-II are preceded by a C-rich domain. However, the sequence between −100 and −82 of the CDI promoter is not similar to the C-rich domain and the homologous sequence in the CDI promoter lacks an ACGT core sequence shared by the G-box and hexamer motifs (Fig. 7B). Studies with more mutant promoters and analyses of nuclear factors that bind to the MJA-responsive region are required to clarify the nature of multiple factors that are involved in the MJA-responsive expression of the CDI promoter.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research on Priority areas ('Metabolite Accumulation in Higher Plants' for T.Y. and K.N. and 'Molecular Mechanism of Photosynthetic Responses to Environment' to K.N.) from the Ministry of Educations, Science and Culture of Japan.

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