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Monitoring of Methyl Jasmonate-responsive Genes in Arabidopsis by cDNA Macroarray: Self-activation of Jasmonic Acid Biosynthesis and Crosstalk with Other Phytohormone Signaling Pathways

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Abstract

Jasmonates mediate various physiological events in plant cells such as defense responses, flowering, and senescence through intracellular and intercellular signaling pathways, and the expression of a large number of genes appears to be regulated by jasmonates. In order to obtain information on the regulatory network of jasmonate-responsive genes (JRGs) in *Arabidopsis thaliana* (Arabidopsis), we screened 2880 cDNA clones for jasmonate responsiveness by a cDNA macroarray procedure. Since many of the JRGs reported so far have been identified in leaf tissues, the cDNA clones used were chosen from a non-redundant EST library that was prepared from above-ground organs. Hybridization to the filters was achieved using α-^{33}P-labeled single-strand DNAs synthesized from mRNAs obtained from methyl jasmonate (MeJA)-treated and untreated Arabidopsis seedlings. Data analysis identified 41 JRGs whose mRNA levels were changed by more than three fold in response to MeJA. This was confirmed by Northern blot analysis by using eight representatives. Among the 41 JRGs identified, 5 genes were JA biosynthesis genes and 3 genes were involved in other signaling pathways (ethylene, auxin, and salicylic acid). These results suggest the existence of a positive feedback regulatory system for JA biosynthesis and the possibility of crosstalk between JA signaling and other signaling pathways.

Key words: Arabidopsis; cDNA macroarray; DNA array; jasmonic acid; methyl jasmonate

1. Introduction

Jasmonates modulate various physiological events such as resistance to pathogens and insects, fruit ripening, maturation of pollen, root growth and senescence.[1] Jasmonates are synthesized from linolenic acid by oxygenation with lipoxygenase (LOX), then converted to 12-oxo-phytodienoic acid by allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Fig. 1). Jasmonic acid (JA) is synthesized from 12-oxo-phytodienoic acid through reduction by 12-oxo-phytodienoic acid reductase (OPR) and three steps of β-oxidation. Jasmonic acid carboxyl methyltransferase (JMT) generates methyl jasmonate (MeJA). JA and MeJA are accumulated when plants are wounded.[1] Wounding and MeJA treatment are known to induce the expression of *LOX2*,[2] and *AOS*,[3] and various other stress-related genes,[1,4] which suggests that JA and MeJA are primary intracellular transducers of stress response.

Little information has been accumulated about signaling components downstream of jasmonates, which regulate the expression of jasmonate-responsive genes (JRGs) including those for biosynthesis of JA. Several JA-insensitive mutants, *jar1*, *jin1*, *jin4*, and *coi1*,[5–7] have been isolated so far. Among these, only the gene for coronatine insensitive 1 (*COI1*) has been

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† AOC, allene oxide cyclase; AOS, allene oxide synthase; CLH, chlorophyllase; COI, coronatine insensitive; ESTs, expressed sequence tags; GST, glutathione-*S*-transferase; IAR, IAA-Ala resistant; JA, jasmonic acid; JMT, jasmonic acid carboxyl methyltransferase; JRG, jasmonate-responsive gene; LOX, lipoxygenase; MeJA, methyl jasmonate; MBP myrosinase binding protein; OPR, 12-oxo-phytodienoic acid reductase; PDF, plant defensin; VSP, vegetative storage protein

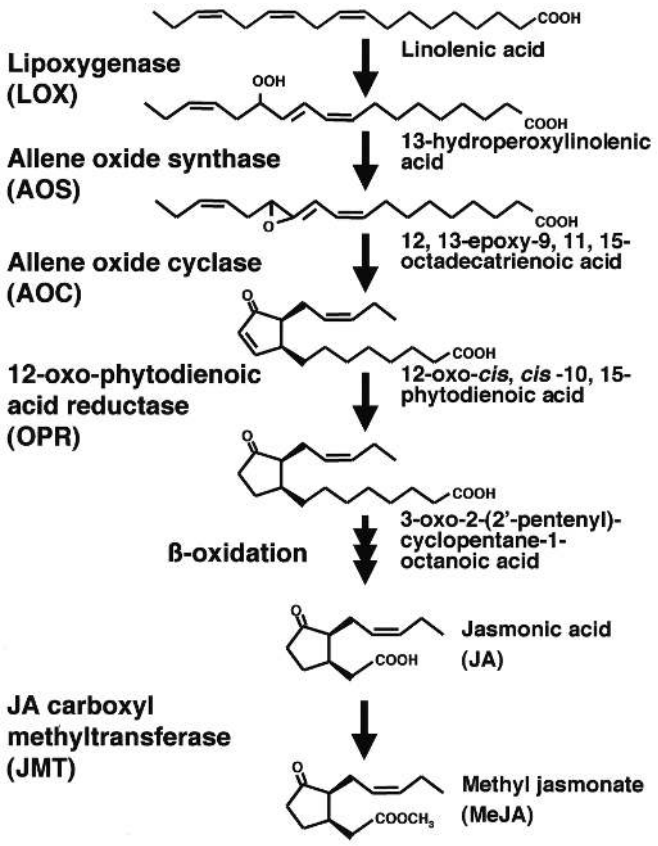

Figure 1. Pathway for the biosynthesis of jasmonates.

cloned and characterized as a signaling component with a leucine-rich repeat motif.[8] Recently, a JRG was identified as a transcription factor which positively regulates MeJA-dependent production of secondary metabolites;[9] this report indicates that components of the jasmonate-signaling pathway itself respond to jasmonate at the mRNA level.

The extent of crosstalk between jasmonate signaling and other signaling pathways is also unclear. It was reported that auxin inhibited JA-dependent induction of genes for vegetative storage protein (*VspB*), lipoxygenase (*LoxA*),[10] and various JRGs.[11] Salicylic acid, is known to inhibit the jasmonate-dependent synthesis of a proteinase inhibitor gene, which encodes a defense protein.[12] On the other hand, concomitant activation of both ethylene and jasmonate signaling pathways is essential for expression of the plant defensin 1.2 (*PDF1.2*) gene which encodes a JA-responsive and antimicrobial plant protein.[13] These findings imply that the signaling pathways downstream of jasmonates interact with other signaling compounds, whereas the molecular mechanism of crosstalk mediated by these substances remains to be clarified.

To obtain information on the genetic mechanism, which underlies the pleiotypic effects of jasmonates and its crosstalk with other signaling pathways, we adopted cDNA macroarray technology. Until now, a total of 12,028 non-redundant ESTs have been generated from five different tissues (above-ground organs, flower buds, roots, green siliques and liquid-cultured seedlings) of *Arabidopsis thaliana* (Arabidopsis).[14] Since many of the JRGs reported up to present have been identified in leaf tissues,[1,4] we made cDNA macroarray filters using the non-redundant 2880 EST clones generated from above-ground organs. Here, we report the identification and characterization of JRGs including components of the jasmonate-signaling pathway using the cDNA macroarray, and discuss the possible physiological functions and the signaling pathway of jasmonates.

2. Materials and Methods

2.1. Plant material

Seeds of Arabidopsis (accession Columbia) were germinated in MS medium,[15] containing 1% (w/v) sucrose. The seedlings were incubated on an orbital shaker under continuous light at 22°C. After 10 days, the plants were treated with 30 μM MeJA. Total RNA was extracted as described.[16]

2.2. Preparation of cDNA macroarray filters

The cDNA clones used for generation of the macroarray had been prepared from above-ground organs of 2- to 6-week-old plants.[14] Inserts of 2880 EST clones were amplified using the following primer set: 5′-GTAATACGACTCACTATAGGGC-3′ and 5′-TCATTAGGCACCCCAGGCTTTACAC-3′. The PCR products were concentrated and spotted onto an 8×12 cm nylon filter, Biodyne A (PALL, U.S.A.) in duplicate using a Biomek 2000 Laboratory Automation Workstation (Beckman Instruments, Inc, U.S.A.). The spotted DNAs were fixed on the nylon filter by UV cross-link. λDNA were also spotted in duplicate as negative controls.

2.3. Hybridization of cDNA macroarray filters

Poly(A)$^+$ RNAs were purified from the total RNA of MeJA-treated and untreated plants using oligotex-dT30 (TaKaRa, Japan). Each mRNA sample was reverse-transcribed in the reaction mixture containing 0.1 μg of mRNA, 2 μg of oligo dT primer (Amersham Pharmacia Biotech, U.S.A.), and 7.5 U of AMV reverse transcriptase (TaKaRa, Japan) in the presence of [α-^{33}P]dCTP, incubated at 37°C for 90 min.

Hybridization with the labeled target was carried out in the presence of 0.5 M Na_2HPO_4, 1 mM EDTA, and 7% SDS[17] at 65°C for 16 hr. After incubation, the filters were washed twice with 0.2 × SSC, 0.1% SDS at 65°C, then exposed to an imaging plate (Fuji Film, Japan) for detection.

2.4. Identification of MeJA-responsive genes

Radioactive images were obtained with a high-resolution scanner (Storm, Amersham Pharmacia Biotech, U.S.A.), and quantification of the signal intensity was carried out using an Array Vision (Amersham Pharmacia Biotech, U.S.A.). Global normalization was adopted for normalizing the difference of signal intensity of each nylon filter: The intensity of all the spots on the filter was averaged and the relative signal intensity was calculated as the ratio of each signal to the average intensity of each filter. The value thus estimated is called the 'expression ratio.' The expression ratios of the duplicated spots were averaged prior to further analysis. Data were obtained from four independent experiments for each experimental condition. Genes that responded to MeJA with reproducible results were identified by one-way analysis of variance (significance level, 0.05). This procedure employs the statistic F, which is used for estimation of a population variance based on the information in two or more random samples, to test the statistical significance of the differences among the obtained mRNA expression levels under each experimental condition. Data analyses were carried out using Microsoft Excel.

2.5. Northern blot analysis

Total RNA (10 μg) was prepared from MeJA-treated and untreated plants as described in Section 2.1. The RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane (Schleicher & Schuell, Germany) by capillary transfer. Insert DNAs of the MeJA-responsive cDNA clones were used as probes. Hybridization was carried out as described in Section 2.3.

3. Results and Discussion

3.1. Identification of JRGs by the cDNA macroarray

To examine the effect of MeJA on the transcription of 2880 genes, a time-course experiment was performed for the plants treated with MeJA for 1, 3, 6, 12, and 24 hr. Scattered plots of 2880 genes, shown in Fig. 2, indicate that MeJA-dependent changes of the mRNA levels mainly occur after 3 to 6 hr of treatment. Among the 2880 genes tested, we identified 41 MeJA-responsive genes (1.5%) whose mRNA levels changed more than three-fold (elevation or decline) within 24 hr of treatment (Fig. 3). Moreover, most of the JRGs identified here were induced transiently, suggesting the presence of a common mechanism for repressing the mRNA expression after the induction. Repression of gene expression by MeJA, on the other hand, was observed for only one gene. The functions of 35 genes out of the 41 genes could be deduced from the translated amino acid sequences of the corresponding EST sequences. The data shown in Fig. 2 also demonstrate the wide detection range of the macroarray system adopted in this study, which allows detection of mRNA levels from 0.1- to 100-fold the average intensity.

To confirm the fidelity of the expression profiles obtained using the cDNA macroarray, Northern blot analysis was performed for the following genes selected from the 41 JRGs detected in this study: *LOX2*, *AOS*, *OPR1*, *OPR3*, *IAR3* (IAA-Ala-resistant 3), *CLH1* for chlorophyllase 1, *GST8* for glutathione-*S*-transferase 8, and a *MBP* homolog encoding a myrosinase binding protein homolog. Comparison of the data obtained from the two methods indicated that the two profiles are fairy consistent for the genes tested (Fig. 4). The expression profiles of *LOX2*, *AOS*, and *CLH1* after treatment of MeJA have been reported in Arabidopsis,[2,3,18] and they are also consistent with those obtained from the macroarray analysis. These results demonstrate that the cDNA macroarray is an effective tool for analyzing gene expression patterns.

Many JRGs are known to be involved in stress responses, but only few of them have been found to be related to other physiological events such as flower and fruit development and senescence.[4] In this study, genes involved in a variety of physiological events were found to respond to jasmonates, in addition to those related to stress responses (putative β-1,3-glucanase, *MBP* homologs), as shown in Fig. 3. These include genes involved in JA biosynthesis,[2,3,19,20] phytohormone-signaling,[21] disease and wounding response,[22,23] amino acid metabolism,[24–26] secondary metabolism,[27] signal transduction,[28] and senescence.[18] Thirty-seven out of 41 JRGs detected by the macroarray, including 6 genes of unknown function, were newly identified as JRGs in Arabidopsis.

3.2. Self-activation of a series of jasmonate biosynthesis genes

A number of genes involved in biosynthesis of JA have been reported in Arabidopsis. These include four homologs of allene oxide cyclase (*AOC*) (accession numbers BAA95763, BAA95764, BAA95765, and AAG09557) which was originally cloned from tomato,[29] three *OPR* genes,[19,20,30] and two *OPR* homologs[31] (accession numbers AAF97278, and AAC33200). The data shown in Fig. 3 indicates that MeJA induced the mRNA expressions of JA biosynthesis genes such as the *AOC* homolog (BAA95764), *OPR1*, and *OPR3*, in addition to previously reported *LOX2*[2] and *AOS*[3] genes, though other *AOC* homologs (BAA95763, BAA95765, and AAG09557), *OPR2*, and other *OPR* homologs (AAF97278, and AAC33200) were not tested in this study. As shown Fig. 3, the mRNA expression profiles of JA biosynthesis genes were different, suggesting that the expressions of JA biosynthesis genes are controlled by different mechanisms. Actually, the presence of multiple jasmonate signal transduction pathways were reported

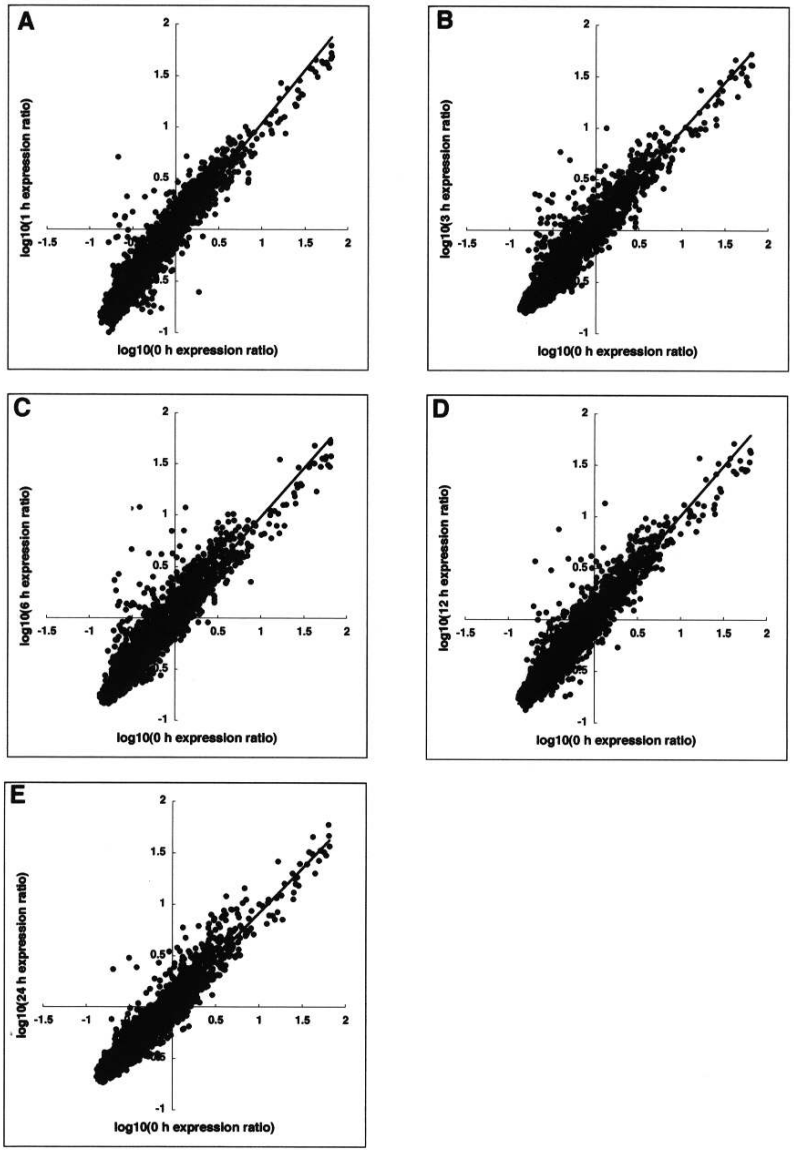

Figure 2. MeJA-dependent changes of mRNA levels in 2880 ESTs. The changes in the mRNA levels after MeJA treatment for 1 to 24 hr are shown. The expression ratio for an EST was calculated as the average value from four independent experiments. The logarithmic values of expression ratios obtained from control and MeJA-treated experiments are presented on the x- and y-axis, respectively. The diagonal in each graph shows the line logY = logX. A, B, C, D, and E indicate the ratios of mRNA levels at 1, 3, 6, 12, and 24 hr of treatment to untreated (0 hr) control, respectively.

by several groups.[32,33]

A recent report indicated that OPR3 converted 12-oxo-*cis*, *cis*-10, 15-phytodienoic acid, an *in vivo* substrate for OPR, more efficiently than OPR1 and OPR2.[20] Moreover, no OPR enzyme activity was detected in the OPR3-deficient mutant. These results indicated that OPR3 is the most important isoform in the biosynthesis of JA.[31] As shown in Figs. 3 and 4, the increase of the mRNA level of *OPR3* (1 hr after treatment with MeJA) preceded the induction of *LOX2*, *AOS*, the *AOC* homolog, and *OPR1* (3 hr after treatment). The earlier induction of *OPR3* than *OPR1* suggests that *OPR3* functions in the early stage of the signaling pathway of jasmonates.

It is noteworthy that MeJA positively induced the expression of all of the genes for JA biosynthesis tested. It has been reported that the gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO*), an enzyme involved in the biosynthesis of ethylene, was induced by ethylene.[34] By contrast, gibberellin 20-oxidase and gibberellin 3β-hydroxylase are repressed by active gibberellin.[35–37] Recently, jasmonic acid car-

Gene identification	GenBank Accession	Peak	Fold
Jasmonate Biosynthesis-related Genes			
allene oxide synthase (*AOS*)	AV441105, AV442349	3	7.4
lipoxygenase 2 (*LOX2*)	AV520879, AV527388	12	12.8
12-oxophytodienoic acid reductase 1 (*OPR1*) *1	AV439642	6	4.9
12-oxophytodienoic acid reductase 1 (*OPR1*) *1	AV440613	3	6.6
12-oxophytodienoic acid reductase 3 (*OPR3*)	AV440671, AV442615	1	7.6
tomato allene oxide cyclase (*AOC*) homolog	AV441185, AV442426	6	16.1
Other Phytohormone-related Genes			
IAA-Ala hydrolase	AV439482, AV441715	1	5.3
tobacco UDP-glucose:salicylic acid glucosyltransferase homolog	AV440279	3	3.4
tomato E8 homolog *2	AV439491	6	4.5
tomato E8 homolog *2	AV518218, AV524915	6	4.9
Disease and Wounding Responsive Genes			
beta-glucosidase homolog	AV440409	6	42.0
putative beta-1,3-glucanase	AV441467	1	5.4
myrosinase-binding protein-like protein	AV525017	3	5.2
myrosinase-binding protein-like	AV439979, AV441884	3	12.1
putative myrosinase-binding protein	AV440772, AV442321	12	3.1
Similar to myrosinase binding protein from Brassica napus	AV520644, AV527049	6	3.8
Amino Acid Metabolism Genes			
anthranilate synthase α-chain 1(*ASA1*)	AV440465	1	4.0
anthranilate synthase β-chain 1 (*ASB1*)	AV521371	6	4.1
tryptophan synthase α-chain 1 (*TSA1*)	AV440924, AV442547	3	4.7
tryptophan synthase β-chain 1 (*TSB1*)	AV442416	3	3.6
tyrosine transaminase homolog	AV440877, AV442456	6	6.7
putative tyrosine aminotransferase	AV440386	1	4.6
glutamate-ammonia ligase (*GSR2*)	AV442472	6	3.2
Secondary Metabolism-related Genes			
arginine decarboxylase 2 (*ADC2*) *3	AV440195	6	4.5
arginine decarboxylase 2 (*ADC2*) *3	AV440359	6	3.6
nicotianamine synthase	AV439641, AV441843	6	3.1
putative catechol-*O* -methyltransferase	AV439530, AV442060	3	8.8
putative flavonol sulfotransferase	AV439692, AV441979	3	4.9
Signal Transduction-related Genes			
ankyrin-repeat protein	AV439917, AV441775	6	8.6
protein kinase-like protein	AV440773, AV442789	6	3.3
Senescence-related Genes			
chlorophyllase 1(*CLH1*)	AV440587	6	12.5
Others			
adenylosuccinate lyase homolog	AV441222, AV442824	6	5.9
glutathione-*S* -transferase 8	AV441242, AV442169	6	6.3
NAD-dependent formate dehydrogenase	AV441244, AV442765	24	4.3
putative cytochrome P450	AV440366	3	3.0
probable selenium-binding protein	AV442112	3	4.2
putative 6-phosphogluconolactonase-like protein	AV441291, AV442216	6	13.1
translationally controlled tumor protein homolog	AV520612, AV527002	24	3.7

Figure 3. List of MeJA-responsive genes. The expression ratio for each EST at the indicated time (1, 3, 6, 12, and 24 hr) was calculated, and then a "ratio" of the maximum or minimum expression ratio to that for control (untreated) was subsequently obtained. The "peak" lane indicates the time showing the maximum or minimum value. In this table, ESTs that showed a three fold elevation or decline in their mRNA level are shown. *1, 2, 3: The ESTs encode different parts of the same gene respectively, and have been recognized as distinct genes. These ESTs were independently spotted on cDNA macroarray filters, and showed the same expression profiles.

boxyl methyltransferase (*JMT*), which catalyzes the conversion of JA to MeJA, was cloned from Arabidopsis.[38] Over-expression of *JMT* caused the constitutive expressions of JRGs including *LOX2* and *AOS*. MeJA is a volatile compound, and considered as an interplant signal transductor like ethylene.[39] These observations and the fact that MeJA induced the expression of the genes for JA biosynthesis may suggest that MeJA is a key compound in the jasmonate-signaling pathway, where MeJA controls its own expression by a positive feedback mechanism.

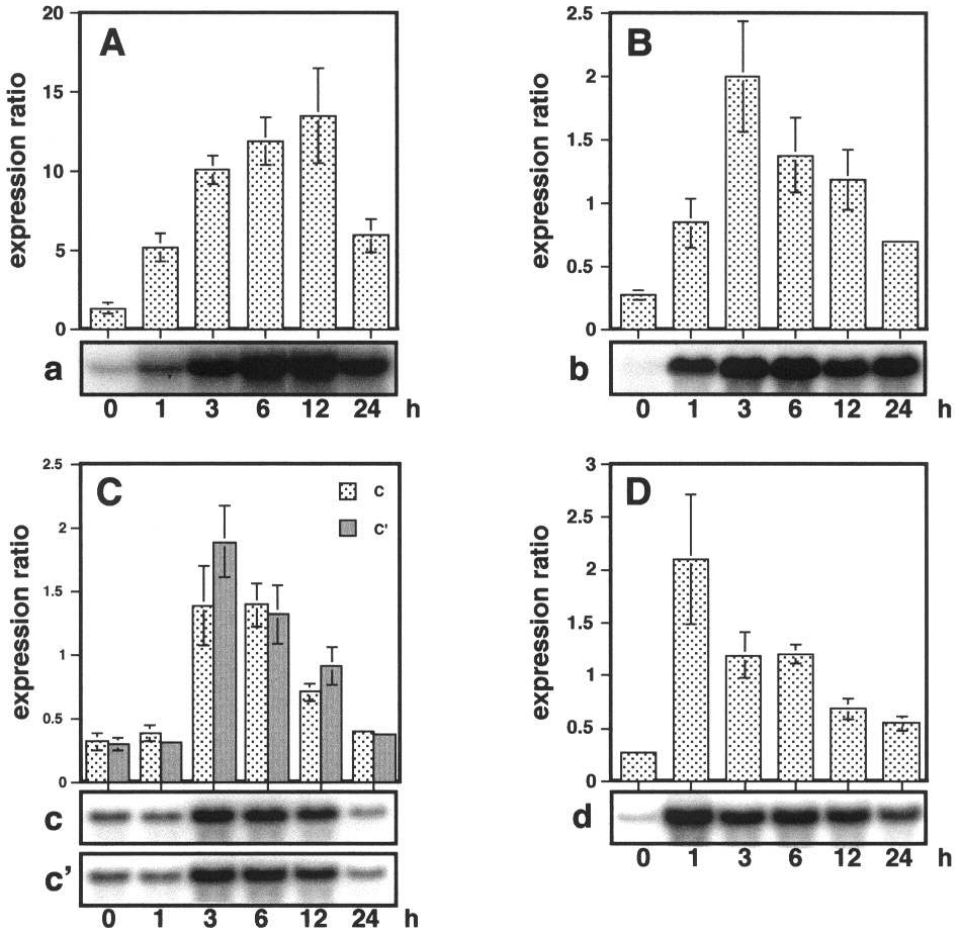

Figure 4. Correlation of expression profiles between the results from cDNA macroarray and Northern blot analysis. The expression ratio of each clone was determined as the ratio of signal intensity to the average of 2880 ESTs at each indicated time. Time for MeJA treatment (0, 1, 3, 6, 12, and 24 hr) was indicated in the figure. A to H show mRNA levels of each EST obtained from the cDNA macroarray, and a to h the results of Northern blot analysis. A, a: *LOX2*. B, b: *AOS*. C, c: *OPR1*, D, d: *OPR3*. E, e: *IAR3*, F, f: *CLH*, G, g: *GST*, H, h: *MBP* homolog. Two ESTs of *OPR1* (c, c′) cover different regions of the gene and were independently spotted on the cDNA macroarray filter. Bars indicate the standard error of four independent experiments.

3.3. Crosstalk of jasmonates with other signaling compounds

As shown in Fig. 3, MeJA induced the expression of a gene encoding dioxygenase-like protein, which is homologous to an E8 protein in tomato with 50% amino acid identity. E8 is known as a protein having a negative effect on ethylene synthesis in ripening of tomato fruits.[40,41] It is, therefore, plausible that MeJA regulates the biosynthesis of ethylene through the function of an E8 homologue. Considering that jasmonates generally have a synergistic effect on the ethylene-signaling pathway,[42] an intricate mechanism of interaction between jasmonates and ethylene signal transduction pathway should be postulated.

Auxin had an antagonistic effect to jasmonates.[10,11] Amide-linked conjugates of IAA are putative storages or inactive forms of the growth hormone auxin.[43–45] *IAR3*, which encodes IAA-Ala hydrolase, was identified as a JRG of unknown function by differential mRNA display,[46] which is further confirmed by the present study, as shown in Figs. 3 and 4. The transcriptional level of *IAR3* was induced with a peak at 1 hr after the treatment of MeJA, whereas expression of 31 JRGs were highest at 3 to 6 hr after the treatment. The interpretation of these observations could be that the production of active IAA was induced by MeJA, and that the produced IAA subsequently repressed transcription of JRGs so that expression of most of the JRGs were transient, as shown in Fig. 2.

It was reported in tobacco that MeJA induced the transcription of a glucosyltransferase, a homologue of UDP-glucose: salicylic acid glucosyltransferase.[47] Salicylic acid is assumed to be a signal molecule that induces the synthesis of pathogenesis-related proteins, and jasmonates show an antagonistic effect to salicylic acid.[1]

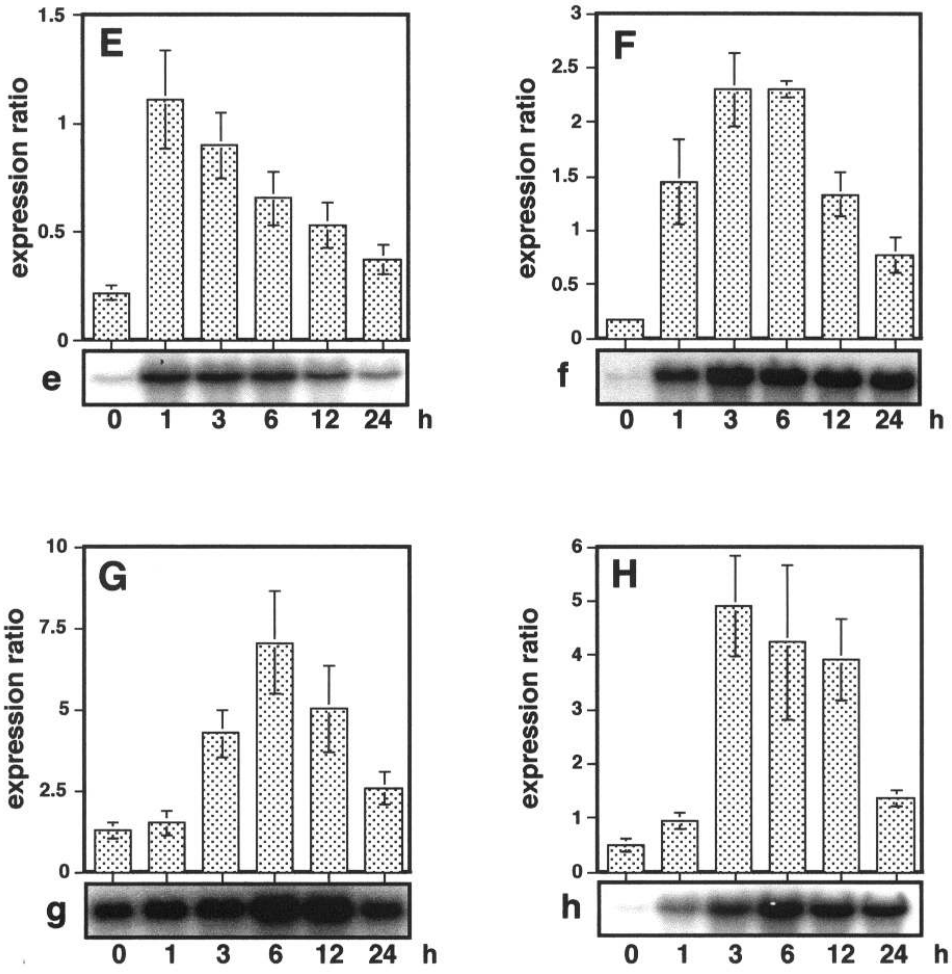

Figure 4. Continued.

The macroarray experiment in this study showed that induction of the glucosyltransferase genes by MeJA was also detected in Arabidopsis. These observations suggest that MeJA inhibits salicylic acid signaling through the induction of the glucosyltransferase, because the sugar-conjugate of salicylic acid is considered to be inactive.[47]

Details of the expression profiles obtained with the cDNA macroarray are posted in the web site: http://www.kazusa.or.jp.

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