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Industrial Applications of *Bupleurum kaoi* Genes Induced by MeJA

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Abstract: Bupleurum has been widely used for the treatment of chronic hepatitis and inflammatory diseases. The plant regulator methyl jasmonate (MeJA) mediates diverse developmental processes and defense responses. In this study, MeJA was applied to root suspension of *Bupleurum kaoi* for its ability to stimulate plant defenses and saponin production. Employing a cDNA microarray containing about 750 ESTs, generated with the PCR-Select cDNA Subtraction kit as probe sets, we profiled the transcriptome of MJ induction at different time intervals. The genes up regulated by MeJA treatment include those involved in jasmonate biosynthesis, secondary metabolism, cell-wall formation, and stress-protection and defenses. Transcript levels of the *BkβAS* and *Bkdef* genes were enhanced by MeJA and affected by developmental stage but not by salicylic acid. The *BkβAS* encodes β-amyrin synthase which is a rate-limiting enzyme of saikosaponins biosynthesis. The *BkDF1* and *BkDF2* encode proteins belonging to group II of plant defensins. Expression of *BkDF1* in root and leaves, and the antifungal activity against at least some tested pathogens indicated that BkDF1 may serve a role in protection of these organs against a range of pathogens.

Keywords: *Bupleurum kaoi; β-amyrin synthase;* cDNA microarray; defensin; methyl jasmonate.

1. Introduction

Methyl jasmonate (MeJA) is a fragrant volatile compound initially identified from flowers of *Jasminum grandiflorum*, and has been reported to be distributed ubiquitously in the plant kingdom (Cheong and Yang, 2003). The volatile nature of MeJA led to the discovery of its role as a signal in plant cellular responses, plant– herbivore interactions (Pare and Tumlinson, 1999) and plant–plant interactions (Arimura et al., 2000). MeJA and its free-acid jasmonic acid (JA), collectively called jasmonates, are plant stress hormones that act as regulators of diverse developmental processes (Staswick et al., 1992; Creelman and Mullet, 1997; Weber et al., 1997; Wasternack and Hause, 2002) and defence responses (Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Reymond and Farmer, 1998; Thomma et al., 1999; Kessler et al., 2004).

Genes upregulated by MeJA treatment include those involved in jasmonate biosynthesis, secondary metabolism, cell-wall formation, and those encoding stress protective

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and defense proteins. The induction of secondary metabolite accumulation is an important stress response that depends on jasmonates as a regulatory signal (Gundlach et al., 1992). Plant secondary compounds are usually classified according to their biosynthetic pathways (Harborne, 1999). Three large molecule families are generally considered: phenolics, terpenes and steroids, and alkaloids. Different strategies, using *in vitro* systems, have been extensively studied with the objective of improving the production of secondary plant metabolites. Undifferentiated cell cultures have been mainly studied, but a large interest has also been shown in hairy roots and other organ cultures. Compared to other biotechnological tools like microorganism or mammalian cell cultures (Hutchinson, 1994; Chartrain et al., 2000), plant secondary metabolism remains poorly understood (Verpoorte and Memelink, 2002). The emergence of recombinant DNA technology has opened a new tool with the possibility of directly modifying the expression of genes related to biosyntheses.

The roots of *Bupleurum* species have been reported to have pharmacological properties such as anti-inflammatory activity (Bermejo Benito et al.,1998; Navarro et al., 2001) and anti-hepatotoxic effect (Lin et al., 1990). Pharmacological properties of *Bupleurum* saikosaponins have been corroborated by *in vivo* and *in vitro* studies (Bermejo Benito et al., 1998; Chiang et al., 2003; Hsu et al., 2004; Shyu et al., 2004). The constituents and levels of saikosaponins are considered as criteria for evaluating the quality of *Bupleurum*. Exogenously applied MeJA induced the biosynthesis of many secondary metabolites including terpenoids, and stimulated the saponin production in cultured *B. falcatum* root fragments (Aoyagi et al., 2001), but a detailed mechanism of its stimulatory process yet remains poorly understood. Recently, the exogenous MeJA was found to induce accumulation of *β-amyrin synthase* (*βAS*) and *squalene synthase* (*SQS*) transcripts in *Glycyrrhiza glabra* and the accumulation of soysaposin (Hayashi et al., 2003). *Bupleurum kaoi* Liu, Chao et Chuang is native to Taiwan and has been widely used for the treatment of chronic hepatitis and inflammatory diseases. The genome of *B. kaoi* is about 7.3×10^8 bp per copy, as determined by flow cytometry (Lin, 2004). Isolation and characterization of these genes in *Bupleurum* may help in unraveling the secondary metabolic pathway and subsequent increase in the production of saikosaponins. To obtain information on the regulatory network of the MeJA responsive genes and the saikosaponin biosynthesis, we constructed partial *B. kaoi* cDNA libraries from adventitious roots and three month old seedlings, using PCR-select cDNA subtraction for exogenous MeJA treatment and then analyzed with a small scale cDNA microarray. The genes, upregulated by MeJA treatment and their potential industrial applications have been discussed.

2. Materials and methods

2.1. Plant growth and stress condition

Adventitious root culture of *B. kaoi* was established by consecutively subculture every six weeks in Gamborg's B5 liquid medium (Gamborg et al., 1968) supplemented with 2 ppm NAA and maintained on a rotary shaker at 100 rpm and 25°C in the dark. The adventitious roots were subcultured as a seed culture by adding 0.2 g roots into a 125 ml flask containing 50 ml of B5 medium with 2 ppm NAA. Four weeks later, the nutrient medium was refreshed with or without MeJA (500 μM) and harvested at intervals of 5 min, 1, 2, 8, 24 hours (BkIII), 2, 4, 6, 8 days (BkI + BkII) and 2, 3, 4, 5 weeks (BkIV) after MJ application (Figure 1). Roots were frozen in liquid nitrogen prior to use.

2.2. Total cellular RNA extraction

This method was adopted from Chang et

al. (1993) with some modifications. Three to five grams of tissue was frozen in liquid nitrogen and ground to fine powder with motor and pestle. The powder was added to 15 ml of pre-warmed (65°C) extraction buffer (2% CTAB (hexadecyltrimethylammonium bromide), 2% PVP (polyvinylpyrrolidinone K 30), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 mg/ml spermidine, 2% β-mercaptoethanol), and the contents were mixed completely by vigorous shaking. The buffer/tissue mix was extracted two times with an equal volume of chloroform:isoamyl alcohol (24:1). The RNA was precipitated by adding 1/4 volume of cold 10 M LiCl to the inorganic phase and sat for 12-18 h at –20°C. After centrifugation at 19,800 × *g* at 4°C, the supernatant was poured out and the RNA pellet was washed twice with 80% ethanol then dissolved in 500 μl DEPC-ddH_2O after drying. The resuspended RNA was extracted with chloroform:isoamyl alcohol (24:1). Three volumes of 100% ethanol and 1/10 volume of 3 M NaOAc (pH 5.2) were added to the inorganic phase, and the solution was precipitated with liquid nitrogen for 15 min. The RNA was spun down at 19,800 × *g* for 30 min at 4°C, then washed with 80% ethanol and spun briefly. The pellet was dried and resuspended in an appropriate volume of DEPC-ddH_2O.

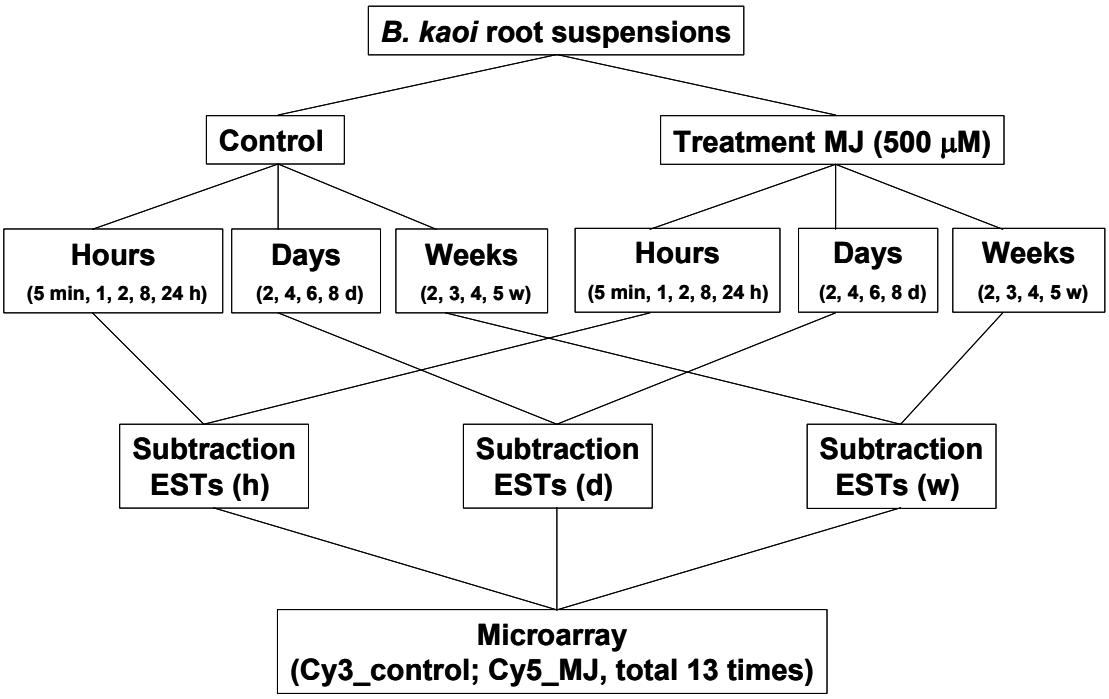

Figure 1. The flow chart of experimental design

2.3. Preparation of PCR-select cDNA subtraction library and DNA sequencing

The polyadenylated RNA was purified from total cellular RNA using an mRNA Purification kit (Amersham Biosciences). Briefly, a maximum of 1.25 mg of total RNA, dissolved in 1 ml elution buffer, was applied to a oligo(dT)-cellulose column. After washing twice with high-salt buffer and three

times with low-salt buffer, the mRNA was eluted in 0.25 ml aliquots of elution buffer four times. The mRNA was used to prepare the subtracted mixture with the PCR-Select™ cDNA Subtraction kit (BD Biosciences Clontech). The subtracted cDNA was proceeded from the mRNA following several steps (first-strand cDNA synthesis, second-strand cDNA synthesis, *Rsa* I digestion, adaptor ligation, first hybridization, second hybridization and PCR amplification) and then the cDNA mixture was directly inserted into the pGEM®-T Easy Vector (Promega) for cloning PCR products before transformation into *ECOS 101* competent cells (Yeastern Biotech., Taiwan). The detailed procedure was followed as given in the manufacturer's instructions manual (BD Biosciences Clontech and Promega).

Isolated cDNA clones were purified using the *Gene-Spin™* Miniprep Purification kit (Protech). cDNAs were sequenced using the cycle sequencing reaction method, BigDye Terminated kit (Applied Biosystems Industries) and analyzed using an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems Industries, Foster City, CA, USA).

Similarity searches were carried out with the BLASTX program.

2.4. DNA microarray fabrication and hybridization

The microarray was designed with four replicates of each probe distributed across the array. Bk EST probe sets derived from subtracted cDNA libraries were amplified with nested primer 1 and 2R. As external and internal control, PCR-amplified fragment from the template λDNA fragment (TX803, Takara) and BkActin were used, respectively. The PCR products were purified with MultiScreen PCR Cleanup kit (MILLIPORE, Massachusetts, MA, USA) and washed with 100 μl sterile deionized water and resuspended in 15 μl sterile deionized water. PCR products were examined and the concentrations were adjusted to or greater than 100 ng/μl in 50% DMSO. The DNA samples were spotted in four replicates successively on the CMT-GAPS II coated glass slides (Corning, New York, NY, USA) with Cartesian SynQUOD PixSys4500 (Genomic Solutions®, Michigan, MI, USA) at 24 to 26°C under RH 60%. After printing, the slides were immobilized by baking and blocking. Preparation of the labeled cDNA and hybridization procedure were followed as described in the instruction manual of 3DNA™ Array 50™ kit (Genisphere, Pennsylvania, PA, USA) including 500 pg polyA$^+$ λRNA (TX802, Takara) as an external control. The hybridization signals for each feature were scanned using the GenePix 4000B and digitized with the GenePix 3.0 software (Axon Instruments, Inc., Union City, CA, USA). Signal intensity of each probe was obtained by average of four replicates and normalized. For further analysis, DNA microarray data files were imported into Spotfire (Genisphere, Pennsylvania, PA, USA).

3. Results and discussion

3.1. Isolation of MeJA induced genes by PCR-select cDNA subtraction

The *B. kaoi* adventitious roots which were treated with MeJA became darker brownish and produced fewer roots than the control, five weeks after the MeJA application (Figure 2). However, no significant effects of MeJA on the growth of adventitious roots were observed within four weeks of culture. A basic database of ~700 unigene ESTs was obtained from PCR-select cDNA subtraction and classified according to the functional organization of the Arabidopsis genome. The insert sizes ranged from 0.2 to 1.2 kb. These genes possibly encode transcription regulators, signal transduction proteins, metabolism related protein and stress related proteins (Figure 3). Significant level of the *β-amyrin synthase* transcripts was detected in plants over 3

months, primarily in roots, while a low level of the *cycloartenol synthase* transcripts was found during all developmental stages. *β-amyrin synthase* mRNA level was enhanced by MeJA treatment but not affected by salicylic acid (Figure 4). Our previous results also showed that the transcript levels of the *B. kaoi defensin* and *β-amyrin synthase* differed at various developmental stages and were modulated by MeJA treatment (data not shown). MeJA also increased transcription of genes that regulate JA synthesis like OPR3 as previously reported (Mussig et al., 2000; Sasaki et al., 2001; Suzuki et al., 2005). The mRNA level of a putative transcription factor *WRKY26* in *B. kaoi* increased within 24 h after the application of MeJA but not SA (Figure 4). The WRKY transcription factors have been implicated in the control of some stress responses (Eulgem et al., 2000).

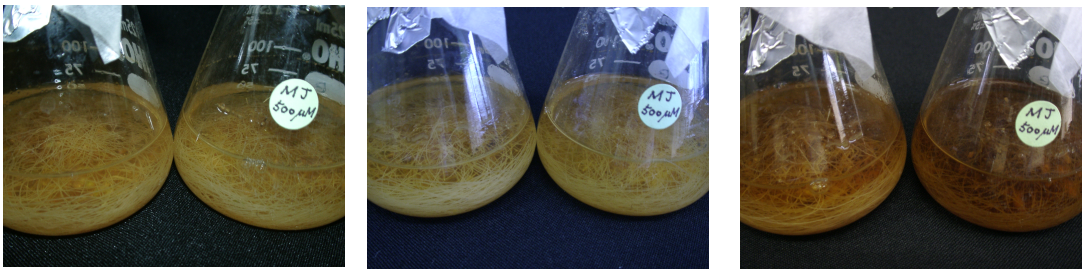

Figure 2. *B kaoi* adventitious root without or with treated MeJA at different time intervals. The adventitious roots which were treated with MeJA became darker brownish and produced fewer roots than the control after 5 weeks.

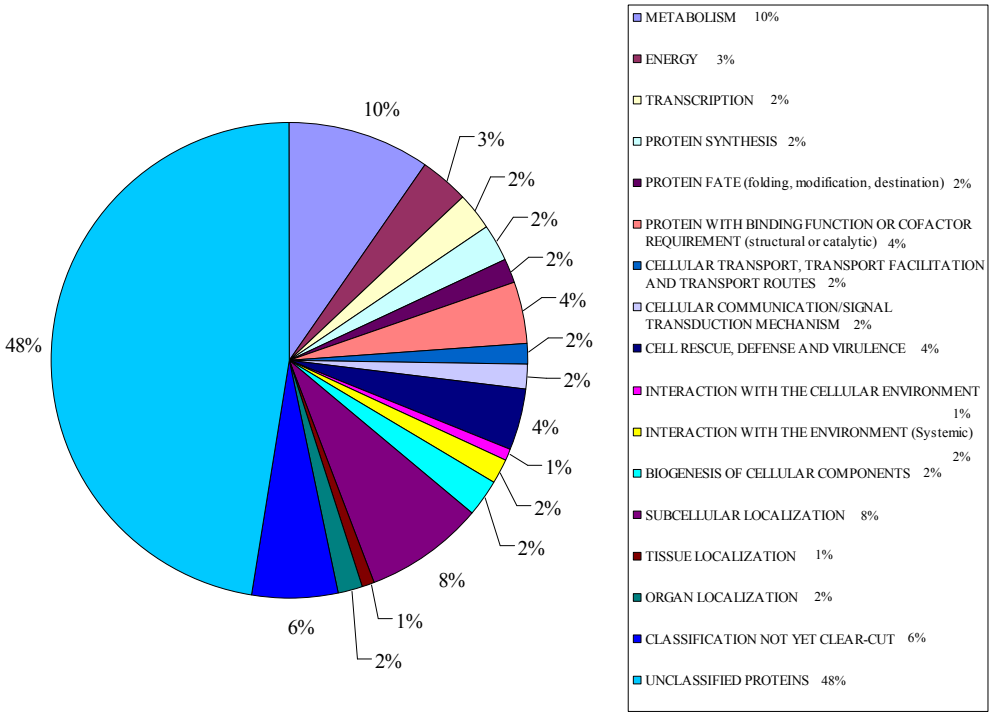

Figure 3. Functional distribution of *B. kaoi* genes in response to MeJA using PCR-select cDNA subtraction. The BLASTX hits of 674 ESTs were classified according to the functional organization of the Arabidopsis genome.

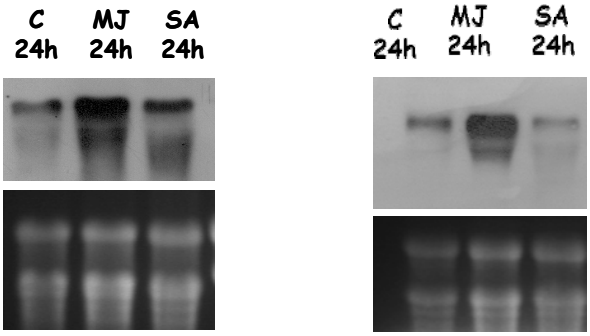

Figure 4. Northern blot analyses of the transcript levels of *β-amyrin synthase* (left, upper) and a putative *WRKY26* (right, upper) in *B. kaoi* plants treated with 100 μM MeJA or 100 μM SA. Total RNA (20 μg per sample) was separated on 1.2 % agarose gel containing formaldehyde, blotted onto a positively charged nylon membrane and then hybridized to DIG-labeled probe. Ethidium bromide stained rRNA is shown as loading control (bottom).

3.2. MeJA induced genes and industrial application

Saponins are attracting interest in view of their multiple biological activities. Despite the interest in facilitating the production of triterpene saponins for development of pharmacological agents, most of the steps in their biosynthesis remain uncharacterized at the molecular level. Recently, the exogenous MeJA has been reported to induce the accumulation of βAS transcripts in *Panax ginseng* (Lee et al., 2004), βAS and SQS transcripts in *Glycyrrhiza glabra* and the accumulation of soysaposin in licorice (Hayashi et al, 2003). The full length βAS cDNA of *B. kaoi* has been cloned and designated as *BkbAS*. Functional analysis of the *BkbAS* is under progress. The MeJA modulated defensin is a potential source of antimicrobial products. The full length *B. kaoi* defensin cDNA designated *BkDF1* was expressed in *Pichia pastoris* to produce matured proteins. The antifungal test of BkDF1 against *B. cinerea* was performed with discs placed on the plate when the radius of fungal colony reached about 3 cm. Sterile water and 40 μg of purified BkDF1 fusion protein were added to each filter paper. The plates were incubated at 25°C and the inhibition of growth was observed (Figure 5).

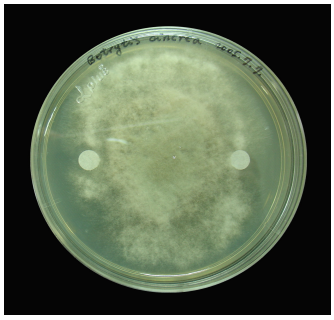

Figure 5. The antifungal test of BkDF1 against *B. cinerea*.
B. cinerea was inoculated on PDA plate. The discs were placed on the plate when the radius of fungal colony reaches about 3 cm. Sterile water (right) and 40 μg of purified BkDF1 fusion protein (left) were added to each filter paper. The plates were incubated at 25°C and the inhibition of growth was observed.

3.3. Identification of MeJA responsive genes by the cDNA microarray

To examine the effect of MeJA on the transcription of 674 genes, a time-course experiment was performed for the plants treated with MeJA for 5 min, 1, 2, 8, 24 hr, 2, 4, 6, 8 day and 2, 3, 4, 5 week. Our data showed that about 100 genes are responsive to MeJA. The levels of MeJA-dependent transcripts primarily occurred during 2 to 8 day after treatment (Figure 6). We also found the mRNA transcript of some MeJA responsive genes had higher levels during 2 to 5 week. To confirm the fidelity of the expression, profiles obtained using the cDNA microarray and real-time RT-PCR will be analysed.

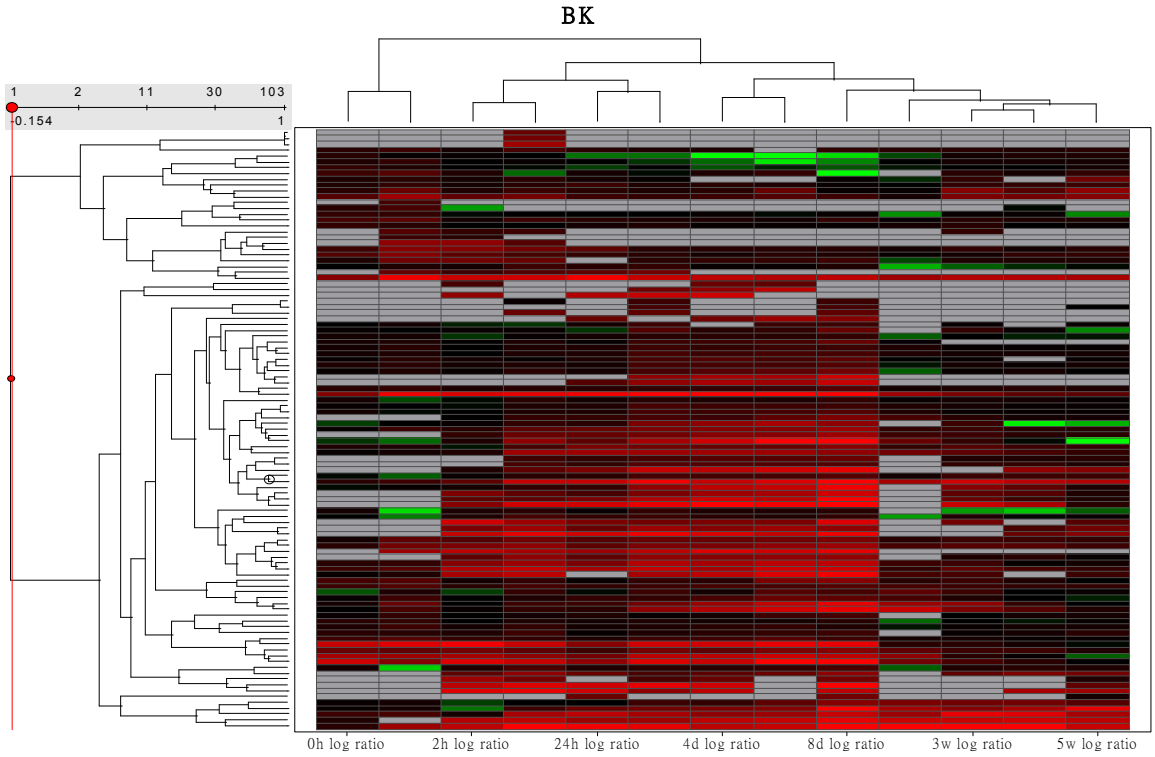

Figure 6. Two-dimensional hierarchical clustering of 103 genes up-regulated by MJ at 13 time points. Colorgram depicts up-regulation (red) and down-regulation (green) array scores for mRNAs.

3.4. Crosstalk of jasmonates with other biosynthesis pathways

Genes up-regulated by MeJA treatment include those involved in jasmonate biosynthesis, amino acid metabolism and disease and wounding response. Our data indicated that MeJA induced the mRNA expressions of genes, possibly related to JA biosynthesis such as the phospholipase D alpha 1, lipoxygenase and allene oxide cyclase family protein. The phospholipase D alpha 1 has been reported to be induced by cold (Vergnolle et al., 2005). It is noteworthy that the gene encoding 1-aminocyclopropane-1-carboxylate oxidase, an enzyme involved in the biosynthesis of ethylene, was induced by ethylene (Whittaker et al., 1997) and also by MeJA in our study. It suggests that jasmonate biosynthesis may have crosstalk with ethylene biosynthesis. MeJA, in fact, has been reported to stimulate ethylene production in many plants such as tomato, Arabidopsis, and tobacco (Xu et al., 1994; O'Donnell et al., 1996; Penninckx et al., 1998).

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