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Gene Note

A stress-responsive multifunctional protein involved in β-oxidation in tobacco plants

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Abstract Biotic and abiotic-induced wounding is one of the severest stresses that plants suffer throughout the growing period. Upon injury, plants rapidly activates a set of genes, which encode diverse proteins to cope with damages. Screening such genes that were transcriptionally activated within 30 min after mechanical wounding in tobacco plants, we identified a particular clone encoding a multfunctional protein, and designated as NtMFP (*Nicotiana tabacum* multifunctional protein). The deduced polypeptide is constituted of 668 amino acids with an apparent molecular mass of 72.3 kDa, and bacterially expressed protein exhibited a clear β-oxidation activity. Fusion proteins with GFP were observed in cytosol, when expressed in onion epidermal cell layers. In addition to wounding, *NtMFP* transcripts were induced by tobacco mosaic virus infection, and by jasmonic acid treatments. When *NtMFP* was suppressed by the RNAi, transgenic tobacco showed dwarfism, early senescence and reduced expresstion of jasmonic acid-responsive genes. Multifunctional protein is generally considered to catalyze multiple steps of the fatty acid β-oxidation. It is also proposed to be involved in the β-oxidation step of jasmonic acid biosynthesis. The present results suggest the possibility that NtMFP commonly functions not only in fatty acid catabolism but also in jasmonic acid biosynthesis pathway.

Key words: β-oxidation, jasmonic acid, multifunctional protein, *Nicotiana tabacum*, wounding.

In plant cell, β-oxidation is involved in fatty acid catabolism, supplying energy by decomposing fatty acids (Kindl 1993). It is also involved in hormone synthesis, including jasmonic acid (JA) and auxins (Baker et al. 2006). Multifunctional protein (MFP) is one of key enzymes that catalyze β-oxidation, being equipped with four enzymatic activities (hydroxyacyl-CoA hydrolyase, hydroxyacyl-dehydrogenase, hydroxyacyl-CoA epimerase and enoyl-CoA isomerase). MFP is primarily localized to peroxisomes, and induced during development and growth (Richmond and Bleeker 1999). For example, the amount of CuMFP from cucumber dynamically increased during seed imbibition (Guhnemma-Schafer and Kindl 1995) Wounding also induced transcripts for MFP proteins, AIM1 and AIM2 in Arabidopsis (unpublished observation; Richmond and Bleeker 1999). Such an increase of MFP was interpreted as that plants cope with energy necessity for germination, and for mending injured areas. In addition, MFP was suggested, but not evidenced to be involved in JA synthesis, catalyzing β-oxidation of trans-Enoyl-CoA (Baker et al. 2008). As MFPs are commonly found in plant cells, it is conceivable that they generally catalyze a broad range of β-oxidation, perhaps on multiple substrates. Here we report isolation and characterization of a tobacco MFP, which is specifically induced upon wound stress and might be involved in JA biosynthesis.

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Tobacco plants (*Nicotiana tabacum* cv Xanthi nc) were grown on soil in a growth cabinet at 23°C under a 14-h light/10-h dark cycle. Healthy leaves from approximately 2 month-old plants were detached, and subjected to stress and chemical treatments. Briefly, wounding was given by punching leaves with a paper puncher; and TMV inoculation was performed by the temperature-shift method (Waller et al. 2006). For chemical treatments, detached leaves were kept in a culture box with a cotton pad containing 10 μl of 100 μM jasmonic acid methyl ester (MeJA), or sprayed with 200 μM salicylic acid (SA). Treated leaf samples were harvested at appropriate time points and immediately frozen in liquid nitrogen before further analyses. The fluorescence differential display was performed essentially as described (Hara et al. 2000), except that samples were fractionated by agarose gel electrophoresis.

Abbreviations: AOS, allene oxide synthase; GFP, green fluorescent protein; JA, jasmonic acid; MeJA, jasmonic acid methyl ester; MFP, multifunctional protein; OPDA, oxo-phytodienoic acid; SA, salicylic acid

Accession number: NtMFP; AB454418
This article can be found at http://www.jspcmb.jp/

DNA and RNA blot hybridization was carried out under the standard method as described (Waller et al. 2006). Subcellular localization was examined by fusion protein with GFP. Full-length cDNA of NtMFP were subcloned into the *Xba*I/*Bam*HI and *Bsr*GI/*Not*I site of the CaMV35S-sGFP (S65T)-nos vector (Chiu et al. 1996) and (S65T)-nos vector alone was used as control. YFP-SKL (containing a C-terminal tripeptide peroxisomal targeting signal, SKL) (Kodama and Sano 2006) was used as the control for peroxisome localization. Gold particle coated plasmids were bombarded into onion epidermal cells using PDS-1000 particle delivery system

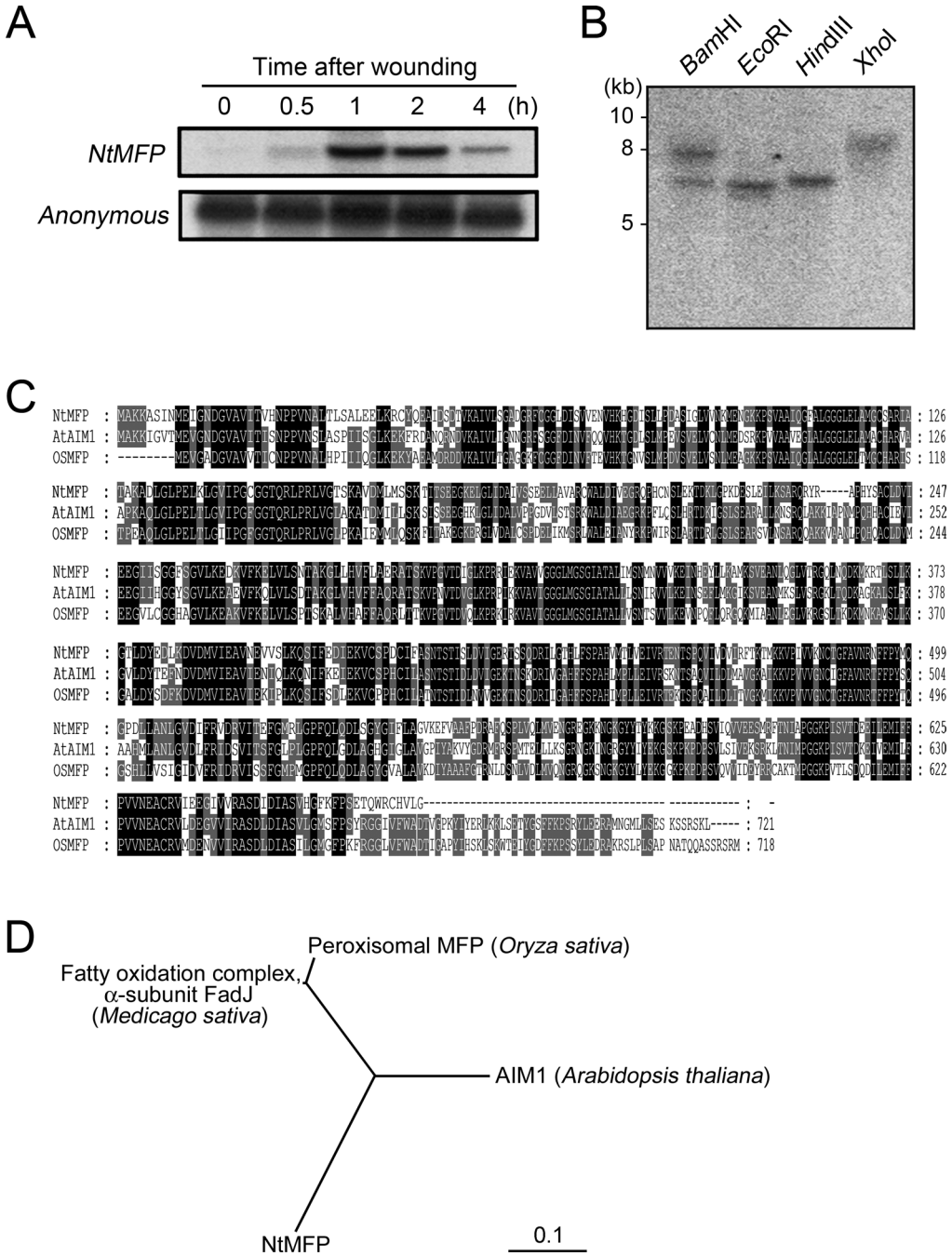

Figure 1. Identification of *NtMFP*. (A) Transcript accumulation. Healthy tobacco leaves were mechanically wounded, and the total RNA was extracted at indicated time points for RNA blot hybridization with the probe of a 1500 bp fragment of *NtMFP* cDNA. As a loading control, constitutively expressed anonymous cDNA was used. (B) Estimation of copy numbers. Total DNA from leaf samples was digested with indicated restriction endonucleases, and subjected to Southern blot hybridization. Molecular size is indicated at the left in kb. (C) Multiple alignment of amino acid sequence with *Arabidopsis* AIM1 (AtAIM1) (accession number NP19630) and *Oryza sativa* peroxisomal MFP (OSMFP) (accession number AAQ13901). Identical and similar amino acids in at least 2 out of 3 samples are framed in black and grey boxes, respectively. (D) Phylogenetic tree. Amino acid sequences were aligned using the ClustalW program including fatty oxidation complex α-subunit FadJ from *Medicago sativa* (accession number, ABE82774).

(Bio-Rad Laboratories). After bombardment, the samples were incubated at 25°C in darkness for 20 to 24 h and GFP was imaged by AX70 Fluorescence microscope (Olympus). The GST-NtMFP recombinant protein was prepared by cloning corresponding full-length cDNA flagment in *Eco*RI and *Not*I sites of pGEX4T-1 vector (Pharmacia), which was then transformed into *E. coli* BL21(DE3) RIL. Recombinant protein was purified through glutathione Sepharose 4B column (GE Healthcare Bio-sciences AB), and subjected to the standard β-oxidase assay using crotonyl-CoA as the substrate. Briefly, hydration of 15 μM crotonoyl-CoA (Sigma) (enoyl-CoA hydratase activity) was determined by measuring absorbancy decrease at 263 nm, and extinction coefficient 6700 $M^{-1} CM^{-1}$ was used to calculate rate of product formation (Binstock and Shulz 1981). Statistical analysis was performed by student's t-test (Snedecor and Cochran 1980). Transgenic RNAi tobacco lines were constructed essentially after the method described (Ogita et al. 2004), using a 315 bp fragment (positions 2059–2313 in *NtMFP* sequence). Thirty nine lines were initially obtained and 3 lines (14, 19 and 22) were selected and used for the present study.

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Screening genes, of which transcripts accumulated within 30 min after mechanical wounding in tobacco leaves by the fluorescence differential display, we initially isolated 9 clones, among which a particular fragment of 1500 bp was selected in this study (Figure 1A). Transcripts of this clone began to accumulate within 30 min after wounding, reaching the maximal level at 1 h, and then gradually declined to the basal level 4 h later (Figure 1A). The copy number of the corresponding gene was estimated by Southern blot hybridization, which showed a single or at most two fragment signals upon digestion with multiple restriction endonucleases (Figure 1B). Although *N. tabacum* is amphidiploid nature, this simple pattern suggests the gene to present as a single copy in its genome. The full length of this clone, which was isolated by screening a cDNA library, was 2398 bp in length, and encoded a polypeptide constituted of 668 amino acids. The putative protein showed a close similarity to multiple functional proteins (MFP) from Arabidopsis AIM1, rice peroxisomal MFP and *Medicago sativa* FadJ (Figure 1C, D). Consequently the protein was designated as NtMFP (*N. tabacum* MFP, accession number AB454418). The structure of NtMFP was predicted after the case of cucumber MFP (CuMFP, CAA55630), which was finely analyzed for enzymatic activities (Preisig-Muller et al. 1994). The first 125 amino acids constitute an isomerase domain, amino acids between 126 and 190 constitute hydratase, and those between 393 and 590 constitute dehyrogenase domain (Figure 2A). All these domains are

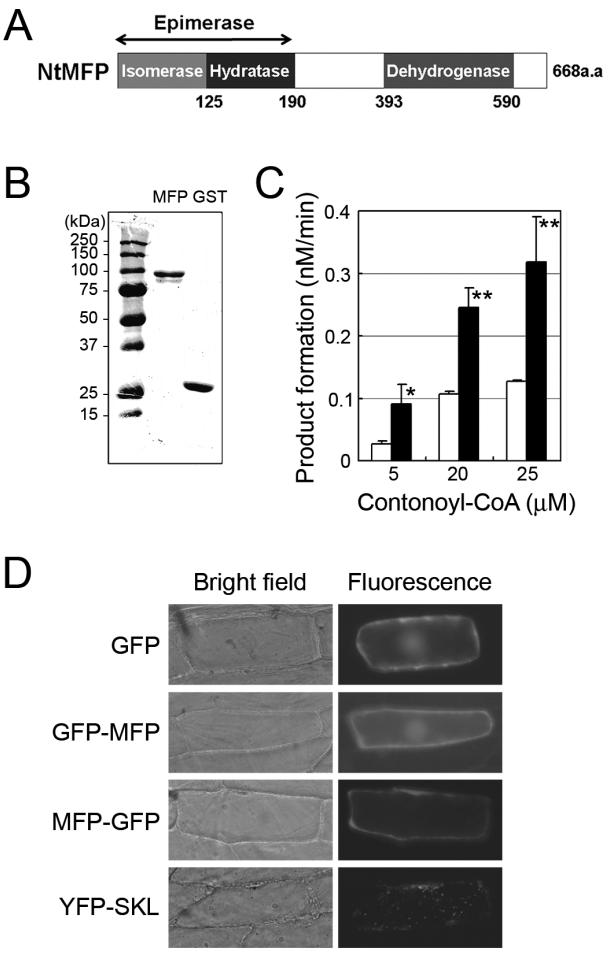

Figure 2. Protein properties and subcellular localization. (A) Pattern diagram of MFP. The catalytic domains were predicted based on CuMFP (accession number, CAA55630), showing the positions of 4 enzymatic activities as indicated. Numbers are amino acid positions from the N-terminus. (B) Protein expression and purification. Recombinant protein (MFP) was bacterially expressed, purified through glutathione-Sepharose column and fractionated by 10% SDS-PAGE. As the control, GST alone was used (GST). Proteins were subjected to immunoblot analysis with anti GST antibodies. Molecular size markers are shown in the left. (C) Enzymatic activity. The β-oxidation activity was estimated by measuring hydration products of contonoyl-CoA in the presence of purified GST-NtMFP (black bar) or GST (open bar) proteins. Values represent the mean of triplicates and standard deviations are shown by bars. Asterisk indicates the significance of difference with the 95% confidence intervals. (C) Cellular localization. Onion epidermal cells were transformed with plasmids expressing green fluorescent protein (GFP), fusion protein with GFP at the N-terminus of NtMFP (GFP-NtMFP), fusion protein with GFP at the C-terminus of NtMFP (NtMFP-GFP) or peroxisome-located SKL protein fused to YFP (YFP-SKL). Fluorescence under UV illumination (Fluorescence) or corresponding differential interference contrast images (Bright field) are shown at the same magnification.

observed in fatty acid β-oxidases (Richmond and Bleecker 1999). To confirm the β-oxidation activity, recombinant protein of GST-NtMFP was bacterially expressed and examined for contrnoyl-CoA hydration activity, a standard indicator activity for the β-oxidation. The purified NtMFP protein through glutathione-column

(Figure 2B) showed a clear enoyl-CoA hydratase activity (Figure 2C), indicating NtMFP to indeed be a β-oxidase.

Since β-oxidation is generally considered to take place in peroxisome (Baker et al. 2006), subcellular localization of NtMFP was determined using fusion proteins with GFP. The transit peptide to peroxisome has been shown to locate at either N-terminus or C-terminus of a protein in question (Subramani 1996). In the case of NtMFP, no clear transit signal was identified, although a potential signal consisted of RIATAKADL (amino acid positions 124–132) was found at the N-terminus region. To avoid interference by fusion, GFP was independently fused to both ends of NtMFP; C-terminus (MFP-GFP) and N-terminus (GFP-MFP). When expressed in onion epidermal cell layers, both constructs gave a clear fluorescence in cytoplasm (Figure 2D). Since the control construct containing a peroxisome-transit signal peptide (SKL) was markedly localized in peroxisomal fraction (Figure 2C), the results suggested that NtMFP is not equipped with a transit peptide to peroxisome, and functions in cytoplasmic fraction.

Expression profile of *NtMFP* upon external stimuli was then examined. As the control, a cDNA for allene oxide synthase (AOS), a key enzyme in JA biosynthesis pathway, was used. Wounding simultaneously induced both transcripts in directly wounded leaves (local) within 1 h, but not in vicinity leaves (systemic) (Figure 3A). Their transcripts were also induced by JA, but not by SA (Figure 3B). These results implied a synchronous regulation of *NtMFP* and *AOS* during stress response. In addition, *NtMFP* was upregulated 3 h after temperature shift upon TMV infection (Figure 3C), suggeting its role in hypersensitive response.

To further investigate biological function, transgenic RNAi lines of tobacco plants were constructed to suppress NtMFP activity, and examined for their feature and stress-response. Among 39 lines initially obtained, the majority showed suppression of *NtMFP* at different levels, and lines R14, R19 and R22 were selected for further analysis (Figure 4A). Phenotypically, the R14 exhibited early senescence and dwafism after 3 months growth (Figure 4B), and these properties were transmitted to the next generation. As R19 and R22 showed similar characterics (data not shown), it was conceivable that NtMFP plays a certain role in growth and stress response. Consequently, leaves from wild-type and transgenic lines were mechanically wounded, and expression of wound-rsponsive genes was periodically examined. Results showed a clear decrease of transcripts for proteinase inhibitor 1a (PI-1a), PI-II and osmotin in transgenic lines (Figure 4C). Densitometric tracing estimated a reduction to up to 40% that of the control (data not shown). Since these genes are well-known JA-responsive markers, these findings suggested that suppression of *NtMFP* resulted in reduction of JA production, and therefore that NtMFP participates in JA biosynthesis.

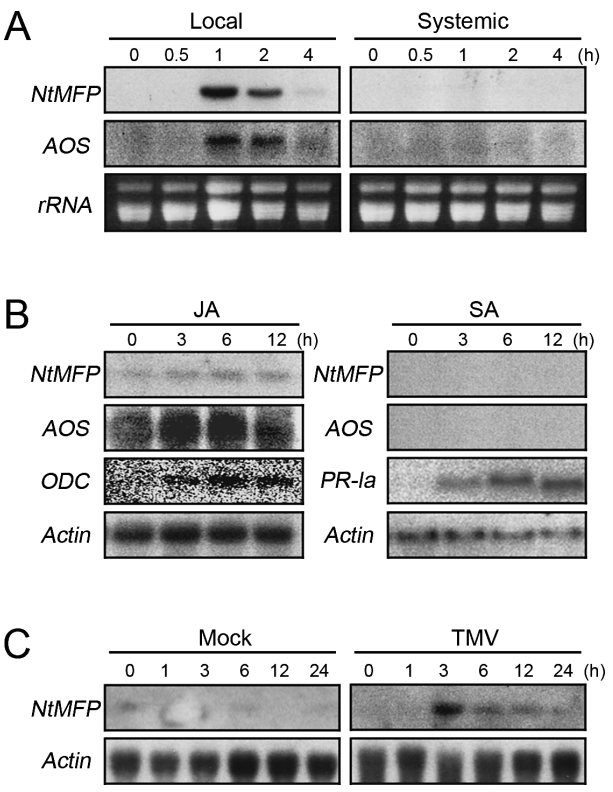

Figure 3. Expression profile. (A) Wounding. Healthy leaves of 3-month-old tobacco plants were mechanically wounded by punching and harvested together with the vicinity unwounded leaves at indicated time points. Total RNA was isolated, and 20 μg aliquots per lane were fractionated with agarose gel electrophoresis, transferred to a nylon membrane and subjected to RNA blot hybridization with the radioactively labeled probe; *NtMFP* or *AOS* (allene oxide synthase). rRNA was used as the loading control. (B) Effects of SA and MeJA. Detached healthy leaves were left for 4 h for acclimatization to the initial wound stress, then treated with MeJA (left) or SA (right) for indicated time intervals. Total RNA was isolated, and successively subjected to RNA blot hybridization as described above with radioactively labeled probes as indicated. For the positive control of drug effects, *ODC* (*ornithine decarboxylase*) and *PR-1a* were used for JA and SA effects, respectively. *Actin* was used as the loading control. (C) Pathogen infection. Detached healthy leaves were treated with 10 mM sodium phosphate buffer alone (Mock), or containing tobacco mosaic virus (10 μg ml^{-1}) (TMV), incubated at 30°C for 48 h, and then transfered to 20°C. Total RNA was isolated at the indicated time points, and subjected to RNA blot hybridization with the radioactively labeled *NtMFP* probe. *Actin* was used as the loading control.

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Since the discovery in 1962 (Demole et al. 1962), jasmonates (jasmonic acid methyl ester, MeJA and JA) have been known to play fundamental roles in various aspects of plant life. For example, they have long been known to be essential in biotic and abiotic stress response as the signaling molecule to activate defense reactions against pathogens, herbivores, wounding, drought and salinity (Cheong and Choi 2003; Balbi and

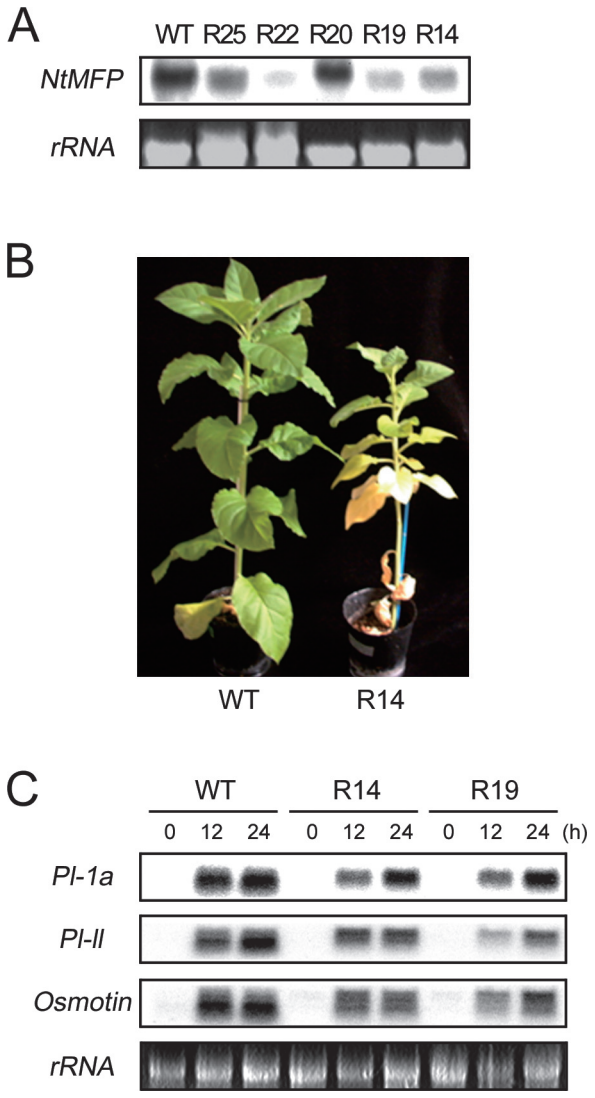

Figure 4. Transgenic RNAi plants. (A) Confirmation of RNAi. Total RNA was isolated from healthy leaves of wild type (WT) or transgenic RNAi lines with indicated line numbers, and subjected to RNA blot hybridization with the radioactively labeled *NtMFP* probe. *rRNA* was used as the loading control. (B) Phenotypes. Features of wild-type (WT) and RNAi line R14 were photographed after 3-month growing. Note the R14 showing dwarfism and senescent leaves. (C) Effects of wounding on JA-responsive gene expression. Healthy leaves of wild-type (WT), RNAi lines R14 or R19 were wounded by punching, and harvested at indicated time points. Total RNA was isolated and used for RNA blot hybridization with radioactively labeled probes, *PI-1a* (proteinase inhibitor 1a), *PI-II* (proteinase inhibitor II) and *Osmotin*. *rRNA* was used as the loading control.

Devoto 2008). Recent surveys have also revealed that jasmonates mediate diverse developmental processes, including seed germination, root growth, fertility, fruit ripening and senescence (Cheong and Choi 2003; Wasternack 2007; Balbi and Devoto 2008). Our observation on NtMFP is consistent with this view, suggesting its role in growth, development and wound response; all assigned to JA function (Cheong and Choi 2003). An unexpected finding was that NtMFP appeared to be localized in cytoplasm, since the known β-oxidation mostly occurs in peroxisomes/glyoxisomes (Schaller 2001; Wasternack 2007). Indeed, recent studies have suggested that JA biosynthesis initiates and proceeds in chloroplast up to synthesis of oxo-phytodienoic acid (OPDA), which is translocated and further converted into JA in peoxisomes (Wasternack 2007; Baker et al. 2008). However, the exact intracellular location is still controvertial: as two enzymes (OPDA reductase and JA carboxyl methyltransferase) catalyzing the last steps were found in cytoplasm (Schaller 2001; Cheong and Choi 2003). The present finding showing NtMFP in cytoplasm provides a simple idea, suggeting all the biosynthesis steps after OPDA take palce in cytoplasm, instead of precursors being in-and-out of peroxisomes. Further analyses including JA quantification in transgenic lines and substrate determination will bring out substantial evidence for NtMFP function. Overall, it is tempting to speculate that NtMFP plays a multiple roles for the β-oxidation step in both fatty acid catabolism and JA biosynthesis pathways.

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