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Floral Nectary-specific Gene *NTR1* Encodes a Jasmonic Acid Carboxyl Methyltransferase

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***NTR1* gene of *Brassica campestris* L. ssp. *perkinensis* encodes a floral nectary-specific methyltransferase. In this study, the *NTR1* cDNA was expressed in *E. coli* to examine the enzymatic characteristics of the protein product. The GST-NTR1 fusion protein was purified to near homogeneity, showing that the size of NTR1 was 44 kDa. The protein reacted specifically with jasmonic acid (JA), consuming methyl group from *S*-adenosyl-L-methionine (SAM). GC-MS analysis revealed that the compound produced was authentic methyl jasmonate (MeJA), suggesting that NTR1 is an *S*-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase. *Km* values of NTR1 for JA and SAM were 38.0 and 6.4 μM, respectively. Optimal activity of the NTR1 was observed at 20°C, pH 7.5, in the presence of 100-150 mM KCl. Thus, kinetic properties, thermal characteristics, optimal pH, and ion-dependency of the NTR1 activity were almost identical to those of *Arabidopsis* JA methyltransferase JMT, indicating that these two proteins are orthologues of each other.**

Key words: Brassica campestris, *jasmonic acid carboxyl methyltransferase, jasmonic acid, methyl jasmonate.*

Methyl jasmonate (MeJA) is a fragrant compound initially identified from the flowers of *Jasminum grandiflorum*,[1] and is ubiquitously distributed in the plant kingdom.[2] Accumulating evidence has suggested that MeJA and its free acid jasmonic acid (JA), collectively referred to as jasmonates, are important cellular regulators modulating diverse developmental processes such as seed germination, flower and fruit development, leaf abscission, and senescence.[3] In addition, jasmonates are signal transducers inducing plant resistance against a group of pathogens[4] and mechanical or insect-derived wounding.[5] In particular, MeJA could be a vapor-phase long distance signal mediating intra- and interplant communications for defense responses[6] and developmental processes.[7]

Jasmonates are synthesized from linolenic acid in plants via the octadecanoid pathway,[3,8] and thus this group of compounds is similar in structure and biogenesis to the animal prostaglandin. Briefly, linolenic acid is oxygenated by lipoxygenase, and then converted to 12-oxo-phytodienoic acid (12-oxo-PDA) by allene oxide synthase (AOS) and allene oxide cyclase. JA is synthesized from the 12-oxo-PDA through reduction and three steps of β-oxidation, and is then catabolyzed further to form its volatile methyl ester MeJA and numerous conjugates.[2]

Very recently, we cloned a gene encoding the enzyme that catalyzes methylation of JA to form MeJA from *Arabidopsis thaliana*.[9] It has been demonstrated that the *Arabidopsis* gene *JMT* encodes an *S*-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase (JMT).[9] The JMT gene was cloned using the *Brassica campestris* floral nectary-specific methyltransferase gene *NTR1* as a probe.[10] The JMT protein showed 85% amino acid sequence similarity with the NTR1. The two proteins share several common features in structural organization, including motifs that are known to be the binding sites (motifs I and III) of SAM,[11,12] a well-known methyl donor in plant cells.

In this paper, we describe the enzymatic characteristics of recombinant NTR1 protein obtained by expressing the *NTR1* cDNA in *E. coli*. Substrate specificity and kinetic characteristics of the recombinant NTR1 protein were found to be almost identical to those of JMT, an indication that these two enzymes are orthologues of each other.

Materials and Methods

Bacterial strains and culture media. Bacterial strain used for the expression of genes in this experiment was *E. coli* BL21 (DE3) [hsdS, gal (cIts857, ind1, Sam7, nin5, lacUV5-T7 gene 1)]. The bacteria were grown in Luria-Bertani (LB)

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Abbreviations: GST, glutathione-*S*-transferase; JA, jasmonic acid; JMT, jasmonic acid carboxyl methyltransferase; MeJA, methyl jasmonate; PAGE, polyacrylamide gel electrophoresis; SAM, *S*-adenosyl-L-methionine; SDS, sodium dodecyl sulfate.

medium (1% tryptone; 0.5% yeast extract; 0.5% NaCl). Solid medium was made with 1.5% (w/v) Bacto-agar.

Enzymes and chemicals. Restriction enzymes were purchased from Boehringer Mannheim and KOSCO. JA, MeJA, SAM, [^{14}C]SAM, acrylamide, and N, N'-bisacrylamide were from Sigma, Dupont, and Serva, respectively.

Expression and purification of the recombinant NTR1 protein. *NTR1* cDNA[10] was inserted into the pGEX-2T vector (Amersham Pharmacia) at *Eco*RI site to fuse with a *GST* gene. The recombinant DNA was transformed into *E. coli* BL21. Orientation of the insert was examined through digestion with diagnostic restriction enzymes and partial DNA sequencing of the transformed plasmids.

To express the recombinant enzyme, transformed *E. coli* BL21 was inoculated into LB medium containing 50 μg/m*l* of ampicillin and grown at 37°C. When A_{590} of the media reached 0.5-0.8, isopropyl β-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM. Incubation was continued at 20°C for additional 5 h, and the cells were harvested using a table-top centrifuge.

The cell pellet was resuspended in 50 m*l* of 4 mM sodium phosphate buffer (pH 7.3) containing 150 mM NaCl, 1% Triton X-100, and 1 mM phenyl methyl-sulfonyl fluoride (PMSF), and was sonicated on ice 10 times for each 30 s. The lysate was centrifuged at 16,000 *g* for 30 min. The supernatant was incubated with glutathione-agarose beads (Sigma) at 4°C for 3 h, and GST fusion proteins were purified as described by Guan and Dixon.[13] Eluted protein fractions were further purified using a Superdex 200 HR 10/30 column as described by Seo *et al.*,[9] and stored at 4°C for 2 months without significant loss of the activity.

Molecular weight determination. Molecular weight of the protein was estimated either through denaturing PAGE or gel-filtration analysis. SDS-PAGE was carried out on 13% polyacrylamide gel and calibrated with molecular weight standards at the range of 6.5-200 kDa (Sigma). For gel filtration analysis with Superdex 200 HR 10/30, the column was calibrated with the following protein standards: aprotinin (6.5 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (68 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). A standard curve was obtained by plotting the retention time (Ve/Vo) of the protein standards against the log of the molecular weights.

Enzyme assay. Enzyme activity of the purified GST-NTR1 fusion protein was assayed by measuring the production of methyl esters from 1 mM substrate and 1 mM SAM including 6.4 μM [^{14}C]SAM. A standard condition for the enzyme assay was established by applying appropriate protein (10 pmoles) in 50 μ*l* of assay buffer (50 mM Tris-HCl, pH 7.5, for standard assay), cation clorides (100 mM KCl for standard assay), and 10 mM β-mercaptoethanol. For standard assay, reactions were conducted at 20°C for 30 min, at which the reaction velocity was linear during the incubation period. After the enzyme reaction, the reaction mixture was extracted with 100 μ*l* of ethyl acetate, and radioactivity at the organic phase was measured using a liquid scintillation counter. In all cases, three independent assays were performed for each point, and the average was calculated. Amounts of MeJA synthesized from JA were determined by comparing the radioactivity and calculated peak area for MeJA on the mass chromatogram through GC-MS analyses calibrated with chemical standard MeJA.

For the kinetics studies, *K*m and Vm values for each substrate were calculated using Lineweaver-Burk plots. Protein concentrations were determined through the method of Bradford[14] using bovine serum albumin as a standard.

GC-MS analysis. For GC-MS analysis, total jasmonates were extracted from the enzyme assay mixture with 1 : 2 (v/v) mixture of hexane/dichloromethane as described by Weber *et al.*[15] The concentrated samples were analyzed directly through GC-MS as described by Creelman *et al.*[5]

Results

Purification of recombinant NTR1 protein. To characterize the biochemical properties of *B. campestris* NTR1, the *NTR1* cDNA[10] was expressed in *E.coli*. The expression vector provides a GST at the N-terminus of the fusion enzyme that can be utilized for rapid purification through glutathione agarose chromatography. Recombinant protein (GST-NTR1) was purified through glutathione agarose column chromatography and Superdex 200 HR 10/30 column chromatography. The purified protein was near homogeneity, showing 70 kDa in the

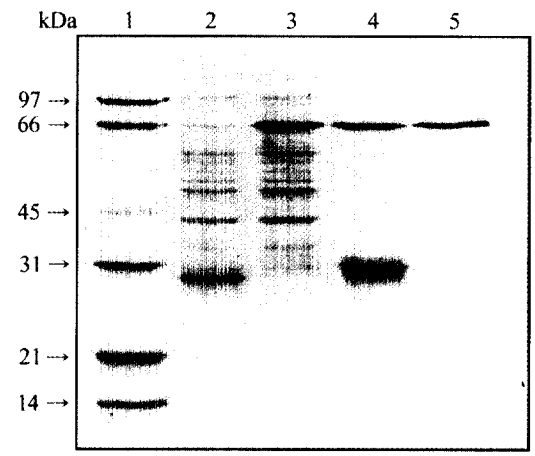

Fig. 1. Purification of recombinant JMT. The recombinant *GST-NTR1* gene was transformed in *E. coli* BL21, and its expression was induced with IPTG. Proteins were purified through glutathione agarose column and Superdex 200 column chromatographies. The purified proteins were analyzed through 12.5% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, protein molecular mass marker; lane 2, 15 μg of total protein from pGEX-2T-transformed *E. coli* harvested after the addition of IPTG; lane 3, 15 μg of total protein from *E. coli* after the induction of GST-NTR1 fusion protein; lane 4, 5 μg of proteins eluted from the glutathione agarose column; lane 5, 5 μg of proteins eluted from the Superdex 200 column. Molecular mass (in kDa) of each protein marker is indicated on the left side of the gel.

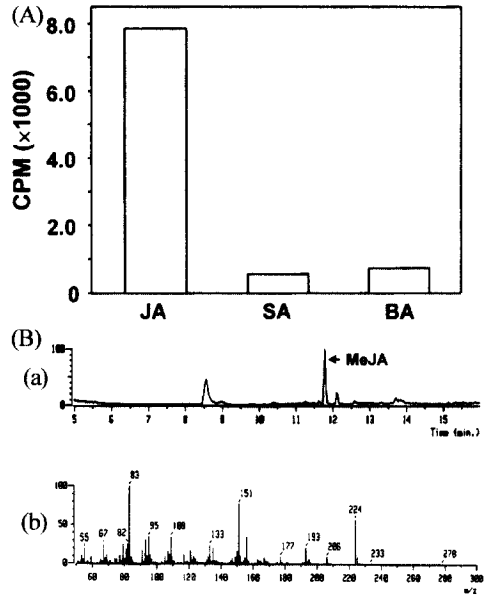

Fig. 2. Methyltransferase activity of NTR1. (A) Substrate specificity of NTR1. Purified GST-NTR1 fusion protein (10 pmoles) was reacted with 1 mM jasmonic acid (JA), salicylic acid (SA) or benzoic acid (BA) and 1 mM SAM including 6.4 μM [^{14}C]SAM at 20°C for 30 min under the standard assay condition described in Materials and Methods. The reaction mixture was extracted with ethyl acetate, and radioactivity in the organic phase was counted using a liquid scintillation counter. Three independent assays were performed for each substrate, and the average was calculated. (B) GC-MS analysis of the reaction product from JA and SAM by NTR1 protein. The reaction products were extracted with ethyl acetate and analyzed through GC-MS. a, mass chromatogram; b, mass spectrum. Molecular mass of MeJA is 224.

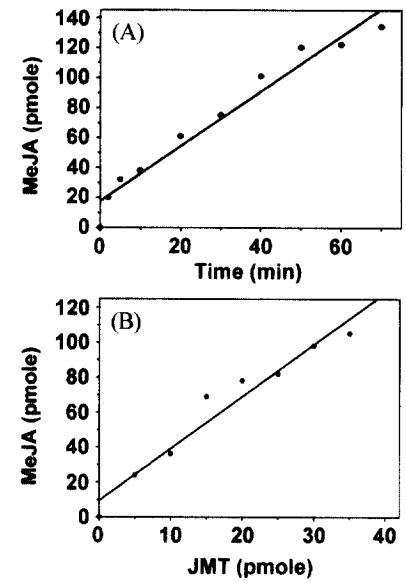

Fig. 3. Kinetic characteristics of NTR1 protein. (A) Time course of steady-state NTR1 activity. Purified GST-NTR1 protein (10 pmoles) was incubated with 1 mM JA and 1 mM SAM including 6.4 μM [^{14}C]SAM at 20°C. Samples were withdrawn at the indicated time points, extracted with ethyl acetate, and the produced [^{14}C]MeJA was determined. (B) Dose dependency of NTR1 activity. Purified GST-NTR1 protein at various concentrations was incubated with 1 mM JA and 1 mM SAM including 6.4 μM [^{14}C]SAM at 20°C for 30 min, and the produced [^{14}C] MeJA was determined. Each data point represents the average of three replicates.

SDS/PAGE analysis (Fig. 1). Taking into account the N-terminal GST (22 kDa), the size of NTR1 was 44 kDa as was expected from its deduced 392 amino acid residues. The molecular weight of the recombinant protein was also confirmed through gel filtration chromatography on an FPLC Superdex 200 HR 10/30 column (data not shown), which indicated that the purified GST-NTR1 fusion enzyme is monomeric.

Methyltransferase activity of NTR1 protein. To test the enzyme activity, affinity-purified recombinant NTR1 was incubated under the standard assay condition described in Materials and Methods with each of various putative substrates in the presence of SAM, in which the methyl group is radio-labeled with [^{14}C]. JA reacted with NTR1 to produce ethyl acetate-extractable product(s), consuming radioactive methyl group from SAM (Fig. 2A). In contrast, NTR1 did not convert salicylic acid or benzoic acid to the corresponding methyl esters. GC-MS analysis demonstrated that the reaction product from JA was authentic MeJA (Fig. 2B).

Kinetic parameters for NTR1 enzyme. For kinetic studies, appropriate enzyme concentration and incubation period were determined so that the reaction velocity was linear during the incubation period. Under the standard assay condition, the amount of MeJA synthesized by the NTR1 enzyme from JA and SAM increased with the reaction time (Fig. 3A). The MeJA production activity was also dependent on the concentration of NTR1 protein (Fig. 3B). To measure the *K*m for each substrate, concentration of one substrate was fixed at a saturated level and the concentration of the other substrate to be measured was varied. Lineweaver-Burk plots were drawn to obtain the *K*m and Vm values. *K*m values of NTR1 for JA and SAM were 38.0 and 6.4 μM, respectively (Table 1). Turnover numbers (*k*cat values) of the enzyme for JA and SAM were 26 and 68 S^{-1}, respectively. The catalytic efficiencies of the JMT enzyme, calculated as k_{cat}/K_m, were 0.7 μMS^{-1} for JA and 11.0 μMS^{-1} for SAM.

Effect of temperature on the activity and stability of NTR1. NTR1 was incubated at various temperatures ranging from 5 to 45°C for 30 min under the standard assay condition. Optimum temperature of JA methyltransferase activity was observed between 15 to 25°C (Fig. 4A).

Thermal stability of the NTR1 protein was also examined by incubating the protein at a broad range of temperatures for 30 min before the activity assay. In this experiment, JA methyltransferase activity of the NTR1 observed at 4°C storage was maintained up to 30°C, but significantly decreased thereafter (Fig. 4B). Upon 30 min of storage at 60°C, JMT activity was completely abolished.

Optimum pH for NTR1 activity. Optimum pH of the

Table 1. Kinetic parameters of NTR1.

Substrate	K_m (μM)	V_m (nmole/min)	k_{cat} (S^{-1})	k_{cat}/K_m (μM^{-1} S^{-1})
S-adenosyl methionine	6.4	81	68	11.0
(±) Jasmonic acid	38.0	31	26	0.7

The values are the averages of three independent measurements. Each value was obtained using Lineweaver-Burk plots, which were linear within experimental error.

NTR1 activity was determined using three buffer systems. Reactions were carried out in 50 mM sodium acetate buffer at pH ranging from 4.0 to 6.0, 50 mM sodium phosphate buffer at pH ranging from 6.0 to 7.5, and 50 mM Tris-HCl buffer at pH ranging from 7.0 to 11.0. JA methyltransferase activity was optimized at pH 6.5 through 8.0, exhibiting the maximal activity at pH 7.5 (Fig. 5).

Effects of various cations on NTR1 activity. Effects of various cations on NTR1 activity were examined. Divalent ions such as Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} did not significantly activate the NTR1 activity at 100 mM each, while monovalent ions such as Na^+, NH_4^+, and K^+ showed better activation effects (Fig. 6A). In particular, NTR1 required K^+ higher than 50 mM concentration for a significant JA methyltransferase activity, exhibiting the maximal activity at 100-150 mM concentration (Fig. 6B). K^+ above this concentration slightly inhibited the enzyme activity.

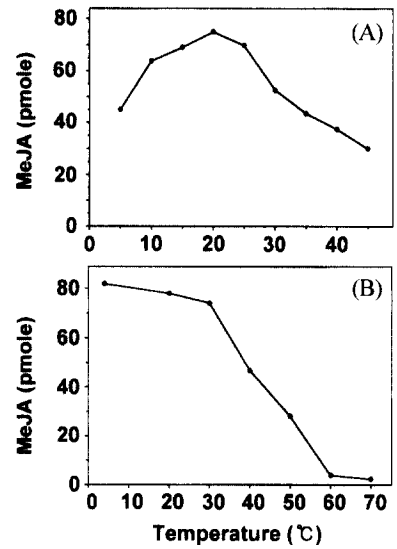

Fig. 4. Thermal properties of NTR1 protein. (A) Effect of temperature on NTR1 activity. Reactions were performed at various temperatures for 30 min in 50 mM sodium phophate buffer, pH 7.5. (B) Effect of temperature on the JMT stability. Purified GST-NTR1 proteins (10 pmoles) were dissolved in assay buffer and incubated at various temperatures for 30 min, and 1 mM each JA and SAM, which included 6.4 μM [^{14}C]SAM, were added to the reaction mixture. The reactions were continued at 20°C for 30 min. Each data point represents the average of three replicates.

Discussion

MeJA is an important plant regulator that modulates diverse develomental processes and activation of defense responses against various biotic and abiotic stresses. In addition, due to its gaseous nature, MeJA has become a strong candidate for signal transducer mediating long distance intra- and interplant communications.[6,7] Furthermore, recent studies have revealed that MeJA enhances the biosynthesis of a group of important secondary metabolites including taxol, an anticancer agent synthesized from *Taxus cuspidate*.[16]

MeJA is a methyl ester of its free acid JA. Recently, we cloned a novel gene that encodes the JA-specific methyl transferase JMT from *Arabidopsis thaliana*.[9] The JMT protein showed strong homology with the amino acid sequences deduced from *Brassica* floral nectary-specific genes *NTR1*,[10] *Clarkia breweri* SA methyltransferase (SAMT) catalyzing methyl salicylate formation from salicylic acid,[17] and a benzoic acid methyltransferase (BAMT) isolated from snap dragon.[18]

In the present study, we examined enzymatic characteristics of the protein product obtained by expressing the *NTR1* cDNA in *E. coli* (Fig. 1) to demonstrate that the *NTR1* also encodes an *S*-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase. NTR1 transferred the methyl group from the methyl donor SAM to specifically produce MeJA from JA, demonstrating its JA-specific carboxyl methyltransferase activity (Fig. 2). Kinetic properties (Fig. 3), thermal characteristics (Fig. 4), optimal pH (Fig. 5), and ion-dependency (Fig. 6) for the enzyme activity were almost identical to those of *Arabidopsis* JMT,[9] suggesting that these two proteins are orthologues of each other. In fact, amino acid identity between them was 87%.

NTR1 gene was initially identified in floral nectarious cells of *Brassica campestris*.[10] *Arabidopsis JMT* was also not detected in young seedlings but expressed in developing flowers.[9] Thus, expression pattern of the two genes are also quite similar to each other.

It appears that NTR1 catalyze MeJA production in the cytoplasm, since the protein contains neither the apparent organ-specific transit signal peptide nor the hydrophobic regions long enough to be integrated in membranes. In fact, it was demonstrated in an immuno-localization experiment that NTR1 was located in the cytoplasm of *Brassica* cells.[10] Cellular organelles such as plastids[19] or peroxisomes[20] are regarded as the primary sites of JA biosynthesis. Activation of JA biosynthesis is not sufficient to explain the complexity in biologi-

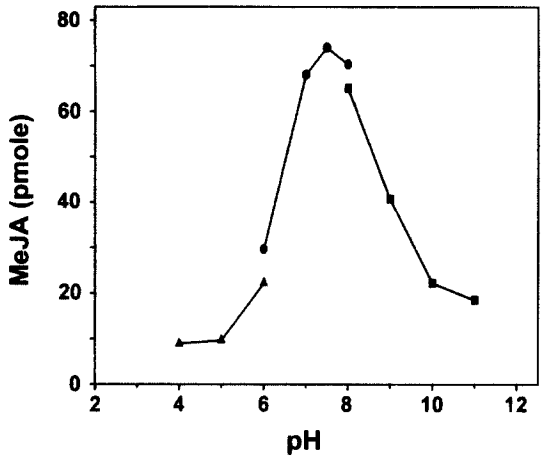

Fig. 5. Effect of pH on NTR1 enzyme activity. Purified GST-NTR1 proteins (10 pmoles) were reacted with 1 mM each JA and SAM (including 6.4 μM [^{14}C]SAM) at 20°C for 30 min in the following 50 mM buffer solutions: (▲) sodium acetate buffer (pH 4.0-6.0), (●) sodium phosphate buffer (pH 6.0-8.0), and (■) Tris/HCl buffer (pH 8.0-11.0). Each data point represents the average of three replicates.

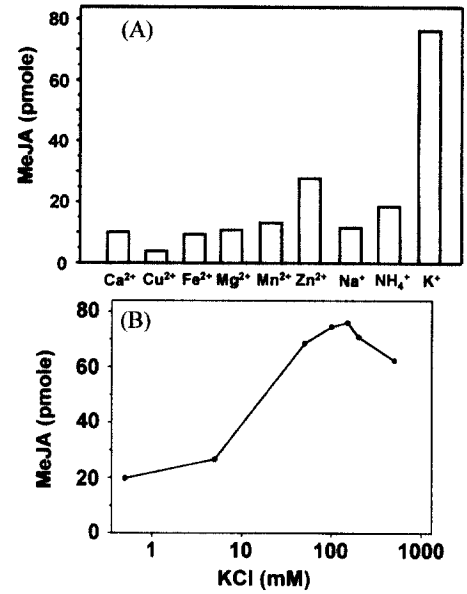

Fig. 6. Effects of cations on NTR1 enzyme activity. (A) Effects of various cations on NTR1 enzyme activity. Purified GST-NTR1 protein (10 pmoles) was incubated with 1 mM jasmonic acid and 1 mM SAM, which included 6.4 μM [^{14}C]SAM, at 20°C for 30 min in the presence of 100 mM each of various cations. (B) Effect of KCl concentration on NTR1 enzyme activity. Each data point represents the average of three replicates.

cal roles of this family of compounds. It was reported that overexpression of the plastidic flax *AOS* cDNA caused an increase in JA levels in transgenic potato plants, while not activating jasmonate-responsive genes.[19] It was thus proposed that mechanical damage or water stress facilitates the movement of JA sequestered in the chloroplast to the cytoplasm, where its receptors are presumably present.[19] However, free acid JA might not be able to move across the cellular membrane without a carrier because of its acidic nature, as observed in an intracellular distribution study of abscisic acid.[21] Thus, it is of interest to investigate further the mechanism of how cytoplasm-localized NTR1 or JMT converts JA accumulated in the organelles into MeJA.

It was also proposed that signals from external stimuli might activate an alternative JA biosynthetic pathway located in the cytoplasm.[22,23] However, overexpression of neither a cytoplasm-localized flax *AOS*[22] nor the *Arabidopsis AOS* cDNA[23] altered the basal level of jasmonates in the transgenic tobacco and *Arabidopsis*. Thus, there might be additional key regulatory points for either the accumulation of jasmonates in the cytoplasm or the generation of signal transducers other than JA.

In contrast, it was observed that transgenic *Arabidopsis* overexpressing the JMT contained threefold elevated level of endogenous methyl jasmonate. The transgenic plants exhibited constitutive expression of JA synthetic genes, jasmonate-responsive genes, and exhibited enhanced level of resistance against a fungal pathogen.[9] Thus, jasmonic acid carboxyl methyltransferases including JMT and NTR1 are key enzymes for the jasmonate-regulated plant responses.

More detailed studies on the modulation of NTR1 expression would open a way toward the comprehensive understanding of how plants control their cellular metabolisms during the reproductive developmental processes and active defense responses.

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