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Phytochemistry 67 (2006) 2002–2008
PHYTOCHEMISTRY
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N-Methyltransferase involved in gramine biosynthesis in barley: Cloning and characterization

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Received 7 April 2006; received in revised form 26 June 2006
Available online 22 August 2006

Abstract

The indole alkaloid gramine occurs in leaves of certain barley (*Hordeum vulgare* L.) cultivars but not in others. A gene sequence in barley that earlier was characterized as a jasmonate-induced *O*-methyltransferase (*MT*) (EC 2.1.1.6, GenBank accession **U54767**) was here found to be absent in some barley cultivars and breeding lines that all lacked gramine. The cDNA was cloned and expressed in *Escherichia coli* and the recombinant protein purified. The purified recombinant protein methylated two substrates in the pathway to gramine: 3-aminomethylindole (AMI) and *N*-methyl-3-aminomethylindole (MAMI) at a high rate, with Km-values of 77 μM and 184 μM, respectively. In contrast, the protein did not exhibit any detectable methylation with the earlier suggested substrate for *O*-methylation, caffeic acid. A number of cultivars and breeding lines of barley were analyzed for presence of the **U54767** gene sequence and MT protein and the enzyme activity *in vitro* with MAMI or caffeic acid as substrates. The results showed a clear relationship between the presence of the *MT* gene, the MT protein and *N*-methyltransferase activity, and confirmed the identification of the gene as coding for an *N*-methyltransferase (NMT, EC 2.1.1) and being involved in gramine biosynthesis.

Keywords: *Hordeum vulgare*; Poaceae; Barley; Gramine pathway; 3-Aminomethylindole; *N*-Methyl-3-aminomethylindole; Caffeic acid; *O*-Methyltransferase; *N*-Methyltransferase

1. Introduction

Plants defend themselves against microorganisms and insects by constitutive and induced defenses, many of them involving secondary metabolites. The indole alkaloid gramine occurs in reed canarygrass (*Phalaris arundinacea*) and in barley (*Hordeum vulgare*). In the former grass, consumption of gramine can adversely affect ruminant performance, whereas in barley, gramine has received attention mainly as a proposed defense compound, especially against aphids (Zúñiga et al., 1985, 1988; Zúñiga and Corcuera, 1986; Kanehisa et al., 1990). Moreover, it has been reported that infestation by the greenbug (*Schizaphis graminum*), (Velozo et al., 1999), pruning, or inoculation with the powdery mildew fungi increased the gramine content in barley leaves (Matsuo et al., 2001).

Gramine occurs in some barley cultivars and is absent or present in very low concentrations in others (Hanson et al., 1981; Lovett et al., 1994; Forslund et al., 1998; Åhman et al., 2000). It is formed from tryptophan, via a pathway involving 3-aminomethylindole (AMI) and *N*-methyl-3-aminomethylindole (MAMI) (Gross et al., 1974). The *N*-methylation of these compounds by an enzyme from barley was first demonstrated by Mudd (1961) and an *N*-methylating enzyme acting on both substrates has been purified and characterized (Leland and Hanson, 1985). However, as yet, the first step in the conversion of tryptophan to 3-aminomethylindole has not been characterized, nor has any gene in the gramine pathway been identified or cloned.

We are investigating the induced defenses to the bird cherry-oat aphid, *Rhopalosiphum padi* in barley. During

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doi:10.1016/j.phytochem.2006.06.036

the work, we identified a differentially regulated clone that was identified in the GenBank database as a gene coding for an *O*-methyltransferase (*OMT*) acting on caffeic acid (accession **U54767**) (Lee et al., 1997a,b). Methyltransferases acting on cinnamic acids such as caffeic acid, their aldehydes, alcoholes or CoA esters have been widely investigated due to their role in lignin biosynthesis (Boerjan et al., 2003). We found however that the *OMT* gene was not expressed in a second barley genotype, which suggested that it might not be involved in primary metabolism, which the proposed function as a caffeic acid methyltransferase indicated, but rather in the biosynthesis of a non-essential secondary metabolite. We therefore hypothesized that the proposed *OMT* gene might be involved in gramine biosynthesis. Earlier reports that the barley cultivar Morex had been shown to lack gramine (Hanson et al., 1981), AMI- and MAMI-methylating activity (Leland et al., 1985), as well as the **U54767** sequence (Lee et al., 1997a), supported this suggestion.

The results, presented here, lead us to redefine the GenBank accession **U54767** as an *N*-methyltransferase (*NMT*), acting on 3-aminomethylindole and *N*-methyl-3-aminomethylindole and being involved in the biosynthesis of the indole alkaloid gramine.

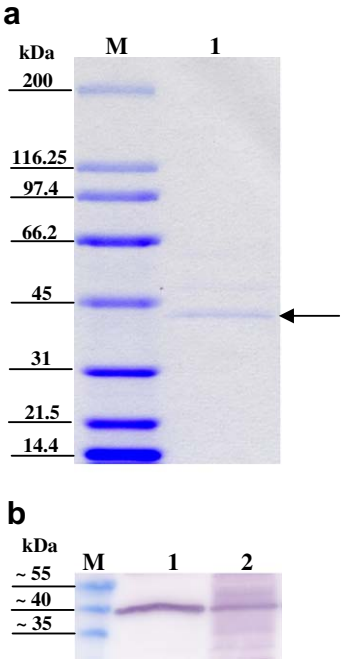

Fig. 1. (a) SDS-PAGE of expressed recombinant MT-protein. M, Protein Marker Broad Range; 1, fraction eluted after DTT-cleavage (50 ng). (b) Western blot using antibodies raised against purified *N*-methyltransferase from barley. Lanes: M, Prestained Protein Marker; 1, fraction eluted after DTT-cleavage (10 ng); 2, crude barley extract (10 μg).

2. Results and discussion

2.1. Purification and characterization of the OMT gene product expressed as a recombinant protein in Escherichia coli

In order to characterize the *OMT* gene product, the cDNA was cloned into the pTYB12 vector and transformed into *E. coli*. Induced cells were harvested and the protein purified using the IMPACT-CN protein purification system. Fig. 1a shows the protein in fractions that eluted after DTT-induced cleavage of the intein-tag that binds to the chitin-column. The fractions contained one protein band, with an apparent molecular mass of about 43 kDa. This is similar to the molecular mass of the purified *N*-methyltransferase acting on AMI and MAMI reported by Leland and Hanson (1985). The protein also cross-reacted with antibodies raised against this protein (Leland and Hanson, 1985) (Fig. 1b).

The methylating activity of the recombinant protein was determined with AMI, MAMI or caffeic acid as substrate and *S*-adenosylmethionine (AdoMet) as a methyldonor. The enzyme exhibited high activity with AMI or MAMI as substrates. The kinetic characteristics of the recombinant protein with regard to AMI, MAMI and AdoMet are shown in Table 1. However, with caffeic acid as substrate, the product formation did not reach above background (Fig. 2). As a control of the caffeic acid enzyme assay, incubations were carried out in parallel with total protein extract from barley cv. Lina. The results showed that the plants contain an OMT acting on caffeic acid which exhibits substrate saturation (Fig. 2). Our results with the purified recombinant protein thus did not confirm the findings by Lee and co-workers, of methyltransferase activity with caffeic acid as substrate

Table 1
Kinetic properties of purified NMT recombinant protein isolated from *E. coli*

Substrate	V_{max} (pkat mg^{-1})	K_m (μM)	V_{max}/K_m
AMI	37.21 ± 1.18	77 ± 0.01	0.48
MAMI	43.54 ± 2.74	184 ± 0.03	0.24
AdoMet	14.50 ± 0.64	148 ± 0.03	0.10

Assays were carried out using 8 μg of NMT, 0.025–0.6 mM of AMI or MAMI, and 0.11–1.1 mM of AdoMet. Values were determined using GraphPad and represent kinetic parameters ± s.d. ($n = 3$).

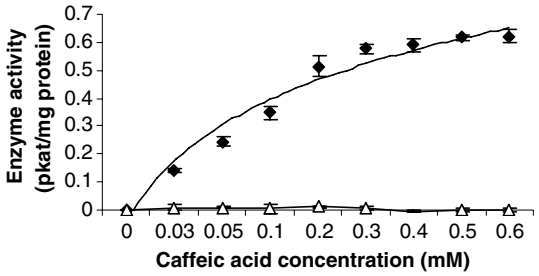

Fig. 2. Methylation of caffeic acid. (◆), total protein extract (10 μg) from barley cv. Lina; (Δ), purified recombinant MT (8 μg) ($n = 3$).

in bacterial lysates from *E. coli* transformed with the **U54767** sequence (Lee et al., 1997b). Based on the enzyme activity of the purified recombinant protein we tentatively characterized the cloned gene as an *NMT*, coding for an *N*-methyltransferase.

2.2. Sequence alignments

The earlier identification of **U54767** as a gene for an OMT was partly based on sequence similarity. However, the sequence identity found between **U54767** and various other OMTs acting on caffeic acid was only between 52% and 59% (Lee et al., 1997b). In an analysis of conserved sequence motifs in plant methyltransferases, the barley **U 54767** was classified as highly unrelated to other OMTs (Joshi and Chiang, 1998). An alignment with our suggested NMT and the caffeic acid OMT consensus in the two regions of importance for substrate specificity identified by Schröder et al. (2002), showed that only 5 out of 14 amino acids are identical in motif 1 and 4 out of 12 amino acids in motif 2 (not shown). In view of the new proposed function of **U54767** gene product as an NMT, sequence alignments were carried out with other *N*-methyltransferases that have been published more recently (Kato and Mizuno, 2004; Raman and Rathinasabapathi, 2003; Uefuji et al., 2003; Choi et al., 2002). However, no significant sequence similarity or common motif was found. Possibly, the barley *NMT* has evolved from an *OMT*, as was proposed for the β-Ala NMT from *Limonium latifolium*, where the protein sequence showed more similarity to OMTs than to other NMTs (Raman and Rathinasabapathi, 2003). In the latter report, it was suggested that sequences annotated as *O*-methyltransferases in the genome databases based on sequence similarity might actually be *N*-methyltransferases (Raman and Rathinasabapathi, 2003). Our results support this suggestion.

2.3. Presence of the MT gene, the NMT protein, gramine content and methyltransferase activity in different barley genotypes

The presence of the *MT* gene and the gramine content was examined in a selection of barley cultivars, accessions and breeding lines. The genotypes were selected to represent barley known from previous investigations either to contain gramine (Lina, Osiris) (Åhman et al., 2000; F. Michoux, personal communication) or to lack this compound or contain only traces of it (Morex, Mona, Golf, Maythorpe, Golf) (Hanson et al., 1981; Forslund et al., 1998; Åhman et al., 2000; F. Michoux, personal communication). Furthermore, the genotype selection represented barley that is resistant (*Hordeum vulgare* ssp. *spontaneum* accession 5, CI 16145, CI 11506, doubled haploid lines 5172-28:4, 5172-39:9 and 5175-50:20) or susceptible (Golf, Lina) to *R. padi*, the resistance having been determined as growth and survival rates of the aphid. The cultivar Barke was included in the screening because it was used to produce the doubled haploid line 5175-50:20.

Southern analyses of several different barley cultivars had shown already that the gene sequence is encoded by a single copy gene (Lee et al., 1997a), and we therefore found it appropriate to use genomic PCR for this analysis. The cultivar Morex has earlier been reported not to contain the gene (Lee et al., 1997a), and this was confirmed (Fig. 3a). The *MT* gene was found to be present in 8 of the 13 investigated barley genotypes (Fig. 3a). Six out of these eight genotypes contained 1–4 mg gramine g^{-1} fr. wt. while the other two cultivars, Barke and Golf, contained very low levels of this compound (about 3 μg g^{-1} fr. wt.). Besides Morex, the cultivars Maythorpe and Mona and the accessions CI 16145 and CI 11506 lacked the gene and contained barely detectable levels of gramine (between 7 and 40 μg g^{-1} fr. wt.) (Fig. 3b). Thus, the results confirmed earlier reports with regard to gramine content, and they clearly showed that only barley plants containing the *MT* gene produce gramine in significant amounts. It was also noted that the *MT* gene as well as gramine was absent in several of the *R. padi*-resistant genotypes.

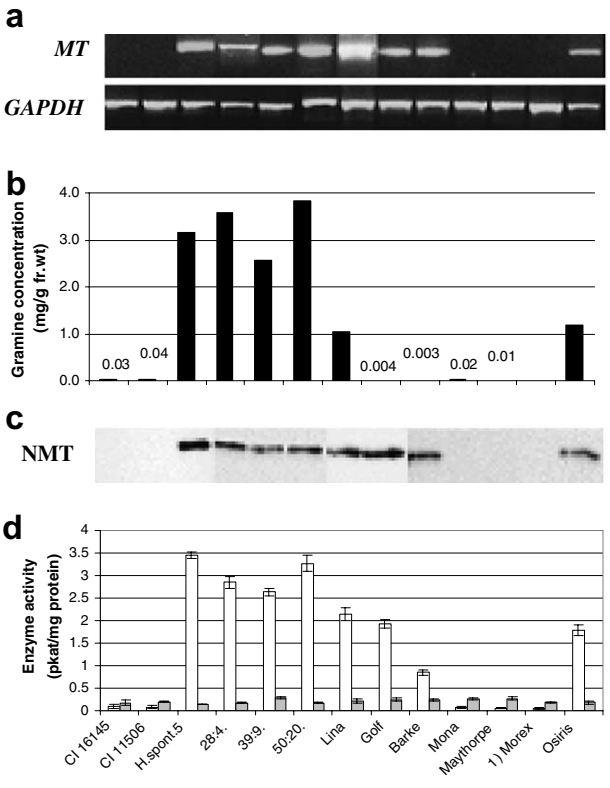

Fig. 3. Presence of the *MT* gene, gramine content, the NMT protein and methyltransferase activity in different barley genotypes. (a) Results of PCR with *MT*-or GAPDH-specific primers. (b) Gramine concentration in tip of first leaf. [1]Results from Hanson et al. (1981). (c) Detection of the NMT protein (10 μg protein/lane) by Western blot with affinity-purified antibodies. (d) Methylating activity *in vitro* with MAMI (white bars) or caffeic acid (gray bars). Error bars show the s.d. ($n = 3$).

Fig. 3c shows the presence of the NMT protein as determined by Western blotting. While the protein could be detected in all genotypes containing the *MT* gene it was absent in all varieties that lacked the gene. The enzyme extracts from the different genotypes were also investigated with regard to methylating activity *in vitro*, using either caffeic acid or MAMI as substrates (Fig. 3d). The results show clearly that significant MAMI-methylating activity is present only in the genotypes carrying the *MT* gene and containing the NMT protein. In contrast, caffeic acid-methylating activity is present in all plants at similar levels.

The cultivars Golf and Barke contain the *MT*-gene and exhibit MAMI-methylating activity but lack gramine. This is probably due to the absence of the gene coding for an as yet unidentified enzyme being involved in the steps preceding the methylating steps in the pathway (Fig. 4). This was also suggested by Leland and co-workers who found three gramine-free barley cultivars to exhibit *N*-methyltransferase activity *in vitro* (Leland et al., 1985). The genotypes lacking the *MT* gene did exhibit detectable, albeit very low MAMI-methylating activity *in vitro*. This could be explained by the presence of other methyltransferases in the enzyme extracts, with low activity towards MAMI. Such non-specific methylating activity might also explain the fact that the investigated genotypes lacking the *MT* gene did contain very low amounts of gramine. Conversely, the *MT* gene product might exhibit low activity on other substrates than AMI and MAMI. This was not investigated, since our results clearly indicated the major role in the biosynthesis of gramine.

In conclusion, our study shows that **U54767**, earlier characterized as coding for a methyljasmonate-inducible *O*-methyltransferase should be re-identified as an *N*-methyltransferase involved in gramine biosynthesis (Fig. 4).

3. Experimental

3.1. Plant material, growth and treatment of plants

Hordeum vulgare cv. Morex (CI 15773) was provided by the National Small Grains Collection, USA. Lina, CI 16145, Barke, Golf, *Hordeum vulgare* ssp. *spontaneum* accession 5, doubled haploid lines 5172-28:4, 5172-39:9 and 5175-50:20 were generously donated by Dr. Inger Åhman, Svalöf Weibull AB, Sweden. CI 11506, Osiris (NGB 11243), Mona (NGB 1499) and Maythorpe (NGB 9608) were obtained from the Nordic Gene Bank (NGB), Sweden. Cultivars Golf (Weibull, 1994) and Lina (Åhman et al., 2000) are susceptible to *Rhopalosiphum padi.* Accessions CI 16145 (van Marrewijk and Dieleman, 1980), *H. spont.* 5, (Åhman et al., 2000), CI 11 506 (Hanson and Charpentier, 1986) and the doubled haploids with *H. spont.* 5 and Lina (for 5175-50:20 also Barke) in their pedigree are resistant to *R. padi* (I. Åhman, personal communication).

Germinated seedlings were planted in soil and grown in a growth chamber at 18 to 22 °C, 16/8-h (light/dark) photoperiod at 160 μmol m^{-2} s^{-1}. Plant age is referred from day of planting, and after 7 days the first leaf is fully expanded and the second leaf is on its way.

For the isolation of the *OMT* cDNA, *H. vulgare*, (cv. Lina) was used. The leaves of 5-day-old plants were immersed in a solution of 45 μM jasmonic acid (Sigma Chemicals, St. Louis, USA) for 24 h in order to induce *OMT* expression (Lee et al., 1997b). Plant material was frozen in liquid nitrogen and stored at −70 °C until further use.

3.2. Differential screening, isolation of proposed OMT clone and sequencing

Seven-day-old barley plants were infested with *R. padi* for 5 days. Subtracted cDNA libraries were constructed

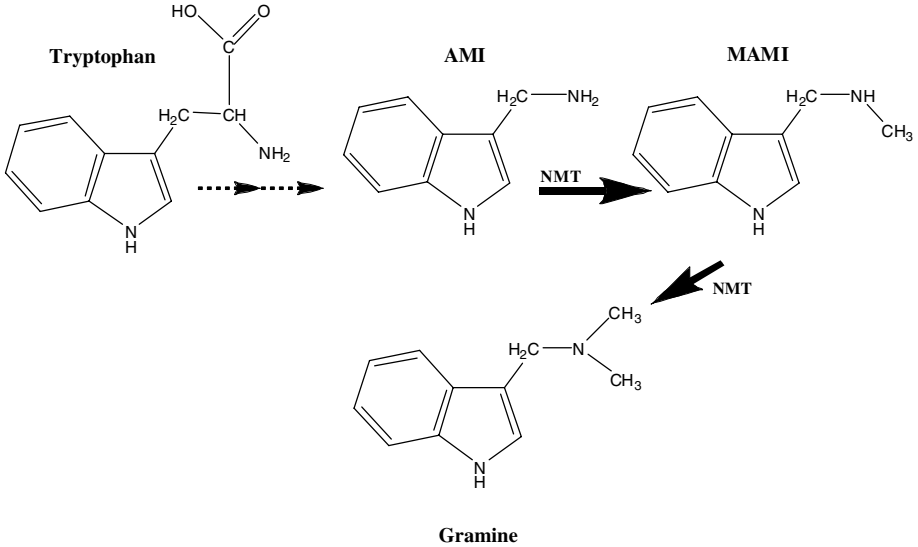

Fig. 4. The pathway from tryptophan to gramine. *N*-Methyltransferase (*NMT*), acting on 3-aminomethylindole (AMI) and *N*-methyl-3-aminomethylindole (MAMI) in the biosynthesis of the indole alkaloid gramine.

from RNA from these plants using the SMART™ mRNA Amplification Kit and PCR-Select™ cDNA Subtraction Kit (both Clontech, USA). Randomly picked clones from the libraries were screened using the PCR-Select Differential Screening Kit (Clontech, USA). Differential expression was confirmed by northern blot analyses. One of the regulated clones turned out to be identical to a 429 bp fragment from the GenBank accession U54767 (Lee et al., 1997b). For this gene there are also a partial cDNA (U43498) and a protein sequence (AAC18643) in the databases.

3.3. DNA extraction and genomic PCR

Genomic DNA was extracted from 100 mg green tissue using the DNeasy Plant Mini Kit from Qiagen (West Sussex, UK). One hundred nanogram of genomic DNA was used as template in the PCR using internal primers OMT F1 (5′-ATATAGCAGAGGCGGTGACT-3′) and OMT R1 (5′-AAGAGAACCGCATCTCCAGT-3′). Cycling conditions were: 94 °C for 4 min, 39 cycles of: 94 °C (30 s), 55 °C (30 s) and 72 °C (1 min). Barley-specific glyceraldehyde-phosphate dehydrogenase (GAPDH) primers 5′-TTCACTGACAAGGACAAGGC-3′ and 5′-CCACCTCTCCAGTCCTTGCT-3′ were used for parallel positive control (Delp et al., 2003).

3.4. Preparation of expression constructs and protein expression in E. coli

Total RNA (2 μg) from leaves treated with jasmonic acid was reverse transcribed using the First-Strand Synthesis System for RT-PCR (Invitrogen life technologies, Carlsbad, USA). The coding region of the *MT* gene was amplified using primers OMTcloneF (5′-GGTGGTCATATGGACAAGATTTCAGCACCTTTCTTTAG-3′) and OMTcloneR2 (5′-CCCGGGCTACTTGGTGAACTCAAGAGCGTA-3′) with Phusion High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland), using 1% of the RT reaction as template. Cycling conditions were: 98 °C for 30 s, 30 cycles of: 98 °C (10 s), 66 °C (30 s) and 72 °C (45 s). The PCR product was purified using the NucleoTrap Nucleic Acid Purification Kit (BD Biosciences, Palo Alto, USA). The plasmid pTYB12 (IMPACT-CN Protein Purification System, New England BioLabs,) was digested with SmaI and NdeI (Fermentas Life Sciences, Burlington, Canada) and the PCR product with NdeI. Ligation was carried out with the Quick Ligation Kit (New England BioLabs Frankfurt am Main, Germany). The new construct pTYB12-*MT* was transformed into *E. coli DH5α-T1* competent cells according to the One Shot Chemical Transformation Protocol (Invitrogen, USA). Colonies were screened for inserts by PCR using primers OMT F1 and OMT R1. Cycling conditions were: 94 °C for 4 min, 35 cycles of: 94 °C (30 s), 55 °C (30 s) and 72 °C (1 min). Clones that gave the expected product were further analysed by digestion with restriction nucleases KpnI, NcoI, NdeI and SapI. The insert of a clone giving the correct restriction pattern was confirmed by sequence analysis (performed by CyberGene AB, Huddinge, Sweden). For protein expression, the *E. coli* strain ER2566 (Impact-CN Protein Purification System, New England BioLabs) was transformed with the plasmid pTYB12-MT according to the One Shot Chemical Transformation Protocol (Invitrogen, USA).

The methyltransferase was purified from 1 l of cell culture according to the protocol supplied with the IMPACT-CN Protein Purification System, New England BioLabs, USA.

3.5. Antibody preparation

A polyclonal rabbit antiserum against purified NMT was kindly provided by Dr. Andrew Hanson. Polyclonal antibodies against the recombinant protein were produced by immunizing chicken. Antibody production and purification were performed by AgriSera (Vännäs, Sweden). The antibody preparation was further purified by affinity chromatography using the recombinant protein coupled to CNBr-activated Sepharose 4B (Amersham Biosciences, UK).

3.6. SDS-PAGE and Western blot

Proteins were separated on 4–20% gradient Tris Glycine gels. The protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, UK) electrophoretically using a Semi Phor™ transfer apparatus (Hoefer Scientific Instruments, San Francisco). The membrane was blocked with 1.25% milk powder in Phosphate Buffered Saline/0.05% Tween buffer (PBS-Tween, pH 7.4) for 1 h. Membranes were incubated with either polyclonal rabbit antiserum (diluted 1:5000) or affinity purified chicken antibodies (diluted 1:500) overnight at 4 °C. Incubation with secondary antibodies (Goat Anti-Rabbit IgG Alkaline Phosphatase, AP diluted 1:30,000 or Rabbit Anti-Chicken HRP diluted 1:10,000) was carried out for 2 h at room temp. Proteins were detected by either adding NBT-BCIP solution (for AP) or by using ECL Plus Western Blotting Detection Reagents kit for HRP, (Amersham Biosciences, UK) in a CCD camera (software ImageReader LAS1000 Pro ver 2.5 and ImageGauge ver 4.0). Protein ladders used were SDS-Page Molecular Weight Standards, Broad Range, Bio-Rad (Fig. 1a), and PageRuler™ Prestained Protein Ladder, Fermentas Life Sciences (Fig. 1b). For more detailed buffer recipes see Sambrook and Russell (2001).

3.7. Preparation of plant protein extracts

Frozen plant material was ground in a mortar in liquid nitrogen. The resulting powder was mixed with 3X (v/w) extraction buffer (0.1 M K-Pi buffer, pH 7.1 with 0.45 M PVPP and 14.3 mM β-mercaptoethanol). Samples were vortexed and then centrifuged at 12,000*g* for 10 min. The

supernatants were kept on ice. Enzyme measurements in crude extracts were carried out the same day since the enzyme activity was unstable upon freezing and thawing.

Protein concentration was determined using the Coomassie Plus Protein assay Reagent (Pierce, UK) with bovine serum albumin as standard.

3.8. Enzyme assays

The indole substrates AMI and MAMI were synthesized according to Gower and Leete (1963) and Neumeyer et al. (1969), respectively. Confirmation of the identity of AMI and MAMI was obtained via ^{1}H-NMR and ^{13}C-NMR using a Bruker DPX (300 MHz) spectrometer. The enzyme assay was based on that described by Leland and Hanson (1985). For assays with crude protein extracts, the mixture contained 10 μl extract (10 μg protein), 10 μl of assay buffer (0.25 M Tris–HCl pH 7.5, 10 mM DTT), 10 μl of 3 mM AMI or MAMI (final concentration 0.6 mM) and 20 μl [methyl-^{3}H] AdoMet (final concentration 0.02 mM, 0.37 GBq/mmol; Amersham Biosciences, UK). For determinations of the kinetic properties of NMT, the incubations contained 8 μg of purified recombinant NMT. For incubations with varying concentrations of AMI and MAMI (between 0.04 and 2.5 mM as final concentration), the final concentration of AdoMet was 1.0 mM, and the specific radioactivity 14.8 MBq/mmol. The kinetic properties with regard to AdoMet were carried out with MAMI as the substrate, at a final concentration of 0.6 mM containing 3 μg of purified NMT from a different batch. The concentrations of AdoMet in the assay varied between 0.11 mM and 1.1 mM and the specific radioactivity between 14.8 MBq/mmol and 0.148 GBq/mmol. After 30 min or 60 min incubation in a shaking water bath at 30 °C, the reactions were stopped by adding 200 μl of 1 M H_3BO_3–Na_2CO_3 pH 10.0. Alkaloids were extracted into 250 μl $CHCl_3$, and 50 μl of the $CHCl_3$ phase counted in a liquid scintillation counter with Emulsifier Scintillator Plus (Perkin Elmer, Boston, USA) as scintillation liquid. For TLC, $CHCl_3$ was evaporated in a speed vacuum centrifuge, the compounds were dissolved in 50 μl MeOH and applied on the TLC-plates (Silica gel 60, Merck, Darmstadt, Germany). The plates were developed in ($CHCl_3$–MeOH–NH_4OH conc. 80:15:1) for 80 min. To visualize indole compounds, the dry TLC-plates were sprayed with van Urk-Salkowski detection reagent (Ehmann, 1977). The regions containing the reaction products were taken to liquid scintillation counting as above. The enzyme activity with caffeic acid was analyzed as described by Pak et al. (2004). The assay mixture with crude protein extracts contained 10 μl extract (10 μg protein), 10 μl assay buffer (0.15 M Tris–HCl pH 7.5, 1 mM $MgCl_2$), 10 μl of 3 mM caffeic acid (Sigma Chemical, St. Louis, USA), and 20 μl [methyl-^{3}H] AdoMet (final concentration 0.02 mM; 0.37 GBq/mmol). For incubations with purified NMT (8 μg in the assay) and varying concentrations of caffeic acid (between 0.06 mM and 2.5 mM as final concentration), the final concentration of AdoMet was 1.0 mM, and the specific radioactivity 14.8 MBq/mmol. After 30 min or 60 min incubation in a shaking water-bath at 30 °C, the reactions were stopped by adding 2.5 μl of 6 M HCl. The methylated products were extracted into 100 μl of EtOAc, and 50 μl was taken for liquid scintillation counting as above. All enzyme assays were carried out in triplicate, with blanks containing buffer instead of enzyme and within initial velocities of the reactions. The kinetic constants (K_m) and maximum velocities (V_{max}) were calculated from non-linear regressions using the PRISM 4 program (GraphPad Software Inc., San Diego, USA). Analyses using linear regression (Lineweaver-Burk plots) gave similar results.

3.9. Gramine extraction and analysis by HPLC

For gramine analysis, the tip from the first leaf was taken from 7 day-old plants. Plant material was frozen in liquid nitrogen and ground into a powder. Circa 20 mg of frozen sample was used for gramine extraction according to Zúñiga et al. (1985) with modifications such as no filtration through glass wool, and pH 10 instead of pH 9. The samples were dissolved in 100 μl solvent according to Matsuo et al. (2001), but with CH_3CN-HAc 1% (1:4) for analysis by HPLC. Gramine (Sigma Chemicals, St. Louis, USA) was used as an internal standard during the extraction procedure. The recovery of the internal standard was ca. 70%.

For HPLC, a ConstaMetric 3200 pump (LDC Analytical, Riviera Beach, USA) equipped with an autosampler MIDAS (Spark Holland Instrument, Emmen, The Netherlands) was used. The samples were separated on a C18-AQ reverse-phase column (5 μm, 4 × 150 mm) at room temp. The elution solvent was CH_3CN-HAc 1% (1:4) with a flow rate of 0.5 ml/min. UV detection occurred at 275 nm (SpectroMonitor 4100, LDC Analytical) and retention time was 5.4 min. The results were analysed using software CSW ver 1.7.

To verify the identity of the analysed peak, eluted fractions with extracted gramine and gramine standard were analysed with an Ion-trap Mass Spectrometer from ThermoFinnigan, San José USA, with direct injection. The same eluant was used as for HPLC described above.

3.10. Sequence alignments

Multiple sequence alignments with other *MT* genes were done using ClustalW (BCM Search Launcher) BOXSHADE 3.21 at the Swiss EMBnet.

Acknowledgements

We are very grateful to Ann-Louise Jonsson at the Karolinska Institute, Huddinge, for the synthesis of AMI and MAMI and to Prof. Andrew D. Hanson, University of

Florida for the gift of antibodies raised against *N*-methyltransferase from barley. Dr. Inger Åhman at Svalöf Weibull AB is sincerely thanked for providing most of the plant material and for comments to an earlier version of the manuscript. Prof. Jan Pettersson, SLU is thanked for providing aphids. We are also grateful to Thérèse Gradin for technical assistance during antibody purification. The work has obtained financial support from Carl Trygger Foundation for Scientific Research, Swedish Farmer's Foundation for Agricultural Research, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas) and Stockholm County Council.

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