# Expression of an Aphid-Induced Barley Methyltransferase in *Escherichia coli*, Purification and Characterisation of the Enzyme

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## *Abstract*

Barley (*Hordeum vulgare*) is the agricultural leading cereal; therefore it is of the high importance to keep the barley crop well protected from various pests and diseases. One of the most serios barley pests in Sweden, causing substantial economical losses due to expenses for insecticides, is the bird cherry-oat aphid (*Rhopalosiphum padi*). This aphid feeds on phloem sap, impairing plant development and transmitting virus infections, i. a. barley yellow dwarf virus. Inflicting little damage to plant tissue, aphids attack sets off the pathogen-defence response in barley. One gene, that has been shown to be induced by the aphid, is encoding an O-methyltransferase, OMT (EC2.1.1.6, GeneBank accession U54767). This gene is also induced by the jasmonic acid signalling pathway, which mediates expression of defense genes. Previous studies demonstrated that not all barley cultivars had the *OMT* gene in their genome, and in the varieties missing the gene, the barley indole alkaloid gramine

was not found either. These facts have led to the hypothesis that the gene, which has been characterized as coding for an O-methyltransferase acting on caffeic acid, might actually be encoding an N-methyltransferase, involved in gramine biosynthesis. Thus the characterization of this aphid-induced enzyme, with the purpose to find out its function in the undamaged plant, has been the aim of the project.

Standard biotechnological methods were applied in this experiment, such as RT-PCR and PCR, Bradford microassay method for protein quantification, agarose and SDS-PAGE gel electrophoresis, Western blotting and TLC analysis. The coding sequence of the *OMT* gene was cloned into an expression vector containing an intein tag. *E.coli* cells were transformed with the construct. The target protein was purified using a chitin column. SDS-PAGE analysis showed a major band at 43 kDa. Purified protein fractions were used in enzyme assays with intermediates in the gramine biosynthesis pathway, AMI (3-aminomethylindole) and MAMI (N-methyl-3-aminomethylindole) as substrate, as well as with caffeic acid. The enzyme activity with caffeic acid as substrate was obscure, the experiment results showed little activity, but they can be questioned. On the other hand both AMI and MAMI were acting as substrates and transformed to MAMI and gramine respectively. This work supports the idea that the methyltransferase gene accession number U54767 could be classified as an *NMT*-gene involved in gramine biosynthesis.

# *Introduction*

The damage caused by herbivores to plants is dependent on the mode of herbivore feeding. Phloem-feeders produce little wound, in contrast to chewing and cell-content feeding insects, which cause more extensive tissue injury. Plant response is adjusted to different types of herbivore assault [1]. The pathogen-defence response pathways are induced by phloem-feeding insects, whereas chewing insects start out wound-signalling pathways. Among the numerous interactions between plants and insects, the interaction, chosen in these studies, is that between barley (*Hordeum vulgare*) and the bird cherry-oat aphid (*Rhopalosiphum padi*).

## Barley

Barley (*Hordeum vulgare*) is an important cereal in Sweden, cultivated on a large area of arable land, about 400 000 ha [2]. Barley is used in the malting industry as well as for livestock feed. The barley cultivar used in this work, Lina, is a two-rowed spring-sown cultivar, which mainly is used for cattle feed. Barley is the most common forage cereal and all kind of farm animals can be fed on it. Therefore it is of high significance to keep the barley crop well protected from various pests and diseases.

## Bird cherry-oat aphid

One of the most serious barley pests, causing substantial economical losses due to insecticide expenses, is the bird cherry-oat aphid *Rhopalosiphum padi*. These aphids overwinter as eggs on its primary host, bird cherry (*Prunus padus* L.), and in summer make use of diverse grasses as secondary hosts, among them barley. Aphids are phloem-feeding insects. In contrast to the effect of chewing herbivores, tissue damage caused by aphids is not significant. Piercing plant foliage with their stylet, aphids pass through the cuticle, epidermis and mesophyll to attain veins of the phloem. Their stylet is in continuous contact with plant cells during feeding, which can continue for several days [1]. Aphids do not only feed on the barley phloem sap and thereby withdraw nutrients from the plant; they can also transmit different infections. In special cells, mycetocytes, aphids carry bacteria, belonging to the genus of *Buchnera,* which are synthesizing B vitamins for their host. These bacteria are transmitted maternally from parent to offspring and colonize the mycetocytes in the aphid embryo [3]. *Buchnera* have been implicated in promotion of aphid transmission of circulative viruses [4]. The most common viral disease of cereal crops, the barley yellow dwarf virus (BYDV), is transmitted by aphids. The BYDV symptoms vary with the stage of crop development. In-

fections at the seedling stage may result in death or dwarfing as well as sterile heads [5].

Aphids have a remarkable life cycle with regard to their ability of breeding by sexual reproduction and parthenogenesis. In Sweden, the life cycle of *R. padi* is holocyclic, involving both parthenogenetic and sexual reproduction. The aphids overwinter as eggs on the winter host, bird cherry. In spring the eggs hatch, giving rise to several generations of asexually reproducing females, virginoparae [6]. A winged generation of females leaves bird cherry and moves to grasses, mainly spring-sown cereals. During summer they propagate parthenogenetically moving from one secondary grass host to another according to requirements. Population growth in aphids can be explosive. Under favourable environmental conditions, a newly born aphid becomes a reproducing adult within seven days, and can produce up to five offspring per day for up to 30 days. Aphids can develop wings for migrations, when their population increases and they have to compete for nutrients. In autumn, a specific type of female appears, which gives birth to a winged generation of males and females, called the fall migrants [7]. The fall migrants move back to bird cherry, where they mate and lay eggs. Only these eggs can survive the Swedish winter, thus finishing the aphid life circle [8].

## Plant defence reactions

Plants respond to insect assault with induction of various defence reactions. A type of substances, involved in defence, is secondary metabolites, which subdivide into three main groups: terpenes, phenolic compounds and nitrogen-containing secondary products. In most cases the secondary metabolites are present at non-induced condition, and only some of them are synthesized depending on the circumstances. A big group of N-containing secondary metabolites are water-soluble basic toxins, alkaloids, synthesized from the amino acids aspartic acid, lysine, tyrosine and tryptophan. Alkaloids have one or several basic nitrogen atoms, which are often built into a heterocyclic ring system. Alkaloids have strong physiological effects in defence against herbivores. Some alkaloids interfere with components of the nervous system, especially chemical transmitters; other affect membrane transport, protein synthesis or miscellaneous enzyme activities [9].

A significant roll in plant defence reactions play signal transduction pathways, which amplify the original signal and result in the activation or repression of genes. Phloem-feeding aphids penetrate with the stylet to the veins between cells, damaging the plant tissue only slightly. They are perceived by the plants as pathogens, what is leading to the induction of the pathogen-defence response pathways, the salicylic acid- and jasmonic acid/ethylene-dependent signalling pathways [1]. Jasmonic acid (JA) is a signalling compound involved in multiple aspects of plant responses to their biotic and abiotic environment. Synthesised from membrane lipids, JA can induce expression of a range of early and late functioning defense genes [10]. A barley gene, which was found to be induced by bird cherry-oat aphid and also by jasmonic acid, was identified as an O-methyltransferase gene [11]. In another recent study, an *OMT*-gene was found to be induced furthermore by another aphid species (greenbug, *Schizaphis graminum*) in sorghum [10].

## Gramine

One secondary metabolite, which has been found to be induced in barley upon aphid infestation, is gramine [12]. The indole proto-alkaloid gramine has high physiological effectiveness necessary for antiherbivore defence. Gramine is toxic to aphids in feeding experiments with holidic diets, decreasing aphids' longevity and fecundity. This indole proto-alkaloid is found in epidermis and in mesophyll parenchyma, but it is missing in the vascular bundles. Different factors like photoperiod, temperature and plant age have an effect on the amount of gramine [12]. It has been reported that gramine inhibits the respiratory chain at complex I in rat liver mitochondria and in bovine heart submitochondrial particles. It was shown that gramine can inhibit the photosynthetic phosphorylation in spinach thylakoids, and also reduce energy transfer in photosynthetic units of freshwater blue-green algae *Anabaena* sp. [13].

Gramine has been detected in the grass family *Poaceae* (see Figure 1), i. a. genus *Phalaris*

*spp.*, *Avena*, *Hordeum*, and also in the family *Fabaceae* genus *Lupinus* [14].

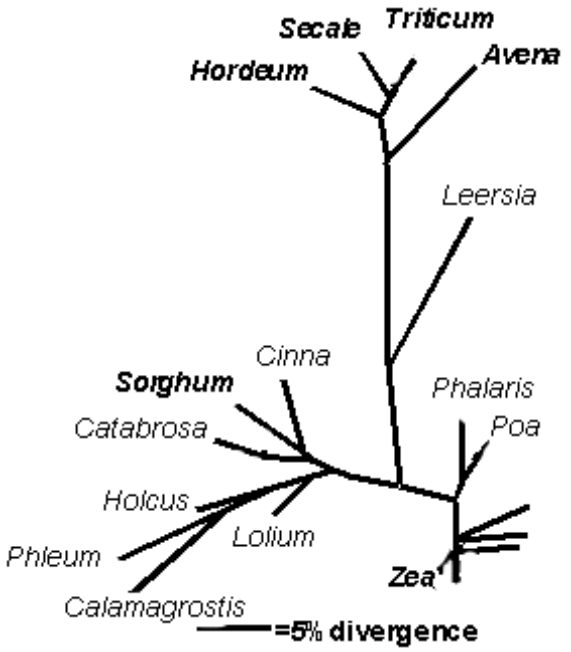


**Figure 1** *The Poaceae Family Tree. Hordeum provides barley, Secale – rye, Triticum – wheat, Avena – oats, Leersia – rice, Zea – corn or maize, Sorghum – millet. Gramine has been found in Hordeum, Avena and Phalaris*

Young plants have a tendency to produce more gramine, but later the gramine content diminishes. The vulnerability of barley to aphids decreases with the increasing gramine amount in the leaves.

The gramine synthesis pathway is not completely understood. In earlier studies [15] it was suggested, that gramine could be synthesized from tryptophan via 3-aminomethylindole (AMI) and N-methyl-3-aminomethylindole (MAMI) (see Figure 2). An N-methyltransferase (NMT) catalyzing S-adenosylmethionine (SAM)-dependent conversion of AMI to MAMI and from MAMI to gramine has previously been identified [15]. Two genes control the gramine synthesis, one (*Ami)* conducting the conversion of tryptophan to AMI, the other (*Nmt)* encoding an NMT enzyme. It has been suggested, that there may be more than two genes determining gramine biosynthesis, because the conversion of tryptophan to AMI probably proceeds in several steps [15].

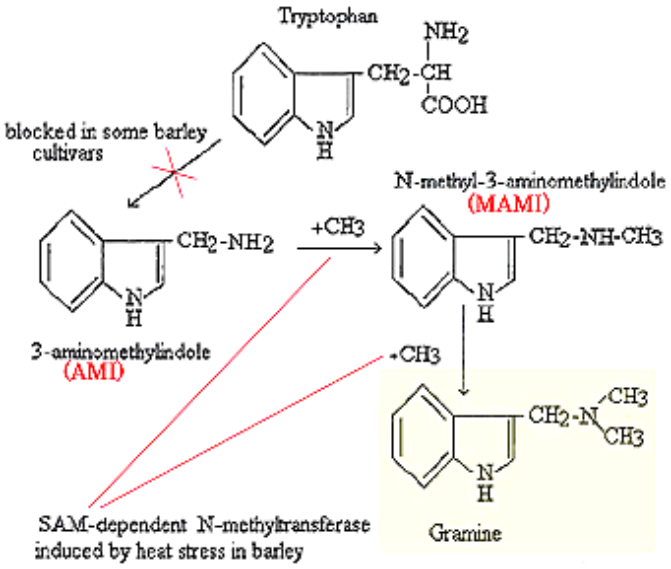


**Figure 2** *NMT catalyzing SAM-dependent conversion of AMI to MAMI and from MAMI to gramine (see more www.hort.purdue.edu)*

## O-methyltransferase

The *OMT* gene, which is the focus of this study (OMT; EC2.1.1.6, GeneBank accession U54767), has been characterized as coding for an O-methyltransferase, acting on the phenolic substrate caffeic acid [16]. In barley it is induced by the signal substance jasmonic acid, regulating a pathway, which increases expression of defence-response genes. OMTs generally methylate caffeic acid and lead to lignin precursors or various classes of flavonoids, some of which are phytoalexins. But for some OMTs caffeic acid was found to be the least effective substrate [17]. OMTs employ SAM as the methyl donor in the methylation of hydroxyl groups [18].

Earlier studies have shown, that feeding of the bird cherry-oat aphid on barley induces the pathogenesis-related proteins chitinase and β-1,3-glucanase [1]. Recently subtracted cDNA libraries from infested and non-infested barley plants have been screened to detect other genes which are up- or down-regulated in response to aphid attack. One of the up-regulated genes, coded for an enzyme, had previously been characterized as an O-methyltransferase [19]. It was proposed, that this enzyme was functioning as caffeic acid O-methyltransferase,

catalyzing the methylation of caffeic acid to ferulic acid [20].
It has recently been shown that not all barley cultivars had the *OMT* gene in their genome [21]. The *OMT* gene was detected in 8 out of 12 barley lines tested (Ph. D. student K. Larsson, personal communication). In the barley varieties missing the gene, the indole alkaloid gramine was not found either. In all gramine-containing lines *OMT* was present. Moreover, presence or absence of the *OMT* gene was not correlated with the barley resistance to aphids [21]. These observations have led to the hypothesis that the enzyme O-methyltransferase could be involved in gramine biosynthesis.

### Aim of the project

Thus the aim of the project is to characterize the aphid-induced methyltransferase with the purpose to:

- test the hypothesis that OMT is involved in gramine biosynthesis;
- clone the *OMT* gene into an expression vector with the purpose to synthesize the enzyme in *E. coli;*
- characterize the enzyme kinetically and
- find out its function in the undamaged plant.

## *Materials and methods*

### Growth and treatment of plants

*Hordeum vulgare*, variety Lina, susceptible to the aphids, was sown in November 2003 and grown in a growth chamber at 26$^{o}$C, long day, (18 h light/6 h darkness). 5-day-old barley plants were harvested. Their green tissue was floated in Petry dishes with 45 μM jasmonic acid solution for 24 hours in order to induce the *OMT*-gene. Plant material was frozen in liquid nitrogen and stored until use at -70$^{o}$C.

### Total RNA isolation

Total RNA was isolated from 100 mg green tissue using the Plant RNeasy Mini kit (Qiagen) according to the kit protocol. The frozen tissue was ground in a mortar to a fine powder and mixed with lysis buffer RLT. DNA was digested during RNA purification by means of the RNase-Free DNase Set (Qiagen) according to the kit protocol.

### RT-PCR

Total RNA from leaves treated with jasmonic acid was reverse transcribed into single-stranded cDNA using the First-Strand Synthesis System for RT-PCR (Invitrogen) and the PCR machine UNO II (Biometra). The forward primer OMTcloneF (5'-GGT GGT CAT ATG GAC AAG ATT TCA GCA CCT TTC TTT AG-3') and the reverse primer OMTcloneR2 (5'-CCC GGG CTA CTT GGT GAA CTC AAG AGC GTA-3') were applied to amplify the coding region of the *OMT* gene using a proof-reading thermostable DNA polymerase (Phusion – High-Fidelity DNA Polymerase, Finnzymes, MJ BioWorks). First strand cDNA served as template for the PCR reaction. The PCR machine used for the sequence amplifying was PTC-100 Programmable Thermal Controller Peltier-Effect Cycling, MJ Research. The temperature program for PCR was: 98$^{o}$C for 30 s, then 30 cycles of 98$^{o}$C (10 s), 66$^{o}$C (30 s), and 72$^{o}$C (45 s). The quality of the synthesized OMT-fragment was controlled by 2% agarose gel electrophoresis. The PCR product was purified using the NucleoTrap Nucleic Acid Purification Kit (BD Biosciences) according to its protocol.

### Cloning of the OMT gene into the pTYB12 vector

The plasmid pTYB12, which allows the fusion of the cleavable intein tag to the N-terminus of a target protein, was chosen as a vector (supplied as part of the IMPACT-CN Protein Purification System, BioLabs, New England). The plasmid was digested with the restriction nucleases *Sma*I and *Nde*I (Fermentas Life Sciences). The DNA fragment was digested with the restriction nuclease *Nde*I. The digesting reactions were carried out at 37$^{o}$C, except for *Sma*I which was incubated at 30 $^{o}$C. The digested DNA was ligated into the pTYB12 using the BioLabs Quick Ligation Kit.

### Transformation of *E. coli DH5α-T1* and screening for recombinants

The new construct pTYB12-OMT was used to transform *E. coli DH5α-T1* competent cells for amplifying of the plasmid according to the One

Shot Chemical Transformation Protocol (Invitrogen). The recombinant cells were selected on Petri dishes with LB/amp medium (1% tryptone, 0,5% yeast extract, 1% NaCl pH 7,0, 100 µg/ml ampicillin). 96 randomly chosen colonies were inoculated in a microtitre plate in LB medium with 100 µg/ml ampicillin. Plates were shaken at 37ºC for 1 hour. 1 µl of this culture was used as template for a PCR test for inserts using internal primers OMT F1 (5'-ATA TAG CAG AGG CGG TGA CT-3') and OMT R1 (5'-AAG AGA ACC GCA TCT CCA GT-3'). PCR conditions were 4 min 94 $^{o}$C, 35 cycles of: 94$^{o}$C 30 s, 55$^{o}$C 30s; and 72 $^{o}$C 1 min (PCR machine used here was PTC-100 Programmable Thermal Controller Peltier-Effect Cycling, MJ Research)**.** Products were analysed by agarose gel electrophoresis. Three clones which gave the expected product for the OMT insert were grown over night in LB medium with ampicillin at 37$^{o}$C with shaking. The plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen) according to its protocol. To confirm the obtained recombinant clones, digesting reactions with restriction nucleases *Kpn*I, *Nco*I, *Nde*I and *Sap*I were carried out over night at 37$^{o}$C. The digest reactions were analyzed by electrophoresis on a 1 % agarose gel. The gel was run at 100 V for 2 hours.

The new construct pTYB12-OMT was controlled for the right insert by PCR (PCR machine PC-960G Gradient Thermal Cycle) with the three pairs of primers: OMT clone F and OMT clone R2; OMT F1 and OMT R1; and Intein Forward (5'-CCC GCC GCT GCT TTT GCA CGT GAG-3') and T7 Terminator Reverse (5'-TAT GCT AGT TAT TGC TCA G-3') and analysed on a 2 % agarose gel. The plasmid DNA from the clone containing the OMT insert was sent to be sequenced at Cybergene, Novum in Huddinge.

## Transformation of the expression strain *E. coli ER2566* and screening for recombinants

The *E. coli* strain ER2566 was provided by Impact-CN Protein Purification System (BioLabs) as a host strain for the expression of a target gene, cloned in the pTYB12 vector. Competent cells of ER2566 were transformed with the plasmid pTYB12-OMT according to the One Shot Chemical Transformation Protocol. The same plasmid preparation, which was sent to be sequenced, was used here. The recombinants were selected on Petri dishes with LB medium and 100 µg/ml ampicillin. Colonies were picked and grown in LB/amp medium with shaking at 37$^{o}$ C over night.

## Transformation of *E. coli ER2566* as the positive control of the induction reaction

To control the protein induction, *E. coli ER2566* was transformed with pMYB5 vector, as it was recommended by the instruction manual of the IMPACT-CN Protein Purification System. The transformation was carried out according to the One Shot Chemical Transformation Protocol. The recombinant cells were selected on Petri dishes with LB medium and 100 µg/ml ampicillin. The control clones ER2566-pMYB5 were inoculated in 5 ml LB medium with 100 µg/ml ampicillin with shaking over night at 37$^{o}$C and used as positive control for the induction of protein expression.

## Induction of protein expression

Different conditions for protein expression were weathered. Expression clones were grown in LB/amp medium (1% tryptone, 0,5% yeast extract, 1% NaCl pH 7,0, 100 µg/ml ampicillin). Protein expression was induced with 0,5 mM or 1 mM IPTG (isopropylthiogalactoside). Temperature conditions at 15$^{o}$C, 20$^{o}$C and 37$^{o}$C were tested for the best result. The expression clones were harvested after 4 and 6 hours and also next morning. $OD_{595}$ was measured spectrophotometrically. The quality of the induction reactions was analysed by SDS-PAGE gel electrophoresis. Two ready-manufactured 4-20% Tris-Glycine gels (4-20 % gradient Tris Glycine gel Cambrex PAGE for polyacrylamide gel electrophoresis, In Vitro Sweden AB) were loaded with 20 µl of the samples mixed with SDS Sample buffer (final conc. 1×, 50 mM Tris, 2% SDS, 0,1% BPB, 10% glycerol). Samples were boiled in 1×SDS sample buffer at 100$^{o}$C for 5 min. The gels were run at 125 V and 60 mA for 95 min at room temperature.

## Western blot

The total protein was separated using SDS-polyacrylamide gel electrophoresis. The gel was run at 125 V and 30 mA for 100 min at room temperature. The protein bands were transferred from the gel onto Hybond-P PVDF membranes electrophoretically by means of semi-dry transfer apparatus at 47 mA during 60 min. Before the incubation with primary antibodies, the membrane was blocked with blocking solution containing 1,25% milk powder dissolved in buffer PBS-T (NaCl 8 g/l, KCl 0,2 g/l, $Na_2HPO_4$ 1,44 g/l, $KH_2PO_4$ 0,24 g/l, Tween 0,5 ml/l) for 1 hour. The PVDF membranes were incubated with the primary antibody Anti-Chitin Binding Domain Serum diluted 1:5000 overnight at 4°C. Then the membranes were washed with PBS-T three times for 15 min on the shaking table. With the secondary antibody Goat Anti-Rabbit HRP diluted 1:3000 the membranes were incubated for 2 h at RT. After that the membranes were washed with PBS-T three times for 15 min and with PBS two times for 10 min. The protein was detected using the ECL Plus kit (ECL Plus Western Blotting Detection Kit, Amersham Biosciences) and chemiluminescence in the CCD-camera.

## Purification of the target protein

The methyltransferase was purified using the IMPACT-CN Protein Purification System, BioLabs, New England. 1 liter of cell culture was grown at 37°C until $OD_{595}$ was 1,23. Protein expression was induced with 1 mM IPTG (isopropylthiogalactoside) at RT overnight. The cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C, and resuspended in Cell Lysis Buffer (20 mM Tris-HCl pH 8,0, 500 mM NaCl, 1 mM EDTA, 0,1% Tween 20). Cell lysis was achieved by sonication. The clarified cell extract was obtained by centrifugation at 17700 rpm for 30 min at 4°C, and then it was partitioned and loaded onto three 15 ml chitin columns (Polypropylene disposable columns, 5 ml, QIAGEN GmbH, Hilden). The columns were washed with the Column Buffer (20 mM Tris-HCl pH 8,0, 500 mM NaCl, 1 mM EDTA), and the on-column cleavage reactions were started by adding Cleavage Buffer (20 mM Tris-HCl pH 7,5, 500 mM NaCl, 1 mM EDTA, 50 mM DTT). The cleavage reactions were carried out at following conditions: one - at 4°C for 24 h, another – at RT for 24 h and the third – at RT for 40 hours. The NaCl concentrations of 0,5 M and 1 M in the buffers were also tested to achieve the highest efficiency of the target protein purification. The protein was eluted using the Column Buffer. The concentration of the obtained protein samples was measured spectrophotometrically using the Bradford microassay method for protein quantification. The efficiency of the cleavage reactions was analyzed by SDS-PAGE gel electrophoresis.

## Assay of the methyltransferase activity with AMI and MAMI

Purified enzyme was used for assays, based on T. J. Leland's and A. D. Hanson's assay for NMT activity [15], employing chemically synthesized (by Ann-Louise Johnson, KI) AMI and MAMI as substrates. Methyltransferase activity was measured by estimation of the amount of $^3$H-labelled product produced with methyl-$^3$H-SAM. For determination of the kinetic parameters of the methyltransferase, the assays were performed for different incubation times (0, 30 and 60 min) and also for different concentrations of AMI and MAMI (0,75, 1,5 and 3 mM). All reactions were done in duplicate. The assay mixture contained 55 µl enzyme, 0,15 M Tris-HCl pH 9,0, 1 mM $MgCl_2$, AMI or MAMI of the required concentration, and 0,6 mM SAM + $^3$H-SAM, 5 nCi/ µl. After 30 and 60 min incubation in a shaking water-bath at 30°C, the reactions were stopped by adding 400 µl of 1 M $H_3BO_3$-$Na_2CO_3$ pH 10,0. Alkaloids were extracted into 500 µl $CHCl_3$, and 400 µl of the chloroform phase were taken for thin layer chromatography (TLC) analysis. After the chloroform evaporation in the speed vacuum centrifuge, alkaloids were dissolved in 50 µl methanol and applied on the TLC-plates (20×20 cm Silica gel 60, Merck KGaA, Darmstadt, Germany). The plates were developed standing in TLC solvent ($CHCl_3$ – MeOH – $NH_4OH$ conc. 80:15:1 v/v) for 80 min. To visualize the alkaloids separation, the dry TLC-plates were sprayed with Urk-Salkowski detection reagent ({A} Van Urk reagent: 1 g p-dimethylaminobenzaldehyde, 50 ml conc. HCl, 50 ml ethanol; {B} Salkowski reagent: 2.03 g

$FeCl_3 \cdot 6\ H_2O$, 500 ml $H_2O$, 300 ml conc. $H_2SO_4$; the TLC spray reagent, was made up of reagent A and B (1:3) [22]). The TLC-plates were heated in the oven at 100°C for 8 min and immersed in 3 l distilled water three times. The regions containing the reaction products were scraped from the dry TLC-plates for liquid scintillation counting. $^3H$ count per minute was recalculated into built product, pmol/min per 1 mg protein, using Microsoft Excel. The coefficient *Rf* for AMI, MAMI and gramine was calculated as

$$Rf = \frac{a}{b},$$

where *a* - space between the start line and the built products position; *b* - space between the start and the solvent front lines on the TLC-plate.

### Assay of the methyltransferase activity with caffeic acid

The enzyme activity with caffeic acid was analyzed as described by F. E. Pak [17]. The assay mixture contained 27,5 µl enzyme, 0,15 M Tris-HCl pH 9,0, 1 mM $MgCl_2$, 3 mM caffeic acid, and 0,6 mM SAM + $^3H$-SAM, 5 nCi/µl. After incubation in a shaking waterbath at 30°C, the reactions were stopped by adding 2,5 µl of 6 M HCl. The methylated products were extracted into 100 µl ethyl acetate, and 20 µl was taken for liquid scintillation counting. To verify the optimal conditions, the reaction was also performed with Tris-HCl pH 7,5. The methyltransferase extract from green tissue of barley variety Lina was taken as a control for this experiment. To investigate the kinetic properties of the enzyme the incubation of the samples was stopped after 30 and 60 min.

## *Results*

### Cloning of the *OMT* gene into the pTYB 12 vector

The total RNA, isolated from barley green tissue, was converted into single-stranded cDNA by reverse transcription. First strand cDNA served as template for the PCR reaction. A product of about 1100 bp was visualized by 2% agarose gel electrophoresis (see Figure 3).

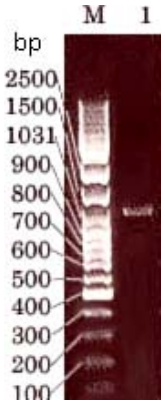


**Figure 3** *Agarose gel electrophoresis of the RT-PCR. Well M is loaded with DNA Ladder mix, well 1 - with the RT-PCR*

The plasmid pTYB12 and the DNA fragment were digested with restriction nucleases as described above and ligated to produce the plasmid pTYB12-OMT.

It became obvious that *E. coli DH5α-T1* was not easy to transform using the pTYB12-OMT plasmid DNA. After four trials merely the last one was successful, which in the end resulted in only one clone with the correct insert. The recombinant cells were selected on Petri dishes, and 96 colonies were inoculated in the microtitre plate in LB medium with ampicillin. A PCR test for inserts was done, and the agarose gel electrophoresis showed that three clones contained the OMT insert. These clones numbered 1, 2, and 3 were grown over night in LB medium with ampicillin, and the plasmid DNA was extracted.

The pTYB12-OMT recombinants were further tested by restriction analysis with restriction nucleases *Kpn*I, *Nco*I, *Nde*I and *Sap*I. The digested DNA was analyzed on 1 % agarose gel. This revealed that one clone gave the expected fragment pattern and thus was chosen as the pTYB12-OMT plasmid (see Figure 4). Lanes 1 – 4 are corresponded to the digesting reactions of the plasmid DNA 1, lanes 7 – 10 belong to the plasmid DNA 2, and the four last lanes to the plasmid 3. The clone 1 has the expected fragment pattern, which corresponds to the pTYB12 plasmid DNA with the insert of the *OMT*-gene coding region. The digesting reac-

tion with the restriction nuclease *Kpn*I (see Figure 4, lane 1) has given two bands of the

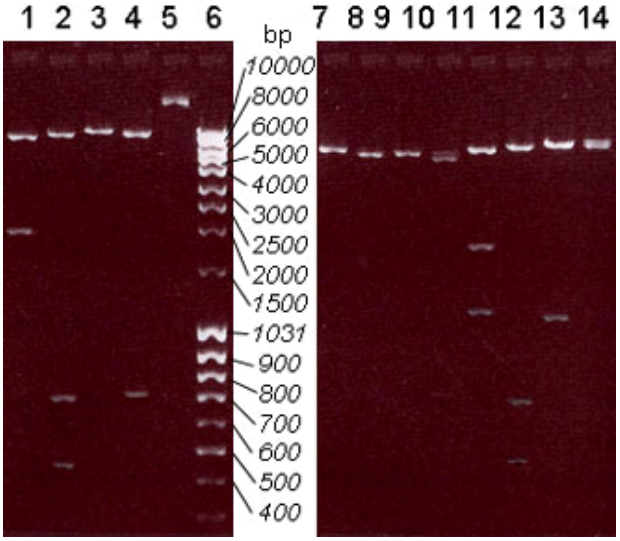


**Figure 4** *Agarose gel electrophoresis of the restriction analysis of putative clones with KpnI, NcoI, NdeI and SapI digesting reactions. Lanes 1 – 4 are corresponded to the digesting reactions of the plasmid DNA 1, lanes 7 – 10 belong to the plasmid DNA 2, and the four last lanes - to the plasmid 3. The wells are loaded with the combinations of clones and restriction enzymes as follows: 1 – clone 1, KpnI; 2 – clone 1, NcoI; 3 – clone 1, NdeI; 4 – clone 1, SapI; 5 – non-digested clone as a control; 6 – DNA Ladder mix; 7 – clone 2, KpnI; 8 – clone 2, NcoI; 9 – clone 2, NdeI; 10 – clone 2, SapI; 11 – clone 3, KpnI; 12 – clone 3, NcoI; 13 – clone 3, NdeI; 14 – clone 3, SapI*

size of about 6700 and 1800 bp. The digesting reaction with the restriction nuclease *Nco*I (see Figure 4, lane 2) resulted in three bands of the sizes 7400, 700 and 450 bp. The digesting reaction with the restriction nuclease *Nde*I (see Figure 4, lane 3) shows a band of about 8500 bp. And finally the digesting reaction with the restriction nuclease *Sap*I (see Figure 4, lane 4) is showing the fragments of 7800 and 700 bp. The clones 2 and 3 have different fragment patterns demonstrating that they do not contain the correct insert. One recombinant possessing the pTYB12-OMT plasmid DNA, the clone 1, had been obtained and was chosen to continue the experiment.

The new construct pTYB12-OMT was controlled for the right insert by PCR with the three pairs of primers: OMT clone F and OMT clone R2; OMT F1 and OMT R1, and Intein Forward and T7 Terminator Reverse. Bands of the correct sizes were visible on 2 % agarose gel (see Figure 5). The product of the PCR with the primers OMT clone F and OMT clone R2 (see Figure 5, lane 1) is about 1100 bp. The PCR with the primers OMT F1 and OMT R1 (see Figure 5, lane 2) has resulted in a band of about 350 bp. The PCR with the primers Intein Forward and T7 Terminator Reverse (see Figure 5, lane 3) produced a band of about 1300 bp.

The PCR reactions have proved that the coding region of the *OMT*-gene was inserted into the expression vector pTYB12.

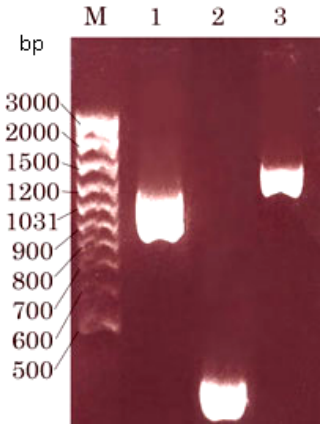


**Figure 5** *Agarose gel electrophoresis of the PCR reaction. The first well to the left, M is loaded with DNA Ladder Plus. Wells 1 – 3 are loaded with PCR product with following primers: 1 - OMT clone F and OMT clone R2; 2 - OMT F1 and OMT R1; 3 - Intein Forward and T7 Terminator Reverse.*

To make sure that there was no error in the sequence of the cloned fragment, the plasmid pTYB12-OMT was sequenced at Cybergene. The sequence proved to be identical to the one published earlier [19] (see Figure 6, page 10).

## Transformation of the expression strain *E. coli* ER2566

Transformed competent cells *E. coli* ER2566 were selected on Petri dishes with LB/amp medium. To control the protein induction *E. coli* ER2566 was also transformed using the pMYB5 vector. Quite a number of colonies were obtained on Petri dishes in both cases. The cultures were inoculated for protein induction.

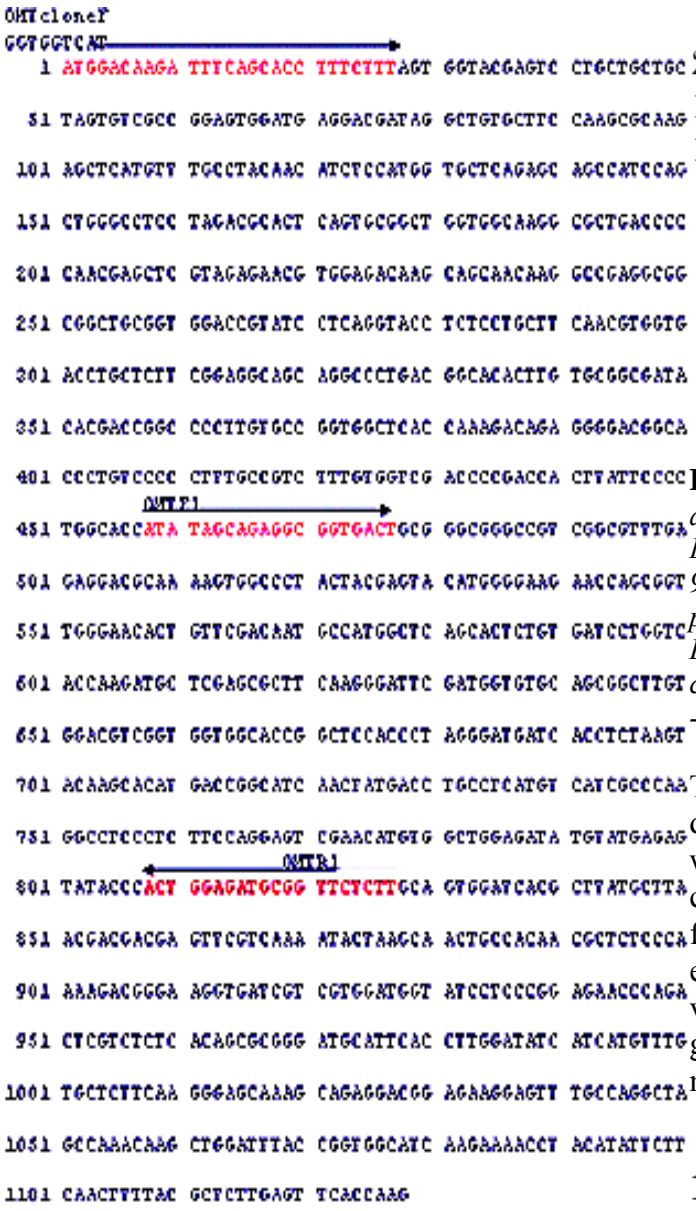


**Figure 6** *Complete insert from pTYB12-OMT. The primers OMTcloneF, OMT F1 and OMTR1 are marked in red. The size of the insert is 1128 bp.*

## Induction of protein expression

The protein expression was induced with 0,5 mM IPTG (see Figure 7). Bands of a size of about 100 kDa, which corresponds to the expected size of the fusion protein intein-methyltransferase, could be seen in all the lanes except the lane M, marker, and lane 2, the non-induced sample. The lanes 3, 7 and 9 show the strongest bands, accounting for the highest synthesis of the fusion protein. Therefore the expression clones E2 and E6 were preferred to continue the trial (see Figure 7, lanes 3 and 7).

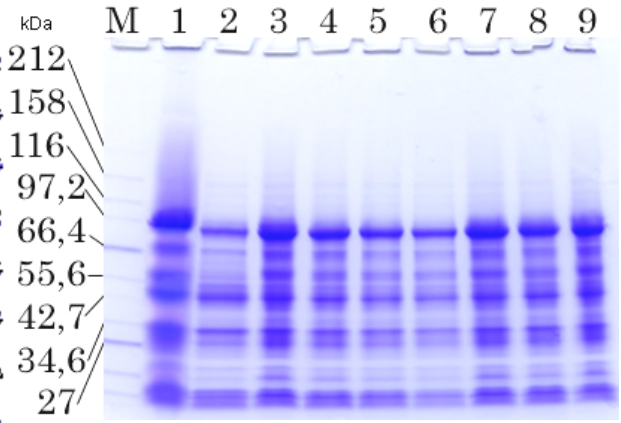


**Figure 7** *SDS-PAGE analysis of the induction reaction. Well M is loaded with Protein Marker Broad Range; the scale is shown in kDa. Wells 1 - 9 are loaded with protein samples as follow: 1 – the positive control clone, ER2566-pMYB5 induced in LB medium at 15°C; 2 – non-induced expression clone E1, 3 – expression clone E2, 4 - E3, 5 – E4, 6 - E5, 7 – E6, 8 - E7, 9 –E8*

To further optimize the protein induction conditions, the protein expression was induced with 0,5 mM or 1 mM IPTG. Temperature conditions at 15°C, 20°C and 37°C were tested for the most optimal result (see Figure 8). The expression clones grown at 37°C were harvested after 4 and 6 hours, and the clones grown at 15°C and RT were harvested next morning.

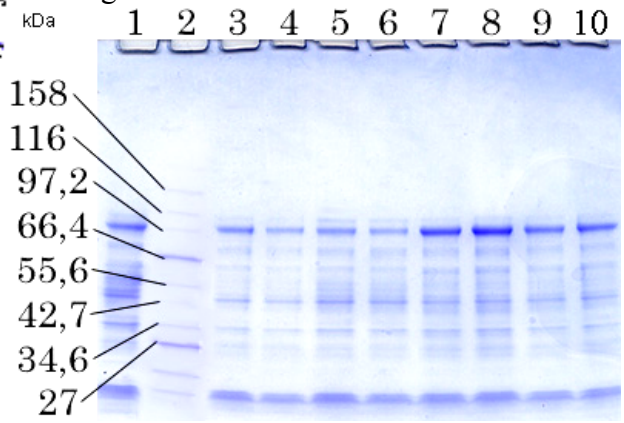


**Figure 8** *Analysis of the different conditions for the induction of protein expression by SDS-PAGE. The gel demonstrates the expression clone E6 induction with 0,5 and 1 mM IPTG at 15°C (lanes 9-10), RT (lanes 7-8) and 37°C (lanes 3-6). The lanes 1 – 10 are corresponded to: 1 - non-induced E1 as a negative control; 2 - Protein Marker Broad Range; 3 – induction with 0,5 mM for 4 h; 4 - 1 mM IPTG, 4 h; 5 - 0,5 mM IPTG, 6 h; 6 - 1 mM IPTG, 6 h; 7 - 0,5 mM IPTG; 8 - 1 mM IPTG; 9 - 0,5 mM IPTG; 10 - 0,5 mM IPTG*

SDS-PAGE gel analysis showed that the strongest band of about 100 kDa, corresponding to the size of the intein-methyltransferase fusion protein, was visible in lane 8, when the protein expression was induced with 1 mM IPTG at RT overnight.

## Western blot

Total protein extracted from induced *E. coli* cells was separated using SDS-polyacrylamide gel electrophoresis, and afterwards the separated bands were transferred electrophoretically onto PVDF membranes. After the immunoblotting using antibodies against the chitin binding domain of the fusion protein, the protein was detected using the ECL Plus Western Blotting kit and chemiluminescence in the CCD-camera (see Figure 9).

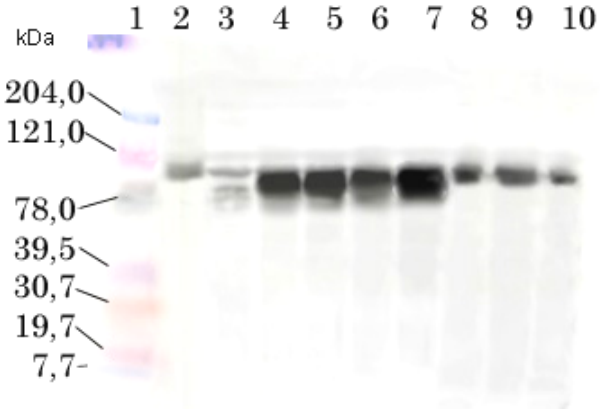


**Figure 9** *Immunoblotting* with *antibodies against the chitin-binding domain. Lane 1 contains size marker (Kaleidoscope Prestained Standards). Lane 2 shows the positive control clone, ER2566-pMYB5 induced in LB medium at 15°C. Lanes 3 – 10 are the results of the following samples: 3 – the non-induced expression clone E1 as a negative control; 4 – E2 induced in LB/amp medium at RT with 0,5 mM IPTG; 5 – E2 induced in LB/amp medium at RT with 1 mM IPTG; 6 – E6 induced in LB/amp medium at RT with 0,5 mM IPTG; 7 – E6 induced in LB/amp medium at RT with 1 mM IPTG; 8 – E8 induced with 0,5 mM IPTG; 9 – expression clone E12 induced with 0,5 mM IPTG; 10 - expression clone E16 induced with 0,5 mM IPTG*

The western blotting showed the strongest bands of about 100 kDa in lanes 4 – 7 and has proved that the induction of the protein expression was successful. The expression clone E6 induced in LB/amp medium at RT with 1 mM IPTG has showed the strongest band, which means that these conditions were the best for the protein expression.

## Purification of the target protein

The target protein was purified using the IMPACT-CN Protein Purification System. 1 l cell culture was grown at 37°C until $OD_{595}$ was 1,23. The expression cells were induced with 1 mM IPTG at RT overnight. The $OD_{595}$ of the harvested cells was 1,995. The cells were broken by sonication, and 50 ml clarified cell extract, obtained by centrifugation, was partitioned and loaded onto three chitin columns. Different conditions for the on-column cleavage reaction were tested: at 4°C and RT for 24 and 40 hours. The highest protein concentration was obtained by elution with the Column Buffer containing 0,5 M NaCl. 6 ml eluant 1, 6 ml eluant 2 and also 6 ml eluant 3 were acquired for each of the three cleavage reactions. The protein concentration of the samples was measured spectrophotometrically, using Bradford microassay method for protein quantification (see Table 1).

**Table 1** *Concentration of the target protein*

| Cleavage reaction conditions | Concentration of the target protein, µg/ml | | |
|---|---|---|---|
| | Eluant 1 | Eluant 2 | Eluant 3 |
| RT, 24 h | 240,00 | 18,80 | 0,00 |
| RT, 40 h | 162,12 | 31,14 | 4,81 |
| 4°C, 24 h | 66,10 | 0,00 | 0,00 |

The efficiency of the protein purification was analyzed by SDS-PAGE gel electrophoresis (see Figure 10). The strong bands of about 100 kDa, corresponding to the fusion protein intein-methyltransferase, can be seen in the lanes 3 and 4 of the samples before loading onto the chitin column. Lane 4 is illustrating the flow-through taken after the chitin column was loaded with the clarified cell extract. The pale band of the size 100 kDa proves that the fusion protein intein-methyltransferase has bound to the chitin beads. The lanes 5 and 6 do not have any bands at all, as expected for the column and DTT wash. The wells 7 - 9 were loaded with the 1 - 3 fractions respectively of the eluant 1. The bands of about 43 kDa corresponding to methyltransferase are clearly visible. The amount of the protein in the eluant 2 was insignificant and could not be detected by SDS-PAGE gel electrophoresis. The cleavage reaction at 4°C was ineffective and the small

amount of the protein could not be detected by SDS-PAGE gel analysis either. Totally 3,138 mg methyltransferase from 1 l cell culture were obtained by the IMPACT-CN Protein Purification System.

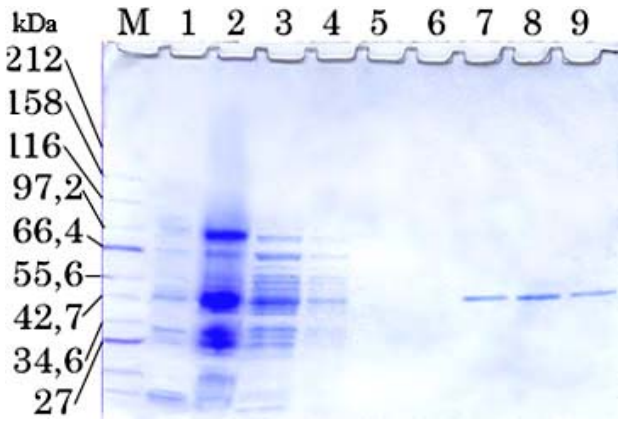


**Figure 10** *Efficiency of the purification of the target protein using buffers with 0,5 M NaCl, the cleavage reaction conditions – 24 h at RT. The lane M is corresponding to the Protein Marker Broad Range. Lane 1 shows the crude extract from uninduced cells. The lanes 2 – 9 are results of the following samples: 2 – the crude extract from the induced cell culture E6; 3 – the clarified cell extract, 4 – the chitin column flow through; 5 – the chitin column wash; 6 - quick DTT wash; 7 - 9 fractions 1 – 3 respectively of the eluant 1 of the cleavage reaction*

## Assay of the methyltransferase activity

For determination of the kinetic parameters of the methyltransferase, AMI, MAMI and caffeic acid were used as substrates. The AMI and MAMI products were separated by means of TLC-plates (see Figure 11). The regions with the reaction products were scraped from the TLC-plates for liquid scintillation counting. $^{3}H$ count per minute was calculated into built product per 1 mg protein (see Table 2)

**Table 2** *AMI and MAMI methylation products built per 1 mg protein, pmol/min, development in time*

| Incubation time, min | 0 min | 30 min | 60 min |
|---|---|---|---|
| AMI | 0 | 226,3±4,6 | 148±2,4 |
| MAMI | 0 | 52,6±7,9 | 71,2±9,8 |
| Lina, AMI | 0 | - | 16,6 |

As it is seen from the Table 2, the methylation of AMI is highest after 30 min incubation, and it decreases later. The methylation of MAMI increases in time and is highest after 60 min incubation. The enzymatic activity was higher with AMI as substrate than with MAMI.

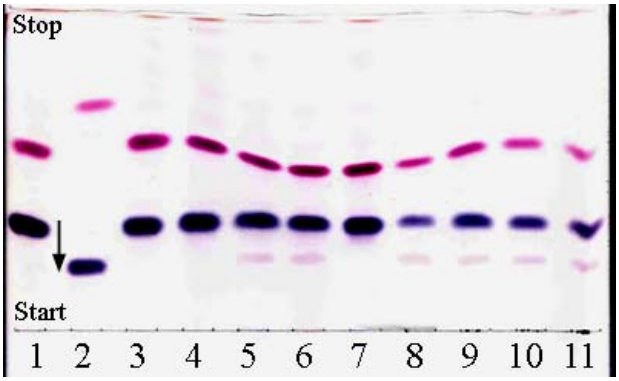


**Figure 11** *TLC analysis of the methyltransferase activity with AMI as substrate. The separation of the methylation products is shown by the arrow. The lanes 1 and 7 are corresponded to AMI standard, the lane 2 – to MAMI standard. the remainder illustrate following samples: 3 and 4 – product at 0 min; 5 and 6 – reactions with 3 mM AMI for 30 min; 8 and 9 – 1,5 mM 30 min; 10 and 11 – 0,75 mM 30 min.*

The methyltransferase activity was analyzed relatively the AMI and MAMI concentration (see Table 3). MAMI built from AMI is inversely proportional to the substrate concentration. The methylation of MAMI has a different pattern, and the experiment data can be questioned.

**Table 3** *AMI and MAMI methylation products built per 1 mg protein, pmol/min, relative to the substrates concentration*

| Concentration, mM | 0,75 mM | 1,5 mM | 3 mM |
|---|---|---|---|
| AMI | 37,03±10,2 | 18,71±1,4 | 21,34±4,7 |
| MAMI | 2,38±0,9 | - | 1,53±0,9 |

The coefficient *Rf* for AMI, MAMI and gramine was calculated (see Table 4, page 13). *Rf* belongs to the interval [0.15; 0.35] and an alignment between its value is as follow:

$$Rf_{\text{MAMI}} < Rf_{\text{AMI}} < Rf_{\text{Gramine}}$$

**Table 4** *Coefficient Rf for AMI, MAMI and Gramine*

| Substrate | Rf |
|---|---|
| AMI | 0,27 |
| MAMI | 0,15 |
| Gramine | 0,35 |

The enzyme activity with caffeic acid was also analyzed. The reactions with the enzyme extract from barley (variety Lina) green tissue did not show any activity. The reactions with the methyltransferase purified by IMPACT-CN Protein Purification System obtained some built product, but the data are questionable because of the experimental problems.

# *Discussion*

## Cloning of the OMT gene into the pTYB12 vector

The purpose of this study was to express the aphid-induced barley methyltransferase in *Escherichia coli* and to characterize the enzyme. With this point of view 5-days-old barley plants green tissue was treated with jasmonic acid (JA) to induce the *OMT*-gene. JA is serving as the intracellular defense signal, mediating expression of a range of early and late functioning defense genes [9]. It had been published earlier that also the *OMT*-gene is induced by JA [20]. The plasmid pTYB12 included in IMPACT Protein Purification System was chosen as a vector. A reason for that was that pTYB12 contained suitable restriction sites, which enable the fusion of the cleavable intein tag to the N-terminus of the target methyltransferase [23]. The plasmid pTYB12 was digested with the restriction nucleases *Sma* I and *NdeI*. *Sma*I recognizes the sequence 5'…CCC GGG…3' digesting between C and G, and its cleavage leaves blunt ends. The cleavage with *NdeI* is staggered and creates cohesive ends. The PCR product was digested only with *NdeI* to create compatible 5' ends for cloning. The 3'end is already blunt ended after the PCR with Phusion DNA polymerase. This enables its ligation with the plasmid DNA. The new plasmid pTYB12-OMT with the size of 8545 bp was obtained after the ligation reaction.

## Transformation of *E. coli* and screening for recombinants

With purpose to amplify pTYB12-OMT, *E. coli DH5α-T1* competent cells were transformed using the new plasmid DNA. The recombinant cells were selected employing the recombinant clones ability of resistance to the antibiotic ampicillin. Only the cells which had taken up the plasmid pTYB12-OMT could survive and grow on the plates with ampicillin. Thus randomly chosen colonies were inoculated in LB/amp medium, and plasmid DNA was obtained by purification.

Several attempts to acquire the positive recombinants had failed. It was already experienced in earlier studies [20] that *E. coli* had difficulties to survive after its uptake of the plasmid with the insert *OMT*. Few recombinant colonies were obtained and the survivors turned out to have mutations in the OMT sequence. In this project the third transformation resulted in a frameshift mutation – deletion of two base pairs with following stop codon leading to a change in reading frame. Eventually the fourth round of transforming *E. coli DH5α-T1* was successful.

To confirm the pTYB12-OMT recombinants, digesting reactions with restriction nucleases *KpnI*, *NcoI*, *NdeI* and *SapI* were performed. The result of the digesting reactions was analyzed by 1 % agarose gel electrophoresis (see Figure 4). It is known that the cutting of the plasmid with insert produces the following fragments: by the restriction nuclease *KpnI* - 6706 and 1801 bp; *NcoI* - 7380, 680 and 447 bp; *NdeI* - 8507 bp; and *SapI* - 7810 and 697 bp. The test of the three putative clones, which gave the expected PCR product during the insert amplification, revealed only one clone with the correct restriction fragment pattern. This once more indicates that *E. coli,* containing *OMT,* forms mutants to survive. The fact, that the clone obtained for pTYB12-OMT, had the correct sequence without any mutation, was finally confirmed by sequence analysis of the insert. This analysis is especially important for the reason, that the protein, purified from *E.*

*coli* for the enzyme measurements, must have the correct amino acid sequence.

## Transformation of the expression strain *E. coli ER2566* and induction of protein expression

Competent cells *E. coli ER2566* were provided by the IMPACT-CN Protein Purification System as a host strain for the expression of a target gene cloned into the pTYB12 vector. *ER2566* cells have a chromosomal copy of the T7 RNA polymerase gene inserted into the *lacZ* gene, and therefore under the control of the *lacZ* promoter. Expression of T7 RNA polymerase is suppressed in the absence of isopropylthiogalactoside (IPTG), by the binding of *lac* I repressor to the *lac* promoter. When IPTG is accessible, the transcription of the T7 polymerase gene is induced. The T7 RNA polymerase will then start transcription of the RNA for the fusion protein, which is under control of the T7 promoter.

The protein expression was induced with 0,5 mM IPTG (see Figure 7). SDS-PAGE analysis showed that this led to the induction of a strong protein band of about 100 kDa, which is in agreement with the expected size of the fusion protein intein-methyltransferase.

Among the different conditions, investigated for the induction of the protein, 20$^{o}$C over night and 1 mM IPTG were the most optimal. SDS-PAGE gel analysis has obtained the strongest band, which corresponded to the size of the intein-methyltransferase fusion protein, for these conditions (see Figure 8). Western blotting has confirmed this result.

## Purification of the target protein

The chitin-binding domain (CBD) in the intein tag allows purification of the fusion protein using a chitin column. The CBD has very high affinity for the chitin beads, which allows effective recovery of the fusion protein from the crude cell extract. The target protein was purified, and SDS-PAGE analysis showed a major band at 43 kDa in the eluant after the on-column cleavage reaction (see Figure 9). As it was expected the strong bands about 100 kDa, corresponding to the induced fusion protein intein-methyltransferase, can be seen at the lanes 3 and 4 of the samples before the loading onto the chitin column. It has been described previously that in an attempt to express the OMT protein in *E. coli* most of the protein was located in inclusion bodies [18]. Also in this experiment the 100 kDa fusion protein band was stronger in the crude cell extract as compared to the clarified lysate. This could be explained that some part of the protein in the *E. coli* cells was in the non-soluble fraction. The flow-through (lane 4) taken after the chitin column was loaded with the clarified cell extract, shows only a pale band of the size 100 kDa, confirming that the fusion protein intein-methyltransferase has bound to the chitin beads. The column and DTT wash (lanes 5 and 6 respectively) do not have any bands at all, proving the high efficiency and specificity of the chitin beads binding capacity. The bands about 43 kDa corresponding to the methyltransferase were obtained at lanes 7 - 9, corresponded to the 1 - 3 fractions respectively of the eluant 1. In addition, higher salt concentration, 1 M NaCl, was employed to reduce non-specific binding, thus increasing purity. But the higher salt concentration did reduce the purified protein concentration. The cleavage reaction at room temperature carried out for 24 h and using buffers containing 0,5 M NaCl was the most effectual resulting in the pure protein.

## Assay of the methyltransferase activity

Purified protein was used in enzyme assays with intermediates in gramine biosynthesis pathway, AMI (3-aminomethylindole) and MAMI (N-methyl-3-aminomethylindole), as substrate, as well as with caffeic acid, because the enzyme was previously described as caffeic acid OMT. AMI and MAMI were both acting as substrates, and transformed to MAMI and gramine, respectively. $^{3}$H count per minute was recalculated to build product, pmol/min, per 1 mg protein (see Table 2). The methylation of AMI was highest after 30 min incubation, decreasing later, which contradicts the kinetic development in time as a logarithmic function. The methylation of MAMI increases in time and is highest after 60 min incubation. The methyltransferase activity was analyzed relatively to AMI and MAMI concentration.

The production of MAMI from AMI was inversely proportional to the substrate concentration, indicating product inhibition.

The experiment has revealed that the coefficient *Rf* for AMI, MAMI and gramine, calculated as a dividend of the interval between the start line - built products and the start - solvent front lines on the TLC-plate, is not constant. It varies for different TLC-plates and seems to be dependent on the silica gel layer quality and the TLC solvent composition. It can be reasonable to apply the substrates samples onto each TLC-plate as a control in further tests.

The experiments, measuring the enzyme activity with caffeic acid as substrate, were difficult to interpret because of the experimental problems. There seems to be very little activity, but the assay must be developed to achieve some reliable results. It has to be noted that the purified enzyme was going through several freeze-thaw cycles between the first measurement with AMI and MAMI as substrate and those with caffeic acid. This could have resulted in the loss of enzymatic activity. Thus these experiments have to be repeated with freshly purified enzyme. However, a freshly extracted protein from green tissue of the barley variety Lina (a variety which contains the OMT gene and has a high gramine content), did not exhibit any activity with caffeic acid, but did methylate AMI and MAMI. This supports the preliminary results from the studies with the in vitro expressed OMT, indicating that it might be involved in gramine synthesis by methylating AMI and MAMI, rather than acting as caffeic acid OMT.

The enzyme of interest has been described as an O-methyltransferase, and a sequence comparison with other sequences in data bases indicates, that it has similarity to other OMTs. But its maximum similarity is only 40% [20]. Thus it could well be, that it carries out the transfer of a methyl group from S-adenosylmethionine to AMI, methylating it to MAMI and a methyl group from SAM to MAMI, with the formation of gramine.

In general terms, this work strongly supports the idea that the methyltransferase gene accession number U54767, could be involved in gramine biosynthesis, possibly acting as an NMT.

## Acknowledgements

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