Research article

Differential regulation of 3-aminomethylindole/N-methyl-3-aminomethylindole N-methyltransferase and gramine in barley by both biotic and abiotic stress conditions

Kristina A.E. Larsson, Sefiu A. Saheed, Therese Gradin, Gabriele Delp, Barbara Karpinska, Christiaan E.J. Botha, Lisbeth M.V. Jonsson

School of Life Sciences, Södertörn University, SE-141 89 Huddinge, Sweden
Department of Botany, Rhodes University, Lucas Avenue, Grahamstown 6140, South Africa

1. Introduction

A 3-aminomethylindole/N-methyl-3-aminomethylindole N-methyltransferase (EC 2.1.1) (NMT) enzyme is responsible for the final steps in the pathway to the indole alkaloid gramine (Fig. 1). The corresponding gene (NMT, GenBank accession no. U54767) has been cloned from barley (Hordeum vulgare L.) [13]. Gramine is thought to be involved in defence against aphids in barley as a feeding deterrent [33,34], and it increases as a consequence of greenbug (Schizaphis graminum Rondanii) feeding [27]. This suggests that, besides constitutive levels of gramine, aphid-induced gramine might be of importance for aphid defence.

Initial knowledge of aphid-induced responses was gained from studies on certain metabolites, defence proteins and RNAs but, more recently, gene-expression profiling has been applied to give a broader understanding of aphid-induced effects [see reviews 11,25,26]. In these studies it is often shown that defence-related genes are upregulated in aphid-infested plants, and it has been suggested that defence mechanisms regulated by methyl jasmonate (MeJA) may be more effective against aphids than other defences [7,26,32].

NMT (earlier believed to be an O-methyltransferase) is induced by methyl jasmonate (MeJA) [14]. If gramine and MeJA-regulated pathways are part of the plant’s effective inducible defence against
aphids, one would expect NMT and gramine induction to be more apparent in resistant than in susceptible genotypes. In order to test this hypothesis, NMT-gene expression and gramine content in aphid-infested plants were examined in different combinations of aphid species and barley genotypes with known resistance characteristics. The effects of bird cherry-oat aphid, *Rhopalosiphum padi* L. and the rose-grain aphid, *Metopolophium dirhodum* (Walker) were studied in a barley breeding line with moderate resistance against *R. padi*, doubled haploid 5172-28:4 (DH28:4), and the susceptible cultivar Lina. A third aphid species, the Russian wheat aphid (*Diuraphis noxia* Mordvilko) was studied on Lina and on the *D. noxia*-resistant line CI 1412 [30]. In order to allow valid comparisons with previous studies on *S. graminum* [27], the combination *R. padi* on cultivar Libra was also included. The results from these experiments prompted us to investigate the effect of MeJA as well as drought treatments and abscisic acid (ABA) on both NMT regulation and gramine content.

2. Methods

2.1. Plant material, growth and treatment of plants

Seeds of barley (*H. vulgare* L.) doubled haploid line 5172-28:4 (DH28:4) were provided by Svalöf Weibull AB. DH28:4 is derived from an F1 DH line obtained from a cross between cv. Lina and a wild *H. vulgare* ssp. *spontaneum* accession. The growth and proliferation of aphids (*R. padi*) on this cv. was decreased by 15% compared to Lina [8]. Seeds of cultivars Lina, Golf, Osiris and Etu were provided by Dr. I. Åhman, SLU, Sweden, and additional seeds of Osiris were provided by NordGen Plants. Seeds of barley line CI 1412 [30] and cv. Libra were provided by Small Grains Collection, United States Department of Agriculture.

Seeds were placed on filter paper soaked with 0.75% hydrogen peroxide in a petri-dish. The dishes were placed in a refrigerator at 6 °C for 3 days and then transferred to room temp. to germinate for 2 days [1]. Germinated seedlings were planted in soil and grown in a growth chamber at 18–22 °C, 16/8-h (light/dark) photoperiod at 160 μmol m⁻² s⁻¹. Plant age is calculated from day of planting, and after 10 days, the second leaf is fully expanded. Plant material was frozen in liquid nitrogen and stored at −70 °C until further use.

For MeJA treatment of cut leaves, the leaf blades of the first leaves (cotyledons) of 7-day-old barley line DH28:4 were cut into 5 cm pieces and placed in a soln. of 45 μM MeJA in H₂O during 24 h at room temp. Control leaves were treated with H₂O only.

For drought treatments, germinating seeds were planted and watered only on the first day, whereas control plants were watered every second day. For ABA treatments, 8-day-old plants were sprayed with a 45 μM ABA soln. in H₂O with 0.001% Triton X-100 twice a day for 48 h. Control plants were sprayed with H₂O containing 0.001% Triton X-100. In both experiments, the blades from the second leaves were harvested from 10-day-old plants.

2.2. Aphid rearing and infestation

Bird cherry-oat aphid, *R. padi* (L.) and rose-grain aphid, *M. dirhodum* (Walker) were collected in a barley field in the vicinity of Uppsala. Aphids were cultivated at Södertörn University in a growth chamber at 18–22 °C, long day (16/8 h photoperiod at 180 μmol m⁻² s⁻¹). The stock cultures of *R. padi* and *M. dirhodum* were kept on barley plants cv.‘s Lina and Kara, respectively. Russian wheat aphids, *D. noxia* (Mordvilko) were obtained from the Agricultural Research Council (ARC) Small Grain Institute, Bethlehem, South Africa. They were maintained on young barley plants, cv. Clipper, in a growth chamber at 18–22 °C, long day (16/8 h photoperiod at 180 μmol m⁻² s⁻¹) at Rhodes University, Grahamstown, South Africa. Plants used for rearing were visually inspected for signs of plant virus infections, and there were no such signs. As infection by *R. padi*-virus (RhpV) has been shown widespread in Swedish populations of *R. padi* [3], the *R. padi* aphids were routinely tested for infection by this virus, as described [3]. The RhpV is transferred to plants, but is not known to cause any effect in plants [3]. The tests were carried out to know the health status of the aphids, since RhpV shortens the lifespan of the aphids and affects their behaviour [4]. All tests were positive. Mixed instars were used for all aphid experiments.

Ten-day-old plants were infested with aphids by placing a plastic cylinder (5 cm high, 2.5 cm wide) around the 2nd leaf of the plant (first true leaf) and carefully placing about 20 aphids per plant within the cylinder using a painter’s brush. The cylinder was enclosed at both ends with foam rubber preventing the aphids from escaping, while allowing the exchange of air within the cylinder. After 1, 2, 3 and 4 days, the aphids were brushed off and the plant tissue within the cylinder was rapidly frozen in liquid nitrogen and stored at −70 °C. Control leaf tissue was treated the same way except for the presence of aphids. Three replicates, each consisting of four to six individual plants, were harvested per treatment. After grinding, each replicate was divided into three aliquots, used for the extraction of RNA, protein and gramine, respectively. Aphid infestation on cv. Libra was carried out as in Velozo et al. [27], except that 20 *R. padi* aphids were used instead of *S. graminum*. Ten-day-old plants were infested with aphids, which were allowed to move freely on the whole plant. After 3 and 6 days the aphids were brushed off and the leaf 2 and 3 were harvested and used to determine the gramine content. Control plants were harvested at day 0, 3 and 6. Three replicates, each consisting of leaf material from two plants, were harvested at each time point and treatment.

2.3. Total RNA isolation and qRT-PCR

Total RNA was isolated from 30 to 100 mg plant tissue using either the Plant RNeasy Mini kit (Qiagen, West Sussex, UK) or the Total RNA Purification From Plant — NucleoSpin® RNA Plant (Macherey-Nagel, Germany) according to the kit protocols. The frozen tissue was ground in a mortar to a fine powder and mixed with lysis buffer. The RNA was DNase treated and the purified RNA was dissolved in RNase-free water. Two types of qRT-PCR were applied during this study. In the aphid infestation experiments, qRT-PCR was performed using iScript™ One-Step RT-PCR Kit with SYBR® Green (BIO-RAD, USA) according to the kit protocol. Thirty
ng of total RNA was used as template. Cycling conditions on MyIQ™ Single-Color qPCR Detection System (BIO-RAD) were: 1 cycle of 50 °C (10 min), 95 °C (5 min), and 45 cycles of 95 °C (10 s) and 59 °C (30 s). A melting curve was run after the PCR starting at 95 °C (1 min), 55 °C (1 min) and then 80 cycles, increasing each cycle by 0.5 °C, starting at 55 °C for 10 s. In the other experiments (MeJA, ABA and drought treatments), cDNA was produced using iScript™ cDNA Synthesis Kit (BIO-RAD) and 1 μl cDNA was taken to a mix with iQTM SYBR® Green Supermix (BIO-RAD). Cycling conditions were: 1 cycle of 95 °C (4.5 min), 50 cycles of 95 °C (20 s), 57 °C (20 s) and 72 °C (30 s), 72 °C (10 min). A melting curve was run after the PCR starting at 95 °C (20 s), 55 °C (1 min) and then 80 cycles, increasing each cycle by 0.5 °C, starting at 53 °C for 10 s. The melting curve at the end of each qRT-PCR run was included to ensure single DNA fragment amplification. In all experiments, three replicates (with 4–6 plant individuals) were analyzed and all reactions were prepared as duplicates. A primer specific for actin was used as reference for relative normalization of transcript levels. A no template control was included. For calculating relative transcription ratio between sample and control compared to reference gene, a formula by Pfaffl [18] was used: \[\frac{\Delta \Delta CT_{target}}{\Delta \Delta CT_{ref}}\]. Results were correlated with primer efficiency by using LinRegPCR software [19]. For statistical evaluation of the results in Fig. 2, an ANOVA was performed on log-transformed ratios, with experimental set, time and treatment as factors. The interaction between time and treatment was also included in the model. Stata version 11 was used for the analysis. All primer sequences are shown in (Table 1).

2.4. Preparation of plant protein extracts and enzyme assays

Frozen plant material was ground in a mortar in liquid nitrogen and protein extracts prepared as described by Larsson et al. [13]. Enzyme assays were carried out as described in [13] with N-methyl-3-aminomethylindole (MAMI) as substrate. 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 (Bradford) Protein assay Reagent (Pierce, UK) with bovine serum albumin as standard.

2.5. SDS-PAGE and Western blot

Protein concentrations were measured as described above and aliquots corresponding to 10 μg were separated on 4–20% gradient Tris–Glycine gels. The protein bands were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, UK) electrophoretically, using a Semi Phor™ transfer apparatus ( Hoefer Scientific Instruments, San Francisco). Western blot was carried out as described by Larsson et al. [13] using affinity purified primary antibodies against the recombinant NMT protein (1:500) and secondary antibody, Anti-Chicken HRP (lgY) (1:10 000). Proteins were detected by 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).

2.6. Gramine extraction and analysis by HPLC

Plant material was frozen in liquid nitrogen and ground to a powder. Ca. 20 mg of frozen sample was used for gramine extraction and analysis as in [13]. For determinations of gramine per dry weight, the tissue powder was divided into two identical samples. One half was used for gramine analysis and the other was used to determine dry weight after drying in a 30 °C oven until the weight was stable.

Fig. 2. The effect of aphid infestation on transcript abundance of NMT in barley leaves. Ten-day-old barley plants of line DH28:4 and cv. Lina were infested for 24, 48, 72 and 96 h with R. padi or M. dirhodum. A) Lina infested with R. padi and B) M. dirhodum, C) DH28:4 infested with R. padi and D) M. dirhodum. The column represent 3 biological replicates (each consisting of 4–6 plants), performed in technical duplicate. The transcript abundance was normalized to actin and is presented as relative values in comparison to day 0 (value set to 1). Error bars indicate s.d. Asterisks indicate statistical significance of infestation in comparison to control (ANOVA. *p < 0.05; ***p < 0.0005).

3. Results

3.1. R. padi and M. dirhodum feeding induces the NMT gene specifically in a barley genotype with moderate resistance against R. padi

The effects of aphid infestation on NMT transcript abundance were studied in six plant–aphid combinations. R. padi and M. dirhodum were infesting the susceptible cultivar Lina and the doubled haploid 5172–28:4 (DH28:4) that has moderate resistance against R.
Neither of the two aphid species had any effect on NMT transcript abundance in Lina, but both caused higher NMT transcript levels in DH28:4 (Fig. 2). In control samples, the amount of NMT transcript decreased with time during the experimental period while it had increased by day 4 in aphid-infested DH28:4 plants. The effect of D. noxia feeding on NMT was examined in Lina and the D. noxia-resistant barley genotype CI 1412 [30]. There was no regulation of NMT-gene expression in these genotypes upon infestation by D. noxia (data not shown).

3.2. Gramine content remains unchanged in all aphid-infested barley lines

The differences between DH28:4 and Lina in NMT transcript abundance did not lead to differences in gramine content (Fig. 3). During the 96 h experimental period from day 7 to 11, there was no change in gramine content in control leaves or infested leaves in any of the genotypes exposed to R. padi or M. dirhodum (Fig. 3). Gramine content in Lina infested with D. noxia also remained unchanged (data not shown). In CI 1412, gramine content was highly variable (0.1–0.8 mg g⁻¹ fr. wt), but there was no indication that infestation by D. noxia caused increased gramine. A separate experiment was carried out with one of the cultivars, Libra, that showed gramine increase after infestation with S. graminum [27]. When Libra was exposed to R. padi, no gramine increase was detected (not shown).

3.3. Upregulation of the NMT gene has no effect on NMT protein amount or enzyme activity in aphid-infested or MeJA-treated tissue

The apparent discrepancy between higher transcript abundance of NMT and lack of significantly higher gramine content in DH28:4 infested by R. padi or M. dirhodum was addressed. One possible explanation was that NMT transcripts were not translated into NMT protein. This was examined by studying the NMT protein concentration and NMT enzyme activity in extracts from DH28:4 infested by M. dirhodum in which the NMT gene was clearly upregulated (Fig. 2). The results showed that the relative amount of NMT protein and NMT enzyme activity decreased similarly for the duration of the experiments in both infested and control plants (Fig. 4).

The effect of a very strong upregulation of the NMT gene on NMT protein and enzyme activity was then investigated using cotyledons incubated in a solution containing MeJA, which causes induction of NMT [14]. As with transcripts for the MeJA-inducible reference gene allene oxide synthase, AOS2 [10,15], NMT transcript increased strongly (200 times) in the MeJA-treated leaves compared to the control (Fig. 5A, B). A previous study reported that MeJA-treatments lead to an increase in AOS protein detectable 12 h after the commencement of the experiment [15], and we thus expected an increase in NMT protein after 24 h. However, neither the quantity of NMT protein nor NMT enzyme activity increased in DH28:4 MeJA-treated leaves, and there was no increase in gramine (Fig. 5C–E).

3.4. Gramine, but not NMT expression, is increased in barley due to drought and ABA treatment

Having found no gramine induction upon infestation by R. padi, M. dirhodum or D. noxia, possible explanations for the difference between S. graminum and these aphid species were considered. In experiments with S. graminum, Cabrera et al. [6] observed that aphid infestation and drought stress caused similar changes to the plants. To test if drought can induce gramine synthesis, Lina plants were either subjected to mild drought stress during development or treated with 45 μM abscisic acid (ABA) from day 8. Leaves from

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Table 1
Sequences of primers used.

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<th>Gene abbreviation</th>
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Fig. 3. The effect of aphid infestation on gramine concentration in barley leaves. Ten-day-old barley plants, line DH28:4 and cv. Lina were infested for 24, 48, 72 and 96 h with R. padi or M. dirhodum. A) Lina infested with R. padi, and B) M. dirhodum, C) DH28:4 infested with R. padi and D) M. dirhodum. Each column represents 3 biological replicates consisting of 4–6 plants. Error bars show the s.d.

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Fig. 5. A) Cotyledons from 4-day-old barley plants were treated with 10 μM MeJA and incubated in a solution containing MeJA or distilled water for 3 h. The quantity of NMT protein was determined using cotyledons incubated in a solution containing MeJA. B) Cotyledons from 4-day-old barley plants were treated with 10 μM MeJA and incubated in a solution containing MeJA or distilled water for 3 h. The quantity of NMT protein was determined using cotyledons incubated in a solution containing MeJA.
drought stressed plants were smaller than those from the well-watered control plants, but showed no signs of wilting. At harvest, control plants had three visible leaves, whereas drought stressed plants had only reached the two leaf stage. To allow direct comparison with the study of Velozo et al. [27], gramine concentration was first calculated relative to fresh weight. Drought and ABA treatment resulted in higher gramine content (Fig. 6A). When calculated relative to protein amount, the gramine increase was less apparent ($p = 0.08$) (Fig. 6B). The treated plants showed an increase in the transcript abundance of dehydration-responsive factor 1 (HvDRF1), an indicator of drought stress (Fig. 6C). There was, however, no increase in NMT transcript abundance (Fig. 6D), confirming the earlier report [14] that ABA does not induce NMT.

The effect of drought stress on gramine concentration was investigated in four additional genotypes, DH28:4, Golf, Osiris and Etu. An increase in gramine relative to dry weight, the gramine increase was statistically significant in Osiris and Etu (Fig. 7B).

4. Discussion

A general finding in this study is that there is no correlation between the regulation of NMT expression and gramine content under the conditions studied here. The NMT gene is confirmed to be induced by MeJA treatment, but not by ABA [14]. The opposite appears true for the end product of the biosynthetic pathway, gramine, that is not increased after MeJA treatment or aphid infestation, but does increase in response to drought or ABA treatment. The latter increase may be explained by higher amounts of tryptophan in the leaves, earlier reported for ABA treated barley [17]. The NMT activity levels seem not to constitute any bottleneck in the biosynthetic pathway. This would also appear from the accumulation pattern of gramine and its precursors AMI and MAMI in developing barley [24]. In the cited study, gramine was detected in the aerial parts by day 2 during seedling development and no earlier detection of AMI and MAMI was evident. The amounts of AMI and MAMI remained very low during development, indicating that the precursors were readily transformed into gramine.

The initial hypothesis that the MeJA-regulated steps in the pathway to gramine may be more strongly upregulated by aphids...
in resistant than in susceptible barley genotypes, was only confirmed with regard to the higher transcript abundance of NMT in resistant DH28:4 as compared with that in Lina. However, as increased NMT transcripts did not translate to higher NMT protein or higher gramine content, the regulation at transcript level had no consequence for the metabolic composition of the plant tissue, and thus not for the defence against aphids. The results may be explained by suppression of NMT mRNA translation, which was also reported in previous studies with barley, in which MeJA suppressed the translation of specific mRNA sets, mainly for pre-existing proteins [20,21]. An alternative explanation, higher turnover of NMT protein in treated plants, does not seem likely since protein content and enzyme activity decreased over time in a similar manner in both control and in treated plants (Fig. 4).

It has been suggested that MeJA-regulated defence is effective against aphids and that aphids can suppress such defence [32]. This line of thought has substantial experimental support in the case of another type of phloem feeder, whitefly [28,31], but in the case of aphids it is mainly based on transcript analysis [32]. Overall, transcript analyses in plants upon aphid feeding have as yet little support at the protein or metabolite level [25,26]. In the case of R. padi on barley, some of the gene sequences found upregulated in a microarray study [8] could be matched with chitinases and glucanases previously demonstrated as aphid-induced using antibodies [9]. In the area of aphid-induced responses related to secondary metabolism, careful follow-up studies at the metabolite level have been carried out in Arabidopsis with genes related to glucosinolate and camalexin biosynthesis [12,16]. The former study [12] indicated that increased gene expression upon herbivory was not always associated with increased amounts of glucosinolates. Our present results add to this body of knowledge in a monocot species and confirms that the regulation at transcript and metabolite level may diverge.

A third major finding is that whereas gramine is induced under conditions of drought stress, it is not induced by the aphids, R. padi, M. dirhodum or D. noxia. This contrasts with Velozo et al. [27] who report increased gramine in response to S. graminum. The three aphid species that were used here give rise to differing severity of symptoms, where R. padi causes no visible symptoms, M. dirhodum causes intermediate symptoms with chlorotic spots and D. noxia causes serious symptoms of chlorosis and necrosis, white or yellow longitudinal streaks and leaf rolling [22,29]. Gramine has been reported in mesophyll and epidermis [2], and especially in the D. noxia infested plants, the lack of gramine increase may thus be due to the disturbance of tissue integrity and metabolism. This effect is probably especially prominent in the CI 1412 line, where mesophyll and bundle sheath cells collapse upon D. noxia infestation [5] and, in accordance, gramine content showed large variation in CI 1412. Nevertheless, our results from experiments using different combinations with three aphid species and four barley genotypes suggest that the gramine increase reported for S. graminum might be
specific to this aphid. Plants infested by *S. graminum* show symptoms of drought stress [6], which suggest that the gramine induction reported for this aphid [27] may be related to drought stress. Additional evidence for this idea is provided by an analysis of transcriptional regulation in sorghum due to infestation by *S. graminum* that indicated the induction of a drought, salt, low temperature-responsive gene [32]. In contrast, no drought-related gene sequences were found upregulated in four different barley lines after infestation by *R. padi* [8].

The potential for gramine increase in response to drought differed between the genotypes. This may explain that a previous study with drought stress in one barley genotype reported no significant effect on gramine content [23]. It also has wider implications, in that drought is a common physiological stress in barley cultivation, and that this condition might well be aggravated by aphid infestation. Given the complex environmental variables in the field, it is thus likely that gramine concentration could become elevated in field-grown plants and its role in plant response to biotic stress probably differs from what appears in greenhouse-based studies.

Acknowledgments

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