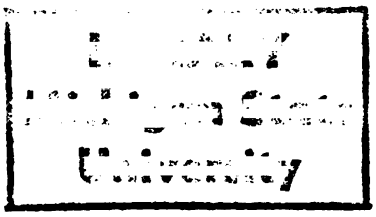

This is to certify that the

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High-Temperature Induction of Gramine Biosynthesis in Barley

presented by

Timothy J. Leland

has been accepted towards fulfillment of the requirements for

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Major professor

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HIGH-TEMPERATURE INDUCTION OF GRAMINE BIOSYNTHESIS IN BARLEY

by

Timothy James Leland

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ABSTRACT

HIGH-TEMPERATURE INDUCTION OF
GRAMINE BIOSYNTHESIS IN BARLEY

BY

TIMOTHY JAMES LELAND

The high-temperature regulation of the N-methyltransferase (NMT) steps of gramine biosynthesis in barley (Hordeum vulgare L) was characterized. In in vivo N-methylation assays using [^{14}C]formate as a methyl precursor, fifth leaves from plants of cv. Proctor and cv. Arimar growing under mild heat stress (30°C day/25°C night) incorporated several-fold more ^{14}C into gramine and its immediate precursor methyl-3-aminomethylindole (MAMI) than did fifth leaf tissue from plants growing in cooler conditions. Crude extracts prepared from Arimar fifth leaves grown at 35°C/30°C and supplied the gramine precursors 3-aminomethylindole (AMI) or MAMI plus S-adenosyl-L-[methyl-^{14}C]methionine ([^{14}C]SAM) had NMT activities at least 20-fold higher than extracts of fifth leaves grown at 15°C/10°C. To study these changes at the protein level, NMT activity from Proctor first leaves was purified >200-fold and used to raise antiserum in a rabbit. Immunoblots showed that growth of fifth leaves at high temperature increased the levels of NMT protein many-fold.

To investigate genetic control of the gramine synthesis pathway, reciprocal crosses were made between three cultivars with distinct phenotypes: Arimar, which contains gramine; Proctor, which contains no indole alkaloids but has normal NMT activity; and Morex, which has

neither alkaloids nor NMT activity. Results from analysis of F_1 and F_2 generations can be explained by hypothesizing that (a) Proctor and Morex carry the same defective allele at a locus (Ami) governing AMI synthesis; (b) Morex also carries a defective allele at a second locus (Nmt) which specifies a NMT enzyme active against both AMI and MAMI; and (c) the Ami and Nmt loci are very tightly linked.

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Special thanks to my major professor, Dr. Andrew Hanson, for his role as teacher in the "art of the soluble". To the members of my committee, Drs. Hans Kende, Norman Good, Stanley Ries, and Shauna Somerville, I would like to express my appreciation for their constructive advise and support. I acknowledge my debt to the faculty, staff, post-docs and graduate students of the MSU/DOE Plant Research Laboratory. The free exchange of ideas, the pursuit of excellence and spirit of goodwill were 'sine qua non'.

Finally and foremost, I acknowledge the support and patience of my wife, Theresa, on whose part was the greater sacrifice.

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LIST OF ABBREVIATIONS

SAM	S-Adenosyl-L-methionine
[^{14}C]SAM	S-Adenosyl-L-[methyl-^{14}C]methionine
AMI	3-Aminomethylindole
BSA	Bovine serum albumin
CRM	Cross-reacting material
Cv	Cultivar
Ci	Curie
d	Day
DEAE	Diethylaminoethylcellulose
DTT	Dithiothreitol
E	Einstein
EDTA	Ethylenediaminetetraacetic acid
equivs	Equivalents
fr wt	Fresh weight
HSP	Heat Shock protein
HPLC	High-performance liquid chromatography
h	Hour
kD	kiloDalton
lf	Leaf
MAMI	Methyl-3-aminomethylindole
NMT	N-methyltransferase
min	Minute
mol wt	Molecular weight
PC	Paper chromatography
PPFD	Photosynthetic photon flux density

PAR	Photosynthetically active radiation
PAGE	Polyacrylamide gel electrophoresis
RH	Relative humidity
s	Second
SDS	Sodium dodecyl sulfate
SE	Standard error
TLC	Thin-layer chromatography
v	Volume

GENERAL INTRODUCTION

Gramine is a simple indole alkaloid found in the shoots of many barley cultivars (1,9). It has also been reported in reed canarygrass (Phalaris arundinacia L) and other Phalaris species (13). Gramine biosynthesis is fairly well characterized: gramine is derived from tryptophan, the first stable intermediate is 3-aminomethylindole (AMI) which is sequentially N-methylated to methyl-3-aminomethylindole (MAMI) and finally to the dimethyl compound, gramine (6,11,15,17,18). The methyl donor in vitro is S-adenosyl-L-methionine (SAM) (15).

There exists considerable genetic diversity within cultivated barley (Hordeum vulgare L) and its wild progenitor (Hordeum spontaneum) for gramine content. Wild barleys and many cultivars (e.g. Arimar) can contain 8 mg g^{-1} dry wt of gramine at the one to three leaf stage (9). In contrast, other cultivars (e.g. Proctor) have no detectable levels of gramine at any time in their life history. In the case of Proctor, there appears to be a lesion early in the pathway because leaf segments fed AMI or MAMI can convert these precursors to gramine (8).

Gramine accumulation is constitutive in first leaves of barley. With each subsequently emerging leaf in plants grown under optimal conditions (21°C, 16 hr d/16°C, 8 hr night) there is less biosynthesis so that total alkaloid content at the fifth to tenth leaf stage may be negligible (7,16,17). If, however, plants are transferred to supra-optimal growth temperatures (30°C/25°C, or higher), young developing leaves are induced to actively synthesize gramine, causing levels to remain high in mature plants (8,9). Although catabolic routes for gramine are known in barley (4,8,16), they are not very active so that turnover of gramine does not exceed 5% d^{-1} (8).

The role of gramine in barley appears to be one of a general defensive compound functioning primarily to deter herbivores. The toxicity of indole alkaloids, of which gramine is a member, towards mammals, is the highest of any class of alkaloids (lethal dose 50 mg/kg body weight) (12). One of the best cases for alkaloid protection against consumption by higher animals can be found in reports that the high levels of gramine and related tryptamines found in certain Phalaris sp. make it unpalatable as a forage for sheep (13). Gramine, fed to meadow voles (5) and aphids (2) at concentrations similar to those found in barley leaves had lethal effects. It is interesting to note, moreover, that gramine accumulation coincides with growth stages at which barley may be most vulnerable to herbivore attack: at first leaf emergence and also under heat stress. Jones (1981) has noted similar cases in cyanogenesis of the bracken fern, Pteridium aquilinum and Sorghum bicolor (10).

The presence of a compound with potent physiological activity at high internal concentration suggests that barley is itself either insensitive to gramine or, more probably, is able to effectively partition gramine away from sensitive metabolic processes. At high temperatures however, gramine becomes autotoxic: application of gramine to plantlets under heat stress inhibited growth and provoked chlorosis and necrosis. In general, high gramine cultivars and wild barleys exhibited more severe heat injury symptoms than did gramine-deficient cultivars (8). It has been suggested that the variation in gramine level among barley genotypes may reflect an evolutionary compromise between herbivore pressure and the probability of encountering high temperatures (8). Similar arguments have been made in the cases of cyanogenesis in Trifolium repens (3) and

chlorogenic acid accumulation in Helianthus annus (14).

As a biological phenomenon, the gramine trait in barley has two interesting features which recommend it for study. Firstly, gramine accumulation is specifically induced by high temperature; it represents a specific metabolic response on the part of the plant to relatively long-term environmental stress. Secondly, the induced response is tissue specific; only those leaves which are expanding and undergoing cell division and elongation in the high temperatures actively accumulate gramine. The regulation of gramine biosynthesis in those cells differentiating under high temperature stress may provide insights into plant responses to other long term stresses, especially where growth and adaptation "decisions" are focused within developing meristems.

The purpose of this work was to characterize the regulation of gramine biosynthesis as a stress response. The means to this end were: 1) gramine biosynthetic activity as a response to high temperature was measured and characterized at the N-methyltransferase steps in the pathway; 2) using natural variants for the gramine trait crosses were made to genetically characterize the pathway; and 3) to assess the specificity of heat stress as an inducer of gramine biosynthesis, barley plants were subjected to a general biological stress (Erysiphe graminis, powdery mildew); the role of gramine as a possible resistance factor was also studied.

REFERENCES

1. BOWDEN E, L MARION 1951 The biogenesis of alkaloids. IV. The formation of gramine from tryptophan in barley. Can J Chem 29: 1037-1042

2. CORCUERA LJ 1984 Effects of indole alkaloids from Gramineae on aphids. Phytochemistry 23:539-541

3. DADAY H 1965 Gene frequencies in wild populations of Trifolium repens L. IV. Mechanism of natural selection. Heredity 20:355-365

4. DIGENIS GA 1969 Metabolic rates of gramine in barley. I: Mechanism of incorporation of gramine into tryptophan in barley shoots. J Pharm Sci 58:39-42

5. GOELZ MFB, H ROTHENBACHER, JP WIGGINS, WA KENDALL, TV HERSHBERGER 1980 Some hematological and histopathological effects of the alkaloids gramine and hordenine on meadow voles (Microtus pennsylvanicus). Toxicology 18:125-131

6. GOWER BG, E LEETE 1963 Biosynthesis of gramine: The immediate precursors of the alkaloid. J Amer Chem Soc 85: 3683-3685

7. GROSS D, H LEHMANN, H-R SCHUTTE 1970 Zur Physiologie der Graminbildung. Z Pflanzenphysiol 63:1-9

8. HANSON AD, KM DITZ, GW SINGLETARY, TJ LELAND 1983 Gramine accumulation in leaves of barley grown under high-temperature stress. Plant Physiol 62:305-312

9. HANSON AD, PH TRAYNOR, KM DITZ, DA REICOSKY 1981 Gramine in barley forage effects of genotype and environment. Crop Sci 21:726-730

10. JONES DA 1981 Cyanide and coevolution. In B. Vennesland et al., ed, Cyanide in Biology. Academic Press, London, pp. 509-516

11. LEETE E, L MARION 1953 The biogenesis of alkaloids. IX. Further investigations on the formation of gramine from tryptophan. Can J Chem 31:1195-1202

12. LEVIN DA, BM YORK JR 1978 The toxixity of plant alkaloids: an Ecogeographic perspective. Biochem System Ecology 6:61-76

13. MARTEN GC 1973 Alkaloids in reed canarygrass. In AG Matches, ed, Anti-Quality Components of Forages. Crop Science Society of America, Madison, pp. 15-31

14. MORAL R del 1971 On the variability of chlorogenic acid concentration. Oecologia (Berl) 9:289-300

15. MUDD SH 1961 3-Aminomethylindole and 3-methylaminomethylindole: New constituents of barley. Nature 189:489

16. SCHALLENBERG J, E MEYER 1981 Degradation of gramine by cell suspension cultures of barley (Hordeum vulgare). Planta Med 42:133

17. SCHNEIDER EA, F WIGHTMAN 1974 Amino acid metabolism in plants. V. Changes in basic indole compounds and the developments of tryptophan decarboxylase in barley (Hordeum vulgare) during germination and seedling growth. Can J Biochem 52:698-703

18. WIGHTMAN F, MD CHISHOLM, AC NEISH 1961 Biosynthesis of tryptophan and gramine in young barley shoots. Phytochemistry 1:30-37

Chapter 1

Induction of a Specific N-Methyltransferase Enzyme by Long-term Heat Stress During Barley Leaf Growth

Title: Induction of a Specific N-Methyltransferase Enzyme by Long-term Heat Stress During Barley Leaf Growth[1]

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[3]Abbreviations: AMI, 3-aminomethylindole; MAMI, N-methyl-3-aminomethylindole; [^{14}C]SAM, S-adenosyl-L-[methyl-^{14}C]methionine; NMT, N-methyltransferase; HSP, heat shock protein.

ABSTRACT

Previous work showed that the indole alkaloid gramine accumulates in the upper leaves (e.g. the fifth) of barley as a response to high growth temperatures. The biosynthesis of gramine proceeds from tryptophan to 3-aminomethylindole (AMI); sequential N-methylations of AMI then yield N-methyl-3-aminomethylindole (MAMI) and gramine.

To determine whether high-temperature stress increases the activity of gramine biosynthetic enzymes, leaf tissue from plants grown at various temperatures was assayed for N-methyltransferase (NMT) activity using AMI and MAMI as substrates in both in vivo and in vitro assays. NMT activity in expanding fifth leaves was increased eight- to 20-fold by growth at high temperatures (35°C day/30°C night) compared to cool temperatures (15°C/10°C). Several days of high temperature were required for full induction of NMT activity. No induction of NMT activity occurred in leaves which had completed expansion in cool conditions before exposure to high temperature.

To investigate NMT induction at the protein level, NMT activity was purified to homogeneity and used to produce polyclonal antibodies. Throughout enzyme purification, relative NMT activities towards AMI and MAMI remained constant, consistent with a single NMT enzyme. Immunoblot analysis showed that a large increase in NMT polypeptide coincided with induction of NMT activity by heat stress. Our results point to a type of high-temperature regulation of gene expression that is quite distinct from heat shock.

INTRODUCTION

Gramine is a simple indole alkaloid found in the shoots of many barley (Hordeum vulgare L.) cultivars and wild barley lines (3,12). Gramine biosynthesis involves the steps shown in Scheme 1. The indole nucleus and the methylene side chain of tryptophan are incorporated into the first stable intermediate of the pathway, AMI[3] (7,9). AMI is then methylated at the amino nitrogen to form the secondary amine, MAMI, which is in turn N-methylated to produce the tertiary amine, gramine (7,19,23). Indirect evidence indicates that these methylations are catalyzed by an NMT enzyme (or enzymes) specific to the gramine pathway (11), for which SAM acts as the methyl donor (19). Although degradative pathways for gramine are known (6), gramine catabolism is very slow (11) so that accumulation is controlled mainly by the rate of synthesis.

Some barley cultivars are gramine-free (11,12). In the case of cv. Proctor, the synthesis pathway is known to be blocked prior to AMI, because Proctor leaf tissue can methylate AMI and MAMI as actively as cultivars which contain gramine (11).

Accumulation of gramine in barley is subject to both developmental and environmental control (11,12). Developmental control is evident from a short phase of constitutive accumulation of gramine in young plants, particularly in the first leaf (11,23). In plants grown at or below optimal temperatures, each successive leaf contains less gramine so that alkaloid levels are negligible in the fifth to tenth leaves (8, 11,23). Gramine accumulation in these upper leaves can be elicited by growth at supra-optimal temperatures (11,12). Only those leaves which

actually emerge during the exposure to high temperature accumulate the alkaloid: previously-expanded leaves do not (11).

Here, we establish by in vivo and in vitro assay methods that a large increase in activity of a specific NMT enzyme accompanies the induction of gramine accumulation by heat-stress. Using antibodies directed against purified NMT protein, we also demonstrate that high-temperature induction of NMT activity is paralleled by an increase in NMT protein level.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Sources for the spring barley cultivars Proctor (CI 11806) and Arimar (CI 13626) were as given previously (12). Plants were grown in pots of soil mix (12) and irrigated with half-strength Hoagland's solution. Standard growth chamber conditions were: day, 16 h, 200 umol $m^{-2}s^{-1}$ PPFD, vapor pressure deficit 10 mbar; night, 8 h. For growth temperature experiments on the fifth leaf, plants were grown for the first 10 d at 15°C/10°C or 21°C/16°C (day/night) and thinned to two or three seedlings per pot. Some pots were then transferred to higher temperature regimes (30°C/25°C, 33°C/28°C, 35°C/30°C) and the experiment was continued until the fifth leaf was at the half-emerged stage, at which time it was harvested. The time between transfer and harvest varied according to temperature and cultivar. To obtain etiolated first leaves for enzyme characterization and purification, seedlings of cv. Proctor were grown at 25°C for 6 to 8 d in darkness in flats of vermiculite.

Radiochemicals and Alkaloid Precursors. [^{14}C]Formate (57 or 59 uCi

umol^{-1}) and S-adenosyl-L-[methyl-^{14}C]methionine (59 or 60 uCi umol^{-1}) were from Amersham Corp. The radiochemical purity of [^{14}C]SAM was checked by TLC on silica gel G in N-butanol:1M HCl:ethanol (5:5:2). [^{14}C]Gramine (0.16 or 0.20 uCi umol^{-1}) and [^{14}C]MAMI (0.19 or 0.25 uCi umol^{-1}) were isolated from Arimar first leaves fed [^{14}C]formate (11).

Gramine (Sigma) was recrystallized from acetone. Tryptamine HCl (NBC) and tyramine (Sigma) were recrystallized from 96% ethanol and checked for purity by paper chromatography in the 'Isobuff' system (11). AMI was synthesized according to Putochin (20) and Schallenberg and Meyer (21). MAMI was synthesized according to Gower and Leete (7). Both AMI and MAMI were purified on a Sephadex LH-20 column eluted with 50% (v/v) methanol. Confirmation of the identity of AMI and MAMI was obtained with a Hewlett-Packard 5985 quadrapole mass spectrometer by direct probe, as well as by TLC and paper chromatography with known standards. The p-dimethylaminocinnamaldehyde spray reagent was used to visualize indole compounds (11).

In Vivo Assay of NMT Activity. This assay used [^{14}]formate to label pools of methyl groups in vivo (11). For growth-temperature experiments, one or two 1-cm segments were cut from the basal furled portions of half-expanded fifth leaves. Batches of three segments were first vacuum-infiltrated either with 1 ul/segment of unlabeled gramine precursor (AMI or MAMI, 9 nmol/segment) dissolved in K-phosphate buffer (20 mM, pH7), or with buffer alone. After 1.5 to 3 h incubation on moist filter paper in darkness, segments were infiltrated with 1 ul/segment of [^{14}C]formate in H_2O (0.4 to 0.5 uCi/segment). Incubation was then continued for 24 h, in the 21°C/16°C growth chamber. Following the 24-h incubation, segments were frozen in liquid N_2; 100 mg of

freeze-dried, ground 7-d Arimar shoots were added to each three-segment sample to provide carrier for ^{14}C-alkaloids during extraction. Alkaloids were extracted as described previously (12). Representative unlabeled samples were spiked with a small quantity of [^{14}C]gramine (4.0 nCi) or [^{14}C]MAMI (2.5 nCi), for estimation of gramine and MAMI recovery which averaged 65% and 56%, respectively. All results have been corrected for alkaloid recovery. Alkaloid fractions were analyzed in two or more of the following systems: TLC on silica gel G in methanol:acetone:con.HCl (90:10:4, v/v), or n-butanol:ethanol:conc.NH_4OH (40:2:3, v/v), or chloroform:methanol:conc.NH_4OH (80:15:1, v/v) (18); or paper chromatography in the 'Isobuff' system. Radioactive zones were located by autoradiography and eluted for scintillation counting. Identification of labeled alkaloids was based on co-chromatography with authentic standards.

In Vitro Assay of NMT Activity. Half-expanded fifth leaves were harvested; in some cases second leaves were also taken. Leaves were ground at 4°C in a mortar and pestle with acid-washed sand in 50 mM Tris/HCl (pH 8.5) containing 7 mM DTT (2 vol/g fr wt). Homogenates were centrifuged at 25,000 x g for 15 min and the supernatant (crude extract) was used for assays.

The assay was similar to those of Mudd (19), Mack and Slaytor (16) and Meyer (17). The standard assay mixture contained 150 mM glycylglycine/NaOH (pH 9.0), 5 mM DTT, 0.6 mM [methyl-^{14}C]SAM (45 nCi umol^{-1}, 5.4 nCi) and 3 mM AMI or MAMI combined with 150 ul of crude extract in a total volume of 200 ul. After a 30-min incubation at 25°C in a shaking water-bath, 500 nmol each of MAMI and/or gramine were added as carriers for labeled alkaloids, and the reaction was stopped with

0.20 ml of 1 M H_3BO_3/1 M Na_2CO_3 buffer, pH 10. Alkaloids were extracted into 2 ml of $CHCl_3$, 1 ml of which was taken for scintillation counting. The remaining 1 ml was reduced to dryness in an N_2 stream and the residue was then redissolved in 60 mM HCl for chromatographic analysis of labeled products as described above. In blank assays, either without enzyme solution or without AMI/MAMI substrates, partitioning of ^{14}C into the $CHCl_3$ phase was always negligible. To estimate recovery of labeled alkaloids, representative unlabeled reaction mixtures were spiked with [^{14}C]gramine (2 nCi) or [^{14}C]MAMI (1.2 nCi). Recoveries averaged 90% for [^{14}C]gramine and 70% for [^{14}C]MAMI. Reported values have been corrected for recovery.

Enzyme Purification. Protein was determined according to Bradford (2) using BSA as a standard. All operations were carried out at 4°C. Buffer pH refers to the value at 4°C. The following procedure gave the highest-specific activity purified product. Six- to 8-d old Proctor shoots (70 g fr wt) were ground in a mortar and pestle with acid-washed sand in 70 ml of 50 mM Tris/HCl (pH 8.5) containing 10 mM DTT. The homogenate was centrifuged at 145,000 x g for 1 h; the supernantant was concentrated to about 8 ml (8 to 12 h) in an Amicon ultrafiltration cell with a PM-30 membrane; after adding 25 ml of 25 mM histidine/HCl, pH 6.2, containing 5 mM DTT, the sample was reconcentrated to about 7 ml. This concentrate was applied to a PBE 94 (Pharmacia) chromatofocusing column (1.5 cm x 30 cm) equilibrated with 25 mM histidine/HCl, pH 6.2. The column was eluted with Polybuffer 74 (Pharmacia), pH 5.0, at a flow rate of 20 ml h^{-1}. To reduce losses of NMT activity at low pH, fractions (4 ml) were collected in tubes containing 0.2 ml of 2M Tris/HCl, pH 7.8, plus 10 mM β-mercaptoethanol. Active fractions

were combined, concentrated using PM-30 Centricon concentrators (Amicon) and injected onto a HPLC DEAE Bio-Gel TSK (75 x 7.5 mm) column. The column was eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM Tris/HCl, 5 mM DTT (pH 7.5), at a rate of 60 ml h^{-1}. Active fractions were pooled, concentrated using a PM-30 Centricon, and loaded onto the first of two tandemly-arranged HPLC gel filtration columns (Bio-Sil TSK-125, followed by Bio-Sil TSK-250, both columns 300 x 7.5 mm, Bio-Rad), equilibrated in 20 mM Tris/HCl, 100 mM Na_2SO_4 and 2 mM DTT (pH 7.2). Elution was at 30 ml h^{-1}. Active fractions were pooled and concentrated as before and carried through a second DEAE step as detailed above. Purified NMT protein was stored at -60°C in 20 mM Tris/HCl, pH 8.0, containing 5 mM DTT and 50% (v/v) glycerol.

Mol Wt Determination. Mol wt was determined either by HPLC gel filtration with Bio-Sil TSK-125 and TSK-250 columns in tandem using thyroglobulin, gamma globulin, ovalbumin, myoglobulin and cyanocobalamin (Sigma) as standards or by SDS-PAGE using BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor and α-lactalbumin (Sigma) as mol wt markers. SDS-PAGE was carried out in 1.5 mm-thick slab gels according to Laemmli (14), with a separating gel of 13% polyacrylamide. Protein bands were stained with Coomassie Brilliant Blue R 250.

Production of Rabbit Immune Serum. Purified NMT protein was used to immunize a rabit. Two immunizations, 18 d apart, were made with 150 ug protein emulsified in complete Freund's adjuvant. A final injection of 50 ug of protein in incomplete Freund's adjuvant was made 10 d later; serum was collected 8 d after the final injection.

Immunoblots. Samples of crude extract from fifth leaves (50 ug of

soluble protein) were separated by SDS-PAGE as described above, along with purified NMT protein standards. At the end of a run, gels were placed in cold 25 mM Tris/192 mM glycine (pH 8.3) containing 20% (v/v) methanol (Towbin buffer, ref. 24) for 20 to 30 min. Protein was then transferred to a sheet of nitrocellulose using a Transphor cell (Hoefer) at 1 amp for 3 to 4 h in Towbin buffer at 4°C. Gels were removed and stained in Coomassie Brilliant blue R 250 to check the completeness of protein transfer. Nitrocellulose transfer sheets were either stained for protein using Ponceau S, or incubated for several hours with 3% BSA, 20 mM Tris/HCl, 0.9% NaCl, 0.01% NaN_3 (pH 7.4) (BSA-Tris-saline) to block unreacted protein binding sites. Blots were then incubated for 1 h at 37°C or overnight at 4°C in 1% BSA-Tris-saline to which either 1:100 pre-immune serum or 1:5000 antiserum had been added. Free antibody was then removed with 4 to 5 washes in 0.1% BSA-Tris-saline plus 0.5% Triton X-100 over a period of 1 h. Alkaline phosphatase conjugated to protein-A (Sigma) diluted 1:3000 in a solution of 0.1% BSA-Tris-saline plus 0.5% Triton X-100 was then added for 1 h. Blots were then washed thoroughly, first for 40 min in 4 to 5 changes of 100 mM Tris/HCl, 100mM NaCl, 2 mM $MgCl_2$, 0.25% Triton X-100 (pH 7.5) and then similarly in 100 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$ (pH 9.5). For detection of antigen bands, nitrobluetetrazolium (0.34 mg ml^{-1}) and 5-bromo-4-chloro-3-indolylphosphate (0.17 mg ml^{-1}) were dissolved in 10 to 15 ml of the pH 9.5 wash buffer. The nitrocellulose blots were placed in this development solution for 15 min in the dark at which time the reaction was stopped with 10 mM Tris/HCl, 1 mM EDTA (pH 7.5). Developed blots were stored dry or in 20 mM Tris/HCl, 5 mM EDTA (pH 9.5).

RESULTS

The fifth leaf was used to investigate effects of heat stress on NMT activity because this leaf contained ten-fold more gramine when it developed at 30°C/25°C than when it developed at 21°C/16°C (11).

Effect of Growth Temperature on In Vivo NMT Activity. Segments of Arimar fifth leaves grown at 30°C/25°C incorporated three to ten times more [^{14}C]formate label into indole alkaloids than did similar segments from leaves grown at 21°C/16°C (Table I). Similar results were obtained with the gramine-free cultivar Proctor, provided that the precursors AMI or MAMI were supplied (Table I). Because the ^{14}C-incorporation was measured at 21°C/16°C in all cases, these results imply that growth at supraoptimal temperatures increased the level of NMT activity present in leaf tissue. To test the validity of this inference, we developed a method for assaying NMT activity in vitro.

Characterization of In Vitro NMT Activity. First leaves, which synthesize gramine constitutively, were used as a convenient source of NMT activity for defining assay and storage conditions. Under the standard assay conditions, ^{14}C-methylation of both AMI and MAMI substrates was linear for 40 min (Fig. 1). A 30-min incubation was therefor routinely used. Analysis of the labeled alkaloid products showed that when AMI was the methyl acceptor, 90% of the ^{14}C was present in MAMI, with the rest in gramine. When MAMI was the methyl acceptor, all radioactivity was in gramine. The NMT activity of leaf extracts was specific for gramine precursors; tryptamine was not methylated at all, and tyramine only slightly (Fig. 1). Furthermore, extracts prepared from wheat seedlings, a species lacking gramine, did not methylate

supplied AMI or MAMI. The pH profiles for N-methylation of AMI and MAMI were similar between pH 7 and 10.5 (Fig. 2). Maximal catalytic activity with both substrates occurred close to pH 9.0. Sodium carbonate/bicarbonate and potassium phosphate buffers were found to be inhibitory; activities towards AMI and MAMI were affected equally (not shown). NMT activity was quite stable in leaf extracts held at 4°C; after 4 d, activity with AMI or MAMI substrates was about 75% of original. However, a single freeze-thaw cycle reduced both activities to half that of original.

Effect of Growth Temperature on In Vitro NMT Activity. For the fifth leaf of Arimar and Proctor, NMT activity towards both AMI and MAMI increased as growth temperature was increased between 15°C/10°C and 35°C/30°C (Fig. 3); NMT activities at these extremes differed by a factor of about 8 for Arimar, about 20 for Proctor. In contrast, NMT activities in the second leaf remained low at all temperatures, consistent with the failure of the second leaf to accumulate alkaloids in comparable experiments (11). NMT activities in Fig. 3 are expressed on a fresh weight basis, but results for leaf 5 expressed as specific activity are very similar because soluble protein levels were 20-22 mg/g fr wt at all growth temperatures. For both cultivars, growth was markedly poorer at the two higher temperature regimes.

The plants of Figure 3 had been grown in the various temperature regimes for at least 12 d. To determine whether shorter intervals of heat stress would elicit an increase in NMT activity, plant were grown at 15°C/10°C and exposed to heat stress for various times (Table II). None of the shorter stress exposures elicited more than one-third of the NMT activity found after a 14-d exposure.

Purification and Characterization of NMT Protein. NMT protein from dark-grown first leaves was purified to apparent homogeneity (SDS-PAGE, Coomassie Blue R-250 stain); Table III summarizes typical results. Throughout purification, NMT activity towards AMI and MAMI remained in the same ratio ($\sim$1:0.7). On gel filtration under non-denaturing conditions (not shown), NMT activity eluted very close to the ovalbumin standard (mol wt 45,000). SDS-PAGE (Fig. 4) indicated a mol wt of $\sim$43,000. Purified NMT protein often, but not always, ran as a doublet on 13% gels.

Effect of Growth Temperature on NMT Protein Level. In an experiment similar to that of Figure 3, fifth leaf extracts were assayed for NMT activity and analyzed by immunoblotting using antiserum directed against NMT protein. NMT activity levels were comparable to those shown in Figure 3. Because data for Arimar and Proctor were similar, only data for Arimar are given. Immunoblots of crude extracts of fifth leaves grown at 15°C/10°C showed no immunologically-detectable bands (Fig. 5A), or very weak ones (Fig. 5B). Extracts of fifth leaves grown at 21°C/16°C, 30°C/25°C and 35°C/30°C gave progressively stronger bands migrating to the same position as purified NMT protein (Fig. 5A). This steady increase in cross-reacting material parallels the behavior of enzyme activity (Fig. 3). Consistent with the lack of NMT induction and alkaloid synthesis in the second leaf, extracts of this leaf showed very weak immunologically-detectable bands, whether the plants had been grown at 15°C/10°C or 35°C/30°C (Fig. 5B).

DISCUSSION

Coordinate Regulation of Steps in Gramine Biosynthesis. The results demonstrate that NMT activity specific to the gramine pathway is induced in growing leaves, but not in mature leaves, by prolonged exposure to high-temperature stress. This mirrors the pattern of induction of overall gramine pathway activity (11). Because the intermediates AMI and MAMI do not accumulate, the N-methylations of gramine synthesis can never be rate-limiting in the overall pathway (11,23). These observations suggest that NMT activity is regulated coordinately with the activity of the rate-limiting step; nothing is known about this step save that it lies between tryptophan and AMI (11). It is interesting that the cultivar Proctor shows normal NMT induction even though it has an early lesion in the gramine pathway, and is alkaloid-free. This establishes that the NMT induction mechanism is independent of the alkaloid products of the pathway.

Is There a Single NMT Enzyme? Although we cannot exclude the possibility that there are two physically similar NMT enzymes specific for AMI or MAMI substrates, three lines of evidence point to a single enzyme. Firstly, activities towards AMI and MAMI were not resolved by any of the separation methods applied, and the activities co-purified in a constant ratio of $\backsim$1:0.7 (Table III). Secondly, approximately the same ratio of 1:0.7 was found for NMT activities in crude leaf extracts, regardless of genotype, growth temperature and leaf position. Also, leaf age was found not to affect the ratio in experiments with first leaf samples between one and five weeks old (not shown). Lastly, the two activities showed the same pH optima, the same sensitivity to inhibition by buffers,

and the same stability to storage at 4°C or freezing/thawing. Criteria similar to these have established that there are separate NMT enzymes for the sequential N-methylations of tyramine in barley roots (17) and of tryptamine in Phalaris shoots (16). We therefore hypothesize that there is a single NMT enzyme that catalyzes both methylations of gramine biosynthesis. Genetic evidence (15) is consistent with this.

Although purified NMT protein was often resolved into a doublet on SDS gels, it is unlikely that this doublet results from the presence of separate NMT enzymes, since the staining intensity of the lower band varied among experiments whereas the activities towards AMI and MAMI did not. Dissociation of enzyme subunits is likewise improbable, because the mol wt estimates from gel filtration of native enzyme and SDS-PAGE were very close (45,000 and 43,000, respectively). We therefore suggest that the doublet is an artifact.

Indirect evidence from in vivo [^{14}C]formate labeling studies previously led us to postulate separate NMT enzymes (11). An assumption we made was that both methylation steps would draw on a ^{14}C-methyl donor pool of the same specific activity. This assumption would fail were the flux rate through the gramine pathway to slow down to the point where first and second methylations become significantly separated in time; we now suppose this to be the case (see Appendix).

High-temperature Induction of NMT in Relation to Heat Shock. The immunoblot analysis demonstrates that the level of NMT cross-reacting protein in leaf five increases steadily as growth temperature is raised and so implies that the heat-induced increase in NMT activity is due, at least principally, to an increase in enzyme level. Such a heat-induced increase in the abundance of a protein bears some resemblance to

the heat-shock response (1,13). However, NMT induction differs from induction of heat shock proteins (HSP'S) in several ways. First, synthesis of HSP's is strongly induced by brief (minutes to hours) exposure to high temperature and declines during prolonged exposure (4,13), whereas NMT induction apparently requires several days exposure for full expression. Second, the heat-shock response occurs in almost all tissues of the plant (5), but NMT induction is restricted to growing leaves. Third, the NMT protein is highly specific to barley, unlike HSP's, at least some of which show close homologies among all living organisms (22). Last, HSP induction generally has a sharper temperature threshold than does induction of NMT activity.

The conditions for induction of NMT activity and gramine accumulation -- prolonged exposure to high temperature during leaf growth -- imply that there is a window in leaf development when high temperature can enhance the expression of an NMT gene, and perhaps also of a gene governing conversion of tryptophan to AMI. The window coincides with the phase of cell division and elongation of the leaf. We speculate that this NMT induction response is representative of a special class of environmental regulation in plants: the eliciting of genetic information in a time-dependent way when chronic environmental stress is imposed on meristematic cells. We further speculate that the phenomenon of progressive adaptation of dividing cells in culture to long-term osmotic stress (10) is another example of this type of environmental control of plant function.

ACKNOWLEDGMENTS

We thank Mr. Tony Bleecker for his generous help with HPLC techniques. We would also like to thank Dr. Neil Hoffman for his help with the immunology and for many valuable discussions.

LITERATURE CITED

1. ASHBURNER M, JJ BONNER 1979 The induction of gene activity in Drosophila by heat shock. Cell 17:241-254
2. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248-254
3. BRANDT K, HV EULER, H HELLSTROM, N LOFGREN 1935 Gramine und zwei begleiter desselben in laubblattern von gerstensorten. Hoppe-Seyler's Z Physiol Chem 235:37-42
4. COOPER P, T-H D HO 1983 Heat shock proteins in maize. Plant Physiol 71:215-222
5. COOPER P, T-H D HO, RM HAUPTMANN 1984 Tissue-specificity of the heat-shock response in maize. Plant Physiol 75:431-441
6. DIGENIS GA 1969 Metabolic fates of gramine in barley II: Biotransformation of gramine into indole-3-carbinol and indole-3-carboxylic acid in barley. J Pharm Sci 58:42-44
7. GOWER BG, E LEETE 1963 Biosynthesis of gramine: The immediate precursors of the alkaloid. J Am Chem Soc 85:3683-3685
8. GROSS D, H LEHMANN, H-R SCHUTTE 1970 Zur Physiologie der Graminbildung. Z Pflanzenphysiol 63:1-9
9. GROSS D, H LEHMANN, H-R SCHUTTE 1974 Zur biosynthese des gramins. Biochem Physiol Pflanzen 166:281-287
10. HANDA AK, RA BRESSAN, S HANDA, PM HASEGAWA 1982 Characteristics of cultured tomato cells after prolonged exposure to medium containing polyethylene glycol. Plant Physiol 69:514-521
11. HANSON AD, KM DITZ, GW SINGLETARY, TJ LELAND 1983 Gramine accumu-

lation in leaves of barley grown under high-temperature stress. Plant Physiol 71:896-904

12. HANSON AD, PL TRAYNOR, KM DITZ, DA REICOSKY 1981 Gramine in barley forage - effects of genotype and environment. Crop Sci 21: 726-730

13. KEY JL, CY LIN, YM CHEN 1981 Heat shock proteins of higher plants. Proc Natl Acad Sci USA 78:3526-3530

14. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685

15. LELAND TJ, R GRUMET, AD HANSON 1985 Biochemical, immunological and genetic characterization of natural gramine-free variants of Hordeum vulgare L. Plant Sci Lett (in press)

16. MACK JPG, M SLAYTOR 1979 Indolethylamine N-methyltransferases of Phalaris tuberosa, purification and properties. Phytochem 18:1921-1925

17. MEYER E 1982 Separation of two distinct S-adenosylmethionine dependent N-methyltransferases involved in hordenine biosynthesis in Hordeum vulgare. Plant Cell Reports 1:236-239

18. MULVENA DP, M SLAYTOR 1983 N-methyltransferase activities in Phalaris aquatica. Phytochem 22:47-48

19. MUDD SH 1961 3-Aminomethylindole and 3-methylaminomethylindole: New constituents of barley. Nature (London) 189:489

20. PUTOCHIN N 1926 Uber einige verbindungen der pyrrol-und indolfeihe und uber esomerisationen in diesen reihen. Ber Dtsch Chem Ges 59:1987-1998

21. SCHALLENBERG J, E MEYER 1983 Simple syntheses of 3-substituted indoles and their application for high yield ^{14}C-labeling. Z Naturforsch 38b:108-112

22. SCHLESINGER MJ, M ASHBURNER, A TISSIERS 1982 Heat Shock: From Bacteria to Man. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

23. SCHNEIDER EA, F WIGHTMAN 1974 Amino acid metabolism in plants V. Changes in basic indole compounds and the development of tryptophan decarboxylase in barley (Hordeum vulgare) during germination and seedling growth. Can J Biochem 52:698-705

24. TOWBIN H, T STAEHELIN, J GORDON 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354

Table I. Incorporation of ^{14}C from [^{14}C]Formate into Indole Alkaloids by Segments of Fifth Leaves from Plants Grown at 21°C/16°C and 30°C/25°C.

Plants were grown in optimal conditions (21°C/16°C) for 10 days; one-half were then transferred to mild heat-stress (30°C/25°C). Fifth leaves were harvested after a further 12-14 days, as they reached half-emergence. [^{14}C]Alkaloid synthesis was assayed at 21°C/16°C.

Experiment	Cultivar	Methyl Acceptor Supplied	^{14}C-Incorporation into Indole Alkaloids[a]		Fold-Increase
			21°C/16°C	30°C/25°C	
			nCi/3 segments		
1	Arimar	None	15.6	55.9	3.6
	Proctor	None	<0.2	<0.3	-
		AMI	20.2	72.1	3.6
		MAMI	10.4	32.0	3.1
2	Arimar	None	7.2	73.1	10.1
	Proctor	AMI	14.5	96.5	6.7
		MAMI	10.2	41.0	4.0

[a]Chromatography showed that for Proctor leaf segments supplied AMI, ^{14}C was present in MAMI ($\geq$60%) and gramine ($\leq$40%); for Proctor segments supplied MAMI, all ^{14}C was in gramine. Arimar segments contained mainly [^{14}C]gramine.

Table II. NMT Activity in Fifth Leaves Exposed to High Temperatures for Various Times.

Plants were grown at 15°C/10°C and transferred to high temperature conditions for various times. Pulse (6 or 10 h) high temperature treatments were given in daytime growth chamber conditions; plants were then returned to 15°C/10°C. Fifth leaves were harvested when half-emerged.

Treatment	NMT Activity[a]	
	Proctor	Arimar
	nmol/50 mg fr wt·h	
Continuous 15°C/10°C	3.1	6.1
Final 14 d at 33°C/28°C	48.1	60.5
Final 3 d at 33°C/28°C	9.3	20.4
6-h, 34°C pulse 4 d before harvest	8.9	-
10-h, 34°C pulse 1 d before harvest	8.9	-

[a]Assayed with AMI methyl acceptor. Data are means of duplicates.

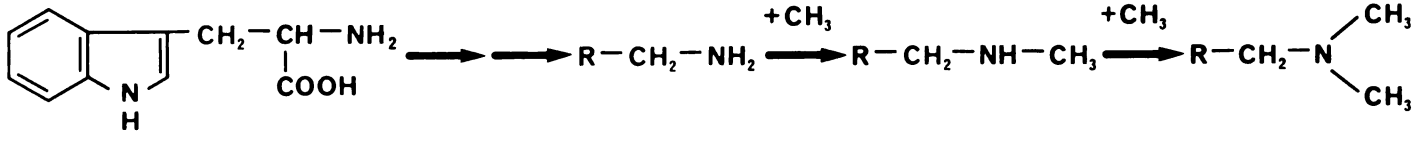
Table III. Purification of N-Methyltransferase from 8d Etoliated Proctor Shoots.

Fraction	Volume	Total Protein	Total Activity[a]	Specific Activity	Recovery	Purification
	ml	mg	Units[b]	Units/mg protein	%	-fold
Crude extract	118	171	42,900	251	-	-
Chromatofocusing	36	5.22	12,100	2,320	28.2	9.2
1st DEAE-HPLC (Bio-Sil TSK)	8	1.57	8,560	5,450	20.0	21.8
Gel Filtration (Bio-Gel TSK)	7	0.228	3,030	13,300	7.0	52.9
2nd DEAE-HPLC	2	0.033	1,840	55,800	4.3	223.0

[a]Assayed with AMI substrate; activities with MAMI substrate were in the range 0.67 to 0.77 x those with AMI substrate for all fractions.

[b]One unit of activity = Incorporation of 1 nmol [^{14}C-methyl] into alkaloids per h.

A



Tryptophan AMI MAMI Gramine

Scheme 1

Fig. 1. Progress curves for ^{14}C-methylation of AMI (●) and MAMI (o) by extracts of dark-grown Proctor barley first leaves. Assay temperature was 25°C; assays contained 120 nmol [^{14}C]SAM, 600 nmol of methyl acceptor and extract equivalent to 50 mg fr wt of leaf. Samples without AMI or MAMI, or with boiled extract, showed no activity. Individual points at 40 min are results for tryptamine (▼) and tyramine (△) methyl acceptors. All data are means of duplicate samples. The experiments were repeated, with similar results.

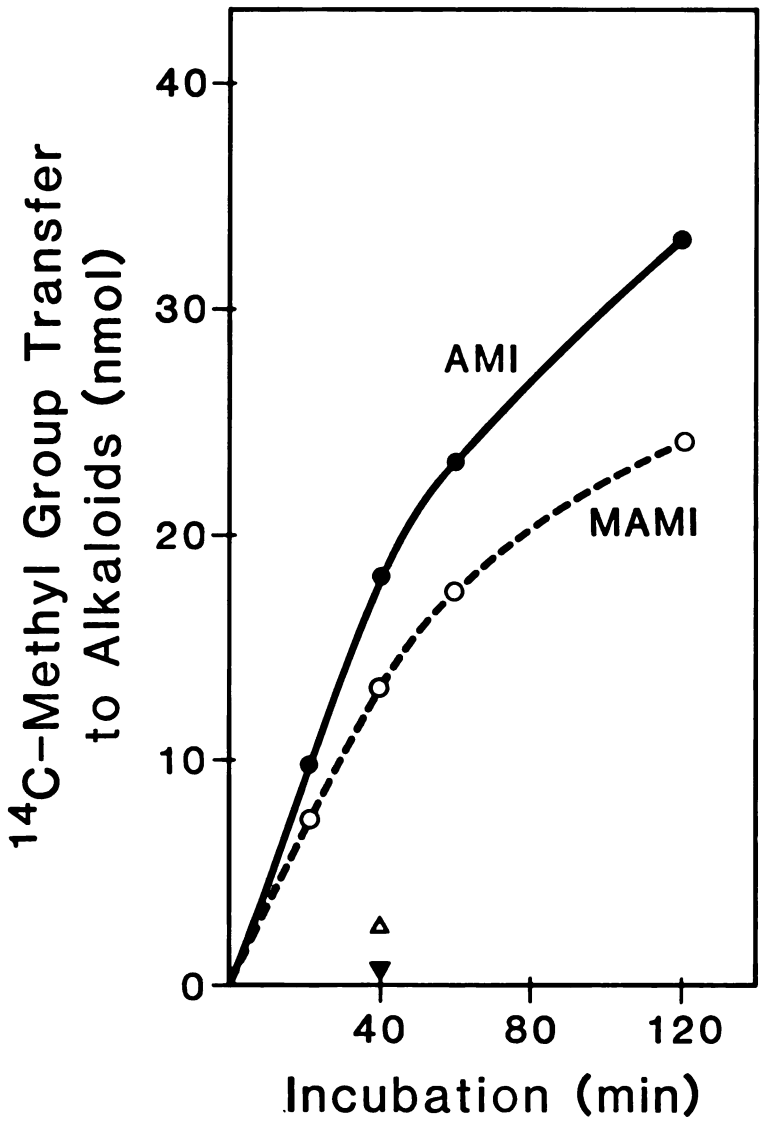

40
30
20
10
0
AMI
MAMI
40
80
120
Incubation (min)
^{14}C-Methyl Group Transfer to Alkaloids (nmol)

Fig. 2. Effect of pH on [^{14}C]SAM-dependent methylation of AMI (left) and MAMI (right) by extracts of dark-grown Proctor barley first leaves. Incubation was for 30 min. Buffers (150 mM) were: ● , HEPES/NaOH; ■, glycylglycine/NaOH; ▲, glycine/NaOH. Plotted pH values were actual measurements of complete assays. Data points are means of duplicate samples. The experiment was repeated, with similar results.

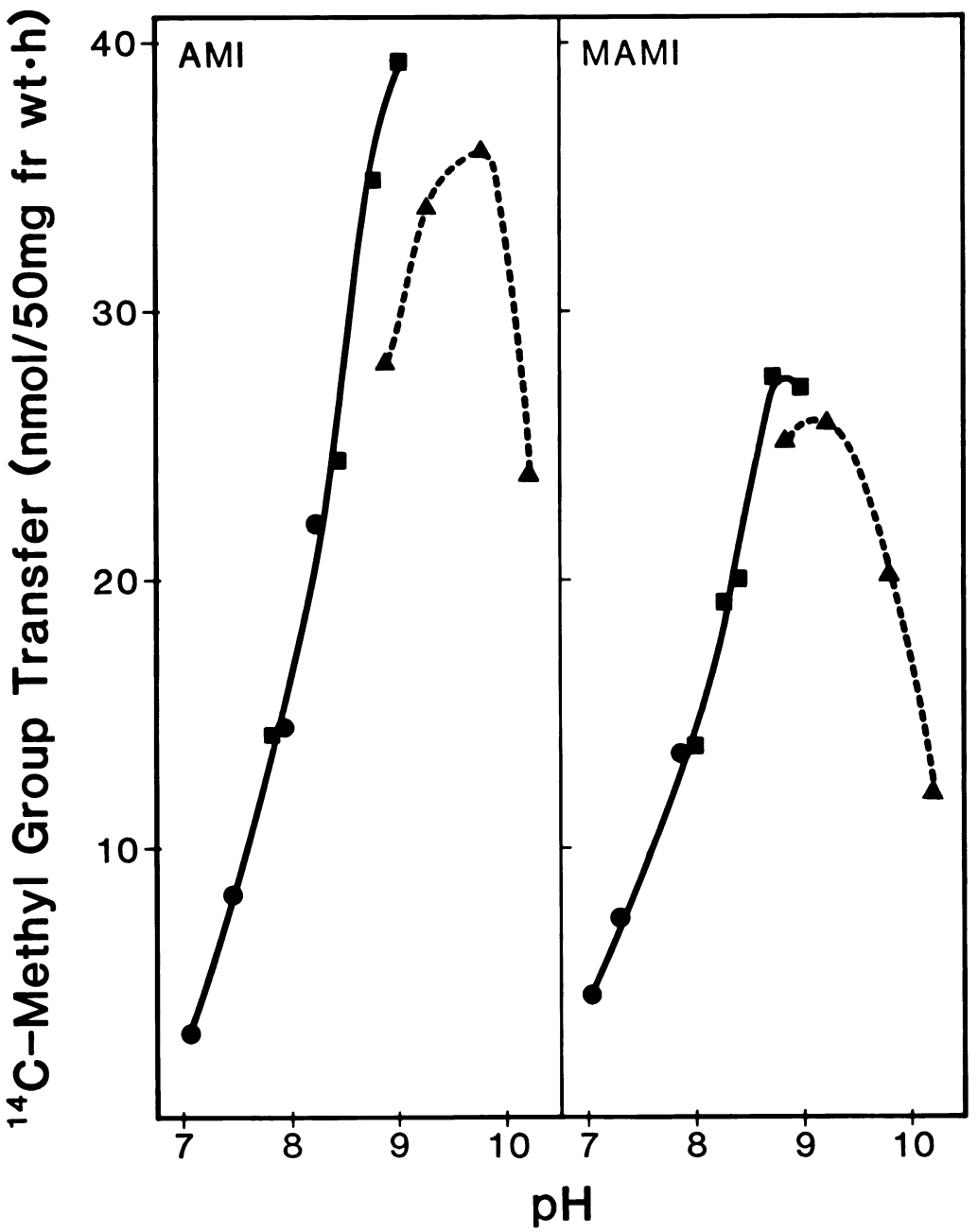

AMI
MAMI
40
30
20
10
7
8
9
10
7
8
9
10
pH
^{14}C-Methyl Group Transfer (nmol/50mg fr wt·h)

Fig. 3. Effect of growth temperature on in vitro NMT activity towards AMI (•) and MAMI (o), for barley cultivars Arimar (left) and Proctor (right). Plants were grown for 10 days in optimal temperature conditions (21°C/16°C) and then transferred to the various temperature regimes; night temperatures were 5°C below day temperatures. Upper frames are results for leaf 5, which accumulates alkaloids when plants are grown at high temperature. Lower frames are results for leaf 2, in which alkaloids do not accumulate at any temperature. Data points are means of 4 replicates. The experiment was repeated twice, with similar results.

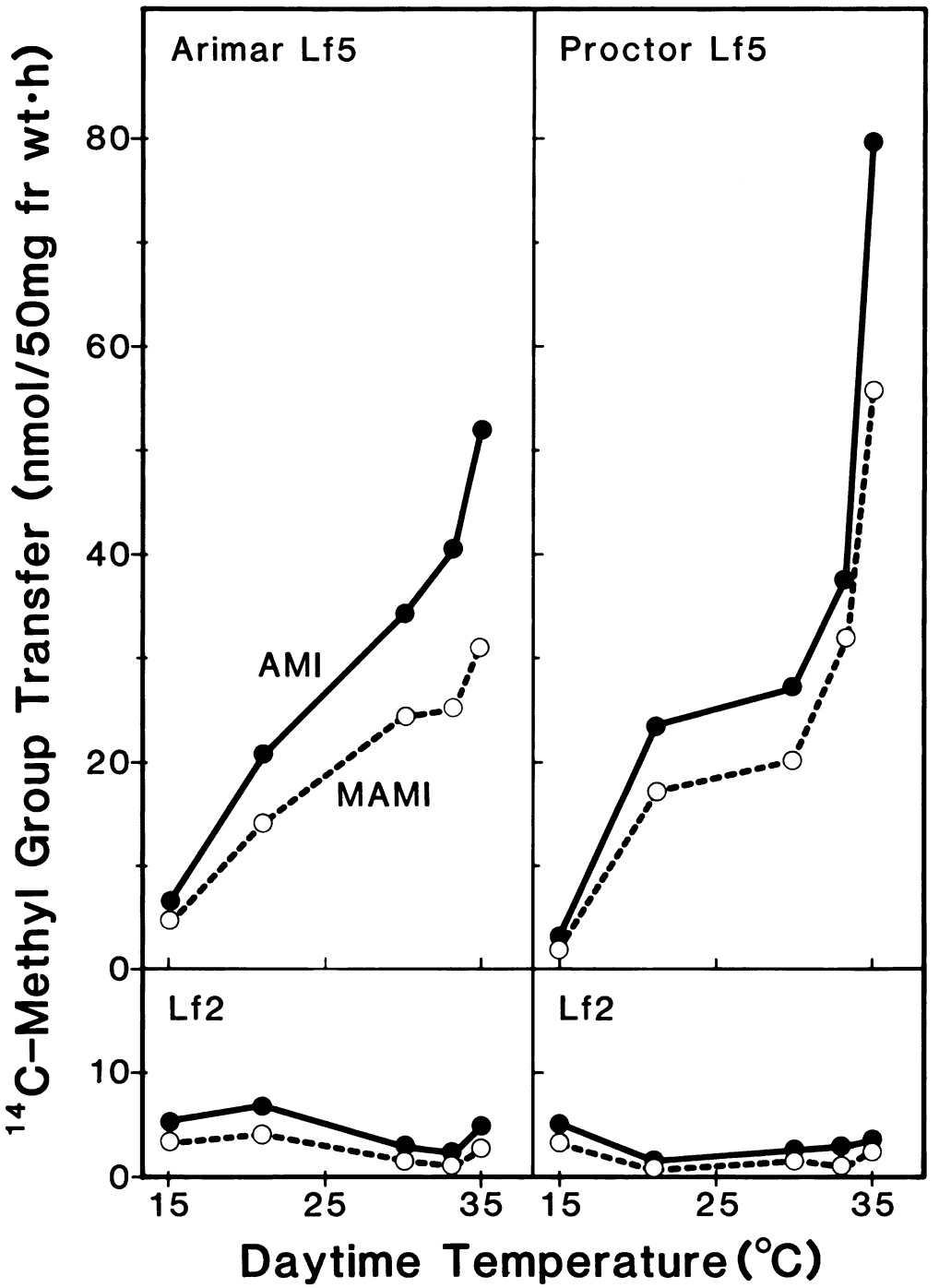

Arimar Lf5
Proctor Lf5
AMI
MAMI
Lf2
Lf2
80
60
40
20
0
10
0
15
25
35
15
25
35
^{14}C-Methyl Group Transfer (nmol/50mg fr wt·h)
Daytime Temperature (°C)

Fig. 4. SDS-PAGE of purified NMT protein and mol wt markers (kD). The NMT sample (~10 ug) was from the second DEAE HPLC step (Table III). Acrylamide concentration was 13%.

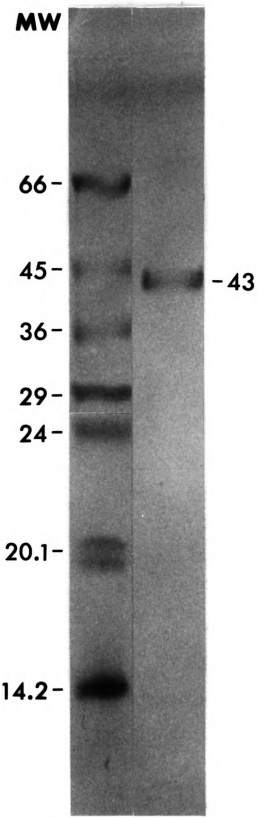
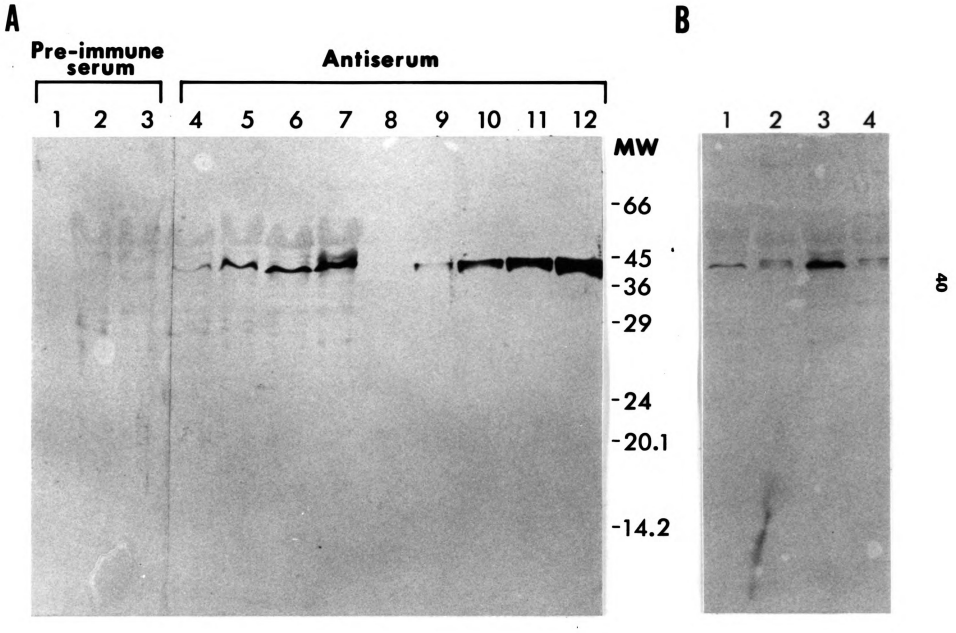

MW
66
45
36
29
24
20.1
14.2
43

Fig. 5. Immunoblot analysis of NMT polypeptide levels in crude extracts of Arimar leaves emerging under various temperature regimes. Plants were grown for 10 d at 15°C/10°C before transfer to other regimes. Lanes with leaf samples contained 50 ug total protein.

A. Fifth leaf samples. Lanes 1-3 were probed with pre-immune serum, lanes 4-12 with antiserum against NMT. Positions of mol wt markers (kD) are indicated on the right. Lane 1: 250 ng of purified NMT protein. Lane 2: leaf 5 grown at 15°C/10°C. Lane 3: leaf 5 grown at 35°C/30°C. Lanes 4, 5, 6, and 7: leaf 5 grown at 15°C/10°C, 21°C/16°C, 30°C/25°C and 35°C/30°C, respectively. Lanes 8, 9, 10, 11, and 12: 10 ng, 50 ng, 100 ng, 250 ng, and 500 ng of purified NMT protein, respectively.

B. Leaf 2 and leaf 5 samples. All lanes were probed with antiserum. Lane 1: leaf 5 grown at 15°C/10°C. Lane 2: leaf 2 grown at 15°C/10°C. Lane 3: leaf 5 grown at 35°C/30°C. Lane 4: leaf 2 grown at 35°C/30°C.


A
Pre-immune serum
Antiserum
1 2 3 4 5 6 7 8 9 10 11 12
MW
66
45
36
29
24
20.1
14.2
B
1 2 3 4

APPENDIX

APPENDIX

N-METHYLTRANSFERASE ACTIVITY IN AGING FIRST LEAVES OF BARLEY

Constitutive gramine accumulation in barley is limited to the early seedling stage of growth and occurs primarily in the first leaf (11,23). The observation that net gramine synthesis declined with time suggested that the enzyme activities of the synthesis pathway might decay at various rates as the first leaf aged. Furthermore, differences in rates of decay would, in the case of separate NMT enzymes, generate a differential decline in N-methylation products (MAMI and gramine) for aging first leaves (11).

When variously aged Proctor first leaves were supplied AMI plus [^{14}C]formate *in vivo*, the amount of label incorporated into gramine declined steadily after 6 d whereas ^{14}C incorporation into MAMI increased until 20 d before declining (see Fig.4 in ref. 11). These results, interpreted on the implicit assumption that the specific activity of the ^{14}C-methyl donor pool was the same for both methylations, were judged consistent with the presence of separate enzymes for the sequential methylations with the second decaying faster than the first (11).

The experiments described here were designed to directly measure both NMT activities as a function of first leaf age, whereas before the second N-methylation step had been measured only indirectly. Variously aged Proctor and Arimar first leaves were assayed *in vivo* by feeding AMI or MAMI plus [^{14}C]formate. Crude extracts prepared from these same leaves were assayed *in vitro* by supplying AMI or MAMI

plus [^{14}C]SAM.

In general, the results illustrated in Fig. A1-A3 show a parallel decline in N-methylation activities as the first leaf aged. In both Proctor (Fig. A1) and Arimar (Fig. A2) in vivo N-methylations of MAMI fell continuously with age. The insets in the respective figures show that in AMI fed samples there were differential declines in the N-methylation products [^{14}C]MAMI and [^{14}C]gramine. These results are similar to those reported previously (11) and indicate that in AMI fed samples, the two N-methylations may be significantly separated in time, thus invalidating our earlier assumption concerning the unchanging specific activity of the methyl donor pool in vivo. The parallel declines in AMI and MAMI N-methylation rates can be most clearly seen in vitro, in Fig. A3. Taken together these results give no indication of separate N-methyltransferase enzymes and are in agreement with the protein purification data given in Chapter 1. As noted before, however, the existence of two coordinately regulated enzymes cannot be ruled out.

MATERIALS AND METHODS

Proctor and Arimar plants were grown at the standard growth chamber conditions given in Chapter 1, Materials and Methods. Plantings were made at regular intervals to allow harvest and assay of variously aged first leaves on the same day. First leaves of the same age were pooled; *in vitro* and *in vivo* assays were drawn from the same pools. Procedures for *in vivo* and *in vitro* assays and extraction were as given in Chapter 1, Materials and Methods.

Fig. A1. Effect of leaf age on incorporation of label from [^{14}C]formate into indole alkaloids by segments of Proctor first leaf blades supplied with AMI or MAMI. Data points are means for at least two samples; standard error bars are shown for data points representing more than two samples. Inset gives the relative incorporation of label into MAMI and gramine in AMI fed samples; MAMI fed samples were labeled in gramine only. Note that Proctor lacks any endogenous pools of AMI or MAMI.

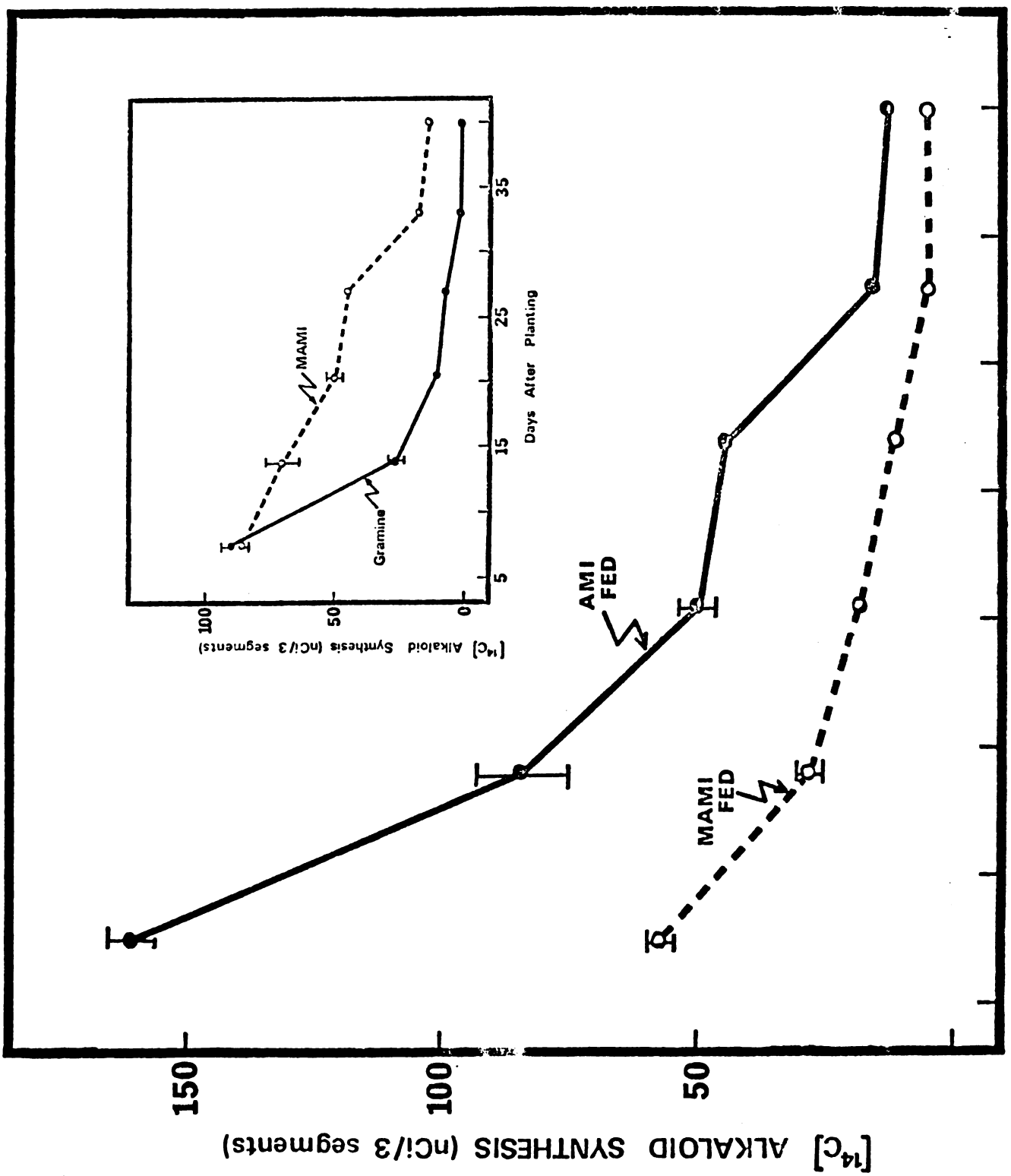
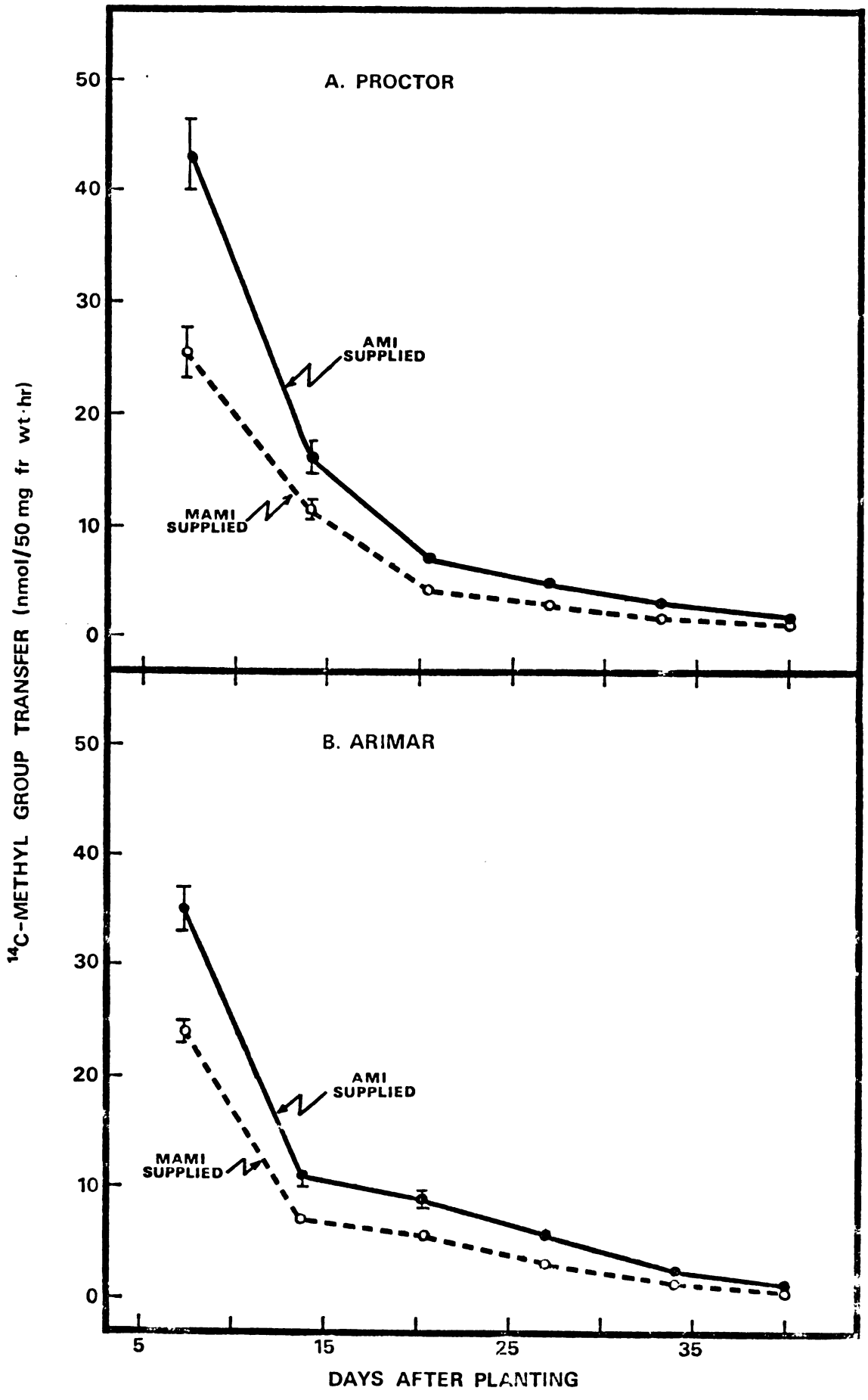

[14C] ALKALOID SYNTHESIS (nCi/3 segments)
150
100
50
5
15
25
35
DAYS AFTER PLANTING
MAMI FED
AMI FED
[14C] Alkaloid Synthesis (nCi/3 segments)
100
50
0
5
15
25
35
Days After Planting
MAMI
Gramine

Fig. A2. Effect of leaf age on incorporation of label from [^{14}C]formate into indole alkaloids by segments of Arimar first leaf blades supplied with AMI, MAMI or K-phosphate buffer. Data points are means for at least two samples; standard error bars are shown for data points representing more than two samples. Inset gives the relative ^{14}C-incorporation into MAMI and gramine in AMI fed segments (A), MAMI fed segments (B), and segments supplied K-phosphate (C). Note that Arimar, as a gramine accumulator, had small endogenous pools of AMI and MAMI.

[14C] ALKALOID SYNTHESIS (nCi/3 Segments)
200
150
100
50
0
5
15
25
35
45
DAYS AFTER PLANTING
AMI FED
MAMI FED
NO SUBSTRATE
A. AMI FED
AMMI
Gramine
B. MAMI FED
Gramine
AMMI
C. NO SUBSTRATE
Gramine
AMMI
Days After Planting

Fig. A3. Effect of leaf age on in vitro NMT activities towards AMI (●) and MAMI (o) in first leaves of Proctor (A) and Arimar (B). Data points are means for at least four assays ± standard error; many standard error values were too small to plot.


A. PROCTOR
B. ARIMAR
AMI SUPPLIED
MAMI SUPPLIED
AMI SUPPLIED
MAMI SUPPLIED
^{14}C-METHYL GROUP TRANSFER (nmol/50 mg fr wt·hr)
DAYS AFTER PLANTING
0
10
20
30
40
50
5
15
25
35

Chapter 2

Biochemical, Immunological and Genetic Characterization of Natural Gramine-free Variants of *Hordeum vulgare* L.

Title: Biochemical, Immunological and Genetic Characterization of Natural Gramine-free Variants of Hordeum vulgare L.*

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Abbreviations: AMI, 3-aminomethylindole; MAMI, N-methyl-3-aminomethylindole; NMT, N-methyltransferase; CRM-, lacking cross reacting material; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SUMMARY

Many barley cultivars (e.g. Arimar) contain the indole alkaloid gramine, but some do not. Among seven gramine-free cultivars tested, two phenotypic classes were found: those with a normal level of the N-methyltransferase (NMT) activity that catalyzes the last two steps of gramine synthesis (e.g. Proctor); and those having neither NMT activity nor protein recognized by polyclonal antibodies raised against purified NMT (e.g. Morex).

A 3 X 3 diallel with reciprocals was made using cultivars Arimar, Proctor and Morex. The pattern of occurrence of gramine and NMT activity among the F_1 hybrids suggested that Proctor and Morex carried defective alleles of the same nuclear gene governing an early step in the indole alkaloid pathway, and that Morex also carried a recessive allele at a nuclear locus encoding NMT activity. However, no non-parental alkaloid phenotypes were found in the F_2 generation from an Arimar X Morex cross and the ratio of progeny with gramine to those with no alkaloids was 3:1. One explanation of these results is tight linkage between genes controlling two of the steps in gramine biosynthesis.

Key words: Biochemical genetics; immunoblot analysis; indole alkaloids; N-methyltransferase.

INTRODUCTION

Gramine is a toxic indole alkaloid which occurs in leaves of certain barley cultivars, but not others (1,2). Gramine is synthesized from tryptophan via 3-aminomethylindole (AMI) and N-methyl-3-aminomethylindole (MAMI) (Fig. 1A). Although the reaction(s) leading from tryptophan to AMI are not yet understood biochemically (3, 5), N-methyltransferase (NMT) activity which catalyzes S-adenosylmethionine-dependent conversion of AMI to MAMI, and MAMI to gramine, has been identified (6). We recently purified this activity >200-fold, and obtained biochemical evidence which was consistent with a single NMT enzyme, but which did not rule out separate enzymes for each step (7).

Our previous work has also shown that the gramine-free cultivar Proctor can convert supplied AMI and MAMI to gramine in vivo (8), and has levels of extractable NMT activity and protein similar to those in gramine-containing (wild type) cultivars (7).

We undertook the present study because pedigree relationahips among gramine-free barley cultivars (1) indicated that they were not all closely related, and so might carry different biochemical lesions in gramine synthesis. Were this the case, it would permit biochemical-genetic dissection of the gramine pathway. We report here on a survey of gramine-free cultivars for variant phenotypes with respect to NMT activity and NMT cross-reacting material, and present a genetic analysis of two representative variants.

MATERIALS AND METHODS

Barley (Hordeum vulgare L.) cultivars Betzes and Robust were obtained from the Crop & Soil Sciences Dept., MSU: others were from sources given previously [1]. Pedigree data were obtained from the USDA Small Grains Collection, Beltsville, MD, U.S.A. Plants for NMT assays, for immunological analysis and for alkaloid screening were grown in growth chambers, 3 or 4 per pot, in soil mix [1] with irrigation on alternate days with half-strength Hoagland's solution. Conditions were: 16 h day, 21°C, RH 60%, 200 $uEm^{-2}s^{-1}$ PAR; 8 h night, 16°C. Cultivars for crossing and F_1 hybrids for selfing were grown in the greenhouse.

First leaves were extracted and assayed for NMT activity using AMI or MAMI as methyl acceptor, as detailed elsewhere [7]. For immunoblot analyses, soluble proteins from first leaves were separated by SDS-PAGE, transferred to nitrocellulose paper and probed with polyclonal rabbit antibodies against purified NMT protein using methods given in [7].

Quantitative determination of alkaloids in F_1 hybrids and their parents was according to [1]. Indole alkaloids in F_2 plants were analyzed qualititatively as follows. Seven- to 9- day old first leaves were harvested individually, cut into 1-cm sections, placed in 3-ml syringes and frozen in liquid N_2. After thawing, about 100 ul of sap was expressed by forcing the piston and made alkaline with 5-10 ul of 2 M NaOH. Alkaloids were then partitioned into 1.5 ml of $CHCl_3$ and the absorbance of the $CHCl_3$ phase was read at 270 nm. Samples with significant absorbance readings were further analyzed by TLC on silica gel G (Polygram, Machery-

Nagel) to determine the types of alkaloid present; developing solvents were n-butanol: ethanol: conc. NH_4OH (80:4:6, v/v) or methanol: acetone: conc. HCl (90:10:4, v/v). Alkaloid zones were detected by UV absorption and the p-dimethylaminocinnamaldehyde spray reagent (1).

We use the following terms to describe alkaloid phenotypes (see also Fig. 1). AMI+ and AMI- denote the presence or absence of AMI synthesis; NMT1+ and NMT1- describe presence or absence of *in vitro* NMT activity with AMI as methyl acceptor; similarly, NMT2+ and NMT2- refer to presence or absence of NMT activity with MAMI as acceptor.

RESULTS

NMT phenotypes of gramine-free cultivars

Seven cultivars, including the previously-studied Proctor, were tested for NMT activity and NMT cross-reacting material. Two cultivars resembled Proctor in having NMT activity with AMI and MAMI substrates (NMT1+/NMT2+), but four cultivars had no activity against either substrate (NMT1-/NMT2-) (Table I). These four cultivars also lacked detectable NMT antigen bands in immunoblots (Fig. 2). Note that phenotypes which were not found include: NMT1+/NMT2-; NMT1-/NMT2+; NMT1-/NMT2- with NMT cross-reacting material present. The pedigrees of the four NMT1-/NMT2- cultivars showed them to be related, closely so in one case (Robust = Morex X Manker). Although pedigree relationships among the three NMT1+/NMT2+ types could not be precisely documented, all

three trace back to old two-row European malting barley stocks and in this sense form a cluster. We therefore judged it likely that cultivars with the same NMT phenotype had the same NMT genotype, and focused genetic studies on one cultivar from each phenotypic class: Arimar, which contains gramine; Proctor which contains no indole alkaloids but which has normal NMT activity: and Morex, which has neither alkaloids nor NMT activity.

Analysis of the diallel cross

Proctor, Morex and the alkaloid-containing cultivar Arimar were entered in a diallel with reciprocals; the F_1 plants were tested for alkaloid type and NMT activity (Fig. 1B). All hybrids with Arimar as a parent had NMT activity and contained gramine as well as small amounts of AMI and MAMI. Quantitative alkaloid analysis of these hybrids (Table II) showed alkaloid levels close to the mid-parent mean value, for both directions of each cross. Hybrids between Proctor and Morex had the same phenotype as Proctor, with NMT activity present but alkaloids absent.

Analysis of F_2 progeny

F_1 hybrids between Arimar and Morex were allowed to self, to give an F_2 population which was tested for alkaloid type. Two models were used as a framework for interpreting the results (Table III). In

the first model (two-gene), Arimar and Morex respectively carry functional allels at two loci: Ami, governing conversion of tryptophan to AMI; and Nmt, specifying an NMT enzyme able to act on both AMI and MAMI. This model predicts a non-parental phenotype containing AMI only. A subsidiary model (three-gene) invokes two NMT loci, encoding enzymes specific for each methylation step, and predicts two classes of non-parental phenotypes containing AMI only, or AMI + MAMI only.

Among the 194 F_2 individuals tested, no non-parental types were found (Table III). The observed segregation between gramine-containing and alkaloid-free classes fits a 3:1 ratio ($\chi^2 = 1.16$, $P = 0.28$). There was no evidence of reduced seed set on F_1 plants, or of low germination of F_2 seed, suggesting that zygotic lethal effects were absent.

DISCUSSION

The existence of variants lacking NMT activity supports the idea, based on the narrow substrate range of NMT, that this activity is specific for gramine biosynthesis (7,8) and hence dispensable. That NMT-deficient cultivars lacked activity against both AMI and MAMI is consistent with the biochemical evidence for a single NMT enzyme (7), as is the failure to recover F_2 individuals with AMI and MAMI but without gramine. We therefore hypothesize that the variant NMT1-/NMT2-, CRM- phenotype is conferred by a null allele at a locus encoding a specific NMT enzyme. The distribution of NMT activity among F_1 hybrids in the diallel cross agrees with this inasmuch as NMT-deficiency behaved

as a recessive trait. The absence of reciprocal differences among the hybrids indicated that NMT activity is nuclear-encoded.

There was no complementation resulting in alkaloid production in the hybrids between Proctor and Morex, indicating that these cultivars may carry lesions at the same locus (loci) governing the conversion of tryptophan to AMI.. Expression of the full alkaloid complement in F_1's from Arimar x Morex and Arimar x Proctor crosses and their reciprocals connotes nuclear control of AMI synthesis in these cultivars by a co-dominant gene. Dominance is not complete because alkaloid levels in the F_1's approximate the midparent value. The intermediate level of alkaloids in the F_1's further suggests that the lesion in AMI- cultivars is at a rate-limiting step in gramine synthesis.

The considerations above suggest in the simplest case that two genes control the ability to produce gramine, one (Ami) governing the conversion of tryptophan to AMI, the other (Nmt) encoding an NMT enzyme. However, the absence of non-parental ditypes from the F_2 population of Morex x Arimar crosses, and the 3:1 ratio of F_2 phenotypes, are inconsistent with this model. The simplest way to resolve the discrepancy is to suppose that Ami and Nmt genes are tightly linked.

We note that there may in fact be more than two genes determining gramine biosynthesis, particularly since conversion of tryptophan to AMI is likely to proceed in several steps (4,5). This possibility can only be addressed by the isolation of further variants or mutants in the gramine pathway.

ACKNOWLEDGMENTS

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REFERENCES

1 A.D. Hanson, P.L. Traynor, K.M. Ditz and D.A. Reicosky, Crop Sci. 21 (1981) 726.

2 E.A. Schneider, R.A. Gibson and F. Wightman, J. Exp. Bot. 23 (1972) 152.

3 E. Leete, Phytochemistry 14 (1975) 471.

4 D. Gross, H. Lehmann and H.R. Schutte, Biochem. Physiol. Pflanzen 166 (1974) 281.

5 A. Breccia and L. Marion, Can. J. Chem. 37 (1959) 1066.

6 S.H. Mudd, Nature 189 (1961) 489.

7 T.J. Leland and A.D. Hanson, Plant Physiol. (in press).

8 A.D. Hanson, K.M. Ditz, G.W. Singletary and T.J. Leland, Plant Physiol 71 (1983) 896.

9 A.K.M.R. Islam, K.W. Shepherd and D.H.B. Sparrow, Heredity 46 (1981) 161.

10 G.E. Hart, A.K.M.R. Islam and K.W. Shepherd, Genet. Res., Camb. 36 (1980) 311.

11 A. Powling, A.K.M.R. Islam and K.W. Shepherd, Biochemical Genetics 19 (1980) 237.

12 G. Wolf and J. Rimpau, Nature 265 (1977) 470.

Table I. NMT activity in first leaves of gramine-free barley cultivars.

Cultivar	Provenance	NMT Activity[a]	
		AMI acceptor	MAMI acceptor
		-- nmol/50 mg fr wt•h --	
CI 11806 Proctor	United Kingdom	16	10
CI 6398 Betzes	Poland	23	14
CI 13852 Coho	Michigan	22	17
CI 15773 Morex	Minnesota	<0.5	<0.5
CI 10648 Larker	North Dakota	<0.5	<0.5
CI 15813 Bowers	Michigan	<0.5	<0.5
PI 476976 Robust	Minnesota	<0.5	<0.5

[a]In gramine-containing cultivars, NMT activity was 10 - 18 or 7 - 11 nmol/50mg fr wt•h, with AMI or MAMI as acceptor, respectively.

Table II. Indole alkaloid levels in leaves of F_1 hybrids. Total indole alkaloids (AMI + MAMI + gramine) are expressed in gramine equivalents; gramine was always the predominant alkaloid, with AMI and MAMI present in small amounts.

Leaf No.[a]	Genotype	Number of Samples	Indole alkaloid level[b]	Midparent value
			mg gramine equivalents/g dry wt	
3	Arimar	5	1.29 ± 0.13	
	Proctor	5	<0.03	0.65
	F_1(Arimar x Proctor)	4	0.52 ± 0.03	
	F_1(Proctor x Arimar)	8	0.75 ± 0.07	
1	Arimar	3	7.04 ± 0.51	
	Morex	4	<0.03	3.52
	F_1(Arimar x Morex)	6	3.56 ± 0.17	
	F_1(Morex x Arimar)	5	3.74 ± 0.35	

[a]First or third leaves were harvested when they reached full expansion (6 - 9 and 16 - 24 days after planting, respectively).

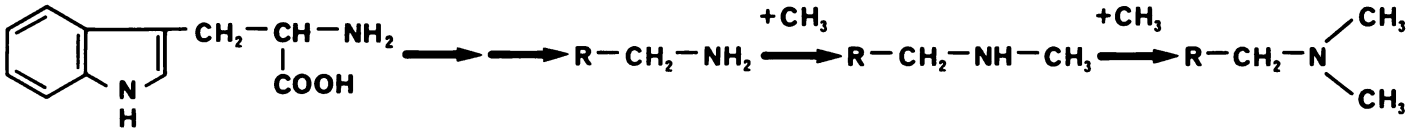
[b]Mean ± S.E.

Table III. Analysis of indole alkaloids in the first leaves of F_2 individuals from Morex x Arimar reciprocal crosses.

Value	Phenotype Classes			
	No Alkaloids (AMI-/NMI1±/NMI2±)	AMI Only (AMI+/NMI1-/NMI2±)	AMI + MAMI (AMI+/NMI1+/NMI2-)	AMI+MAMI+Gramine (AMI+/NMI1+/NMI2+)
Observed	42	0	0	152
Predicted:				
3-gene model	49	36	27	82
2-gene model	49	36	0	109

Fig. 1. A. The gramine biosynthesis pathway and the phentotypes of the cultivars entered in the diallel. B. Phenotypes of the six F_1 hybrids produced in the diallel, and of their parents.

A

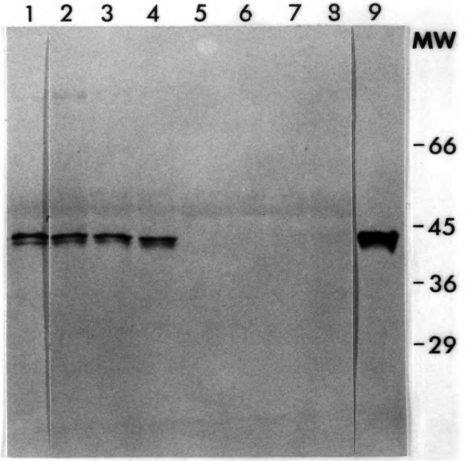


	Tryptophan	AMI	MAMI	Gramine
Arimar	+	+	+	
Proctor	−	+	+	
Morex	−	−	−	

B

♀ \ ♂	Arimar	Proctor	Morex
Arimar	AMI+/NMT1+/NMT2+	AMI+/NMT1+/NMT2+	AMI+/NMT1+/NMT2+
Proctor	AMI+/NMT1+/NMT2+	AMI−/NMT1+/NMT2+	AMI−/NMT1+/NMT2+
Morex	AMI+/NMT1+/NMT2+	AMI−/NMT1+/NMT2+	AMI−/NMT1−/NMT2−

Fig. 2. Immunoblot analysis showing that NMT1-/NMT2- cultivars lack immunologically-detectable NMT polypeptides. Each track contained 50 ug of soluble protein. Following SDS-PAGE on 13% gels, polypeptides were visualized using rabbit antibodies directed against NMT protein. The tendency of NMT to run as a doublet is characteristic also of purified NMT protein (7). Positions of mol wt markers (kD) are indicated. Lane 1, Arimar; Lane 2, Proctor; Lane 3, Betzes; lane 4, Coho; lane 5, Morex; lane 6, Larker; lane 7, Bowers; lane 8, Robust; lane 9, 500 ng purified NMT protein.


1 2 3 4 5 6 7 8 9
MW
66
45
36
29

APPENDIX

APPENDIX

SCREENING OF WHEAT-BARLEY ADDITION LINES FOR NMT ACTIVITIES

Islam et al. (9) have reported the production and identification of six of the seven possible disomic additions of barley (Hordeum vulgare, cv. Betzes; 2n = 14) chromosomes to wheat (Triticum aestivum, Chinese Spring; 2n = 42). Each contained a homologous pair of barley chromosomes together with the complete diploid set of wheat chromosomes in wheat cytoplasm. A line with barley chromosome five was not recovered due to infertility. Subsequently, by use of isozyme analyses, Hart et al. (10) and Powling et al. (11) have shown that the barley chromosomes are expressed in the wheat background and have established markers for each chromosome.

Betzes was tested and shown to have a phenotype similar to that of Proctor (Table I and Fig. 2 in Chapter 2). We reasoned that it might be possible to locate the gene(s) responsible for NMT activities by assaying these addition lines. In subsequent experiments however, no activity was recovered from either first leaves or from heat stressed fifth leaves infiltrated with the gramine precursors AMI or MAMI plus [^{14}C]formate. Extracts from first leaves lacked NMT activity and cross-reacting protein when probed with antiserum against pruified NMT. From these results we concluded that lack of NMT expression could mean either: a) the NMT gene(s) is located on chromosome five, b) there is a regulatory system for NMT and gramine biosynthesis operating in trans, or c) that there is selective repression of NMT expression in the

wheat background. Wolf and Rimpau (1977), working with reciprocal amphidiploid addition lines constructed between wheat and rye (Secale cereale), have reported in trans regulation of phosphodiesterase structural genes. Furthermore these authors present evidence for cytoplasmic control of phosphodiesterase, where the rye gene was expressed in the presence of rye cytoplasm but not with wheat cytoplasm (12).

MATERIALS AND METHODS

Betzes barley and Chinese Spring wheat were obtained through the Dept. of Crop and Soil Sciences, Michigan State University, East Lansing. Six disomic addition lines were the generous gift of Dr. Tony Brown, Division of Plant Industries, CSIRO, Canberra, Australia.

Plants were grown as described in Chapter 2, Materials and Methods. For high-temperature experiments plants were grown for 10 d at 21°C/16°C and either transferred to 33°C/28°C or maintained at 21°C/16°C. Fifth leaves were harvested at half emergence, 14-16 d later. In vivo and in vitro assays and immunological analysis were as described in Chapter 1, Materials and Methods.

Chapter 3

Gramine and Resistance to *Erysiphe graminis*

ABSTRACT

The role of gramine as a factor in resistance to Erysiphe graminis (DC.) Merat hordei Em. Marchal was evaluated. Two barley cultivars, a gramine accumulator (Arimar) and one lacking gramine (Proctor) were compared for resistance to powdery mildew. Both were equally susceptible to infection. In addition, five pairs of isogenic lines differing at a single allele conditioning resistance or susceptibility to E. graminis were screened for gramine content. There was no correlation between resistance and alkaloid content. Infected Arimar plants had only slightly lower alkaloid concentrations than uninfected controls. The results indicated that indole alkaloid levels are not related to resistance to E. graminis.

INTRODUCTION

The production and accumulation of biologically active, if not toxic compounds in plants has led to much speculation about their physiological and ecological significance (14). In the case of the phenolic compounds, and regulation of the key biosynthetic enzyme, phenylalanine ammonia lyase, it has been possible to assign important biological functions, most notable the induction of lignin formation and phytoalexins in response to infection by plant pathogens (6). In other cases, however, including the tryptamine alkaloids in Phalaris (9) and gramine in barley (5) specific functions have not been identified and it is possible only to assign rather general roles in the deterrence of herbivory to these compounds. An interesting exception to this generalized role for alkaloids had been reported in Lupinis angustifolius, where comparisons between largely isogenic genotypes indicated that the quinolizidine alkaloids in wild type lupin plants enable them to produce more seeds than low alkaloid plants under many deleterious environmental conditions (12).

This study was undertaken to investigate a possible role of gramine in resistance to the pathogen Erysiphe graminis. In light of the specific induction of gramine accumulation by heat-stress (4), the possibility of a similar induction occurring in plants under biological stress was studied.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Barley (Hordeum vulgare L.) cultivars Arimar and Proctor were obtained as reported previously (5). Five pairs of barley lines, derived from cv. Manchuria, each pair isogenic except for a specific pair of alleles (Algerian/Ml-a, Long Glumes/Ml-a7(L6), Frangor/Ml-a6, Rupee/Ml-a13, and Ml-a10/Ml-a10) conditioning resistance and susceptibility to culture CR3 of Erysiphe graminis (DC.) Merat hordei Em. Marchal (11), were obtained from Roger Wise, Department of Botany and Plant Pathology, Michigan State University, as was the pathogen E. graminis race CR3.

Plants were planted, 2 to 6 per pot, in a pre-sterilized soil mix (5) and grown at 16-h d, 21°C, RH 60%, 200 uE $m^{-2}s^{-1}$ PAR/ 8-h night, 16°C. Innoculated plants were grown at 15°C with 23 h of light per day. Plants were watered every 2nd day at 21°C/16°C. At 15°C, pots were placed in a shallow pan of water for the duration of treatment.

Inoculation Methods. Arimar and Proctor plants were grown at 21°C/16°C for 7 or 14 d prior to inoculation. For the treatments involving 14 d Arimar plants, pots were covered with a lantern cover (to contain any infection) (Fig.1) and inoculum from previously-infected leaves was shaken down onto the enclosed plants. A double layer of Kimwipe tissue held by a rubber band was used to cap the chimney tops. Control plants were treated identically except for the inoculation step. After inoculation, plants were kept in darkness at a high relative humidity for at least one hour to increase rate of conidia germination.

Seven day Proctor and Arimar plants were inoculated from pre-infected leaves, incubated for an hour in darkness and transferred to 15°C. After observations were made over a period of 8 d, plants were scored and

discarded.

Alkaloid Extraction. Freeze-dried Arimar plants were ground in a Wiley mill. Duplicate samples (0.1 g) were taken from separately pooled infected and control shoots. Extraction of alkaloid fractions was as previously described (5).

Seven day shoots of the paired isogenic lines were harvested, frozen in liquid N_2 and alkaloids were recovered by the cell-sap technique reported previously (8). For each line 100 ul of sap was extracted.

RESULTS AND DISCUSSION

The experiments were conducted to investigate (a) the possible role of gramine as a resistance factor in E. graminis pathogenesis and (b) the effects of E. graminis infection on gramine accumulation in Arimar shoots.

To test the hypothesis that gramine in barley may contribute to resistance to E. graminis, 7 d Proctor (gramine-free) and Arimar (gramine-containing) shoots were exposed to E. graminis. Both Proctor and Arimar leaves showed signs of infection after 4 to 5 d. After 8 to 9 days, sporulating colonies covered the leaves, especially the first leaf; plants in general appeared weak and chlorotic. No significant differences were observed between Proctor and Arimar with regard to rate of infection or to severity of infection at 9 d.

These observations were confirmed by the results shown in Table I. Each pair of isogenic lines has an estimated 99% of their germplasm in common (5), the only significant difference between resistant (R) and sensitive (S) pairs being the allele coding for resistance or susceptibility to E. graminis. No correlation between resistance and gramine presence was shown. These results clearly indicate that gramine was not playing a primary role in resistance.

The final experiment was to determine whether infection with E. graminis would induce gramine accumulation. Observation of the plants at the time of harvest showed much of the E. graminis infection localized to the leaf areas present at time of in oculation. Leaf tissues emerging after spores had settled were largely free of symptoms. The interpretation of the data in Table II therefore refers to a more

systemic response as opposed to a localized response.

The number of replicated samples (duplicates) does not allow a meaningful mean comparison between the alkaloid contents of infected and non-infected shoots. In general, E. graminis infection does not appear to influence gramine levels.

From these experiments it was concluded that gramine plays no significant role in resistance to E. graminis. A similar conclusion was reached by Sherwood et al. (15) in relation to tryptamine alkaloids and to Helminthosporium and Stagonospora leafspot infection in reed canarygrass. Moreover, the typtamine alkaloid concentrations in this species did not change in response to in oculation and infection.

Considering the nature of the pathogen, these results are not surprizing. The host-pathogen response in fungal diseases of cereals is often a very specific one, with single gene resistances often breaking down very rapidly (7). In contrast, the evidence favors biological activity for gramine and other indole alkaloids against a wide array of organisms.

Perhaps the best documented and most cited role of gramine and the tryptamine alkaloids is deterrence against herbivore attack (2,9, 10). Other effects reported specifically for gramine, range from toxicity on meadow voles (3) and aphids (1) to allelopathy (13). The wide ranging biocidal effects of gramine must be interpreted in the context of its regulation. An important point, reinforced by the absence of gramine induction in this experiment, is that the induction of gramine biosynthesis is not a generalized injury response; so far, the only environmental stimulus found to elicit gramine accumulation is high temperature (4,5).

I conclude that gramine does not play a primary role in E. graminis resistance; gramine accumulation was not significantly induced by powdery mildew or the general injury symptoms following in its wake.

LITERATURE CITED

1. CORCUERA LJ 1984 Effects of indole alkaloids from Gramineae on aphids. Phytochemistry 23:539-541

2. GALLAGHER CH, JH KOCH, RM MORE, JD STEEL 1964 Toxicity of Phalaris tuberosa for sheep. Nature 204:542-545

3. GOELZ MFB, H ROTHENBACHER, JP WIGGINS, WS KENDALL, TV HERSHBERGER 1980 Some hematological and histopathological effects of the alkaloids gramine and hordenine on meadow voles (Microtus pennsylvanicus). Toxicology 18:125-131

4. HANSON AD, KM DITZ, GW SINGLETARY, TJ LELAND 1983 Gramine accumulation in leaves of barley grown under high-temperature stress. Plant Physiol 71:896-904

5. HANSON AD, PL TRAYNOR, KM DITZ, DA REICOSKY 1981 Gramine in barley forage - effects of genotype and environment. Crop Sci 21: 726-730

6. JONES HG 1984 Phenylalanine ammonia lyase: regulation of its induction and its role in plant development. Phytochemistry 23: 1349-1359

7. JORGENSEN JH, J TORP 1978 The distribution of spring barley varieties with different powdery mildew resistances in Denmark from 1960 to 1976. Royal Veterinary and Agricultural University Yearbook, Copenhagen

8. LELAND TJ, R GRUMET, AD HANSON 1985 Biochemical, immunological and genetic characterization of natural gramine-free variants of Hordeum vulgare L. (submitted)

9. MARTEN GC 1973 Alkaloids in reed canarygrass. In AG Matches,

ed, Anti-Quality Components of Forages. Crop Science Society of America, Madison, pp. 15-31

10. MARTEN GC, RM JORDAN, AW HOVIN 1976 Biological significance of reed canarygrass alkaloids and associated palatability variation to grazing sheep and cattle. Agron J 68:909-914

11. MOSEMAN JG 1972 Isogenic barley lines for reaction to Erysiphe graminis F. Sp. Hordei. Crop Sci 12:681-682

12. ORAM RN 1983 Selection for higher yield in the presence of the deleterious low alkaloid allele iucundus in Lupinus angustifolius L. Field Crops Res 7:169-180

13. OVERLAND L 1966 The role of allelopathic substances in the 'smother crop' barley. Amer J Bot 53:423-432

14. ROBINSON T 1974 Metabolism and function of alkaloids in plants. Science 184:430-435

15. SHERWOOD RT, KE ZEIDERS, CP VANCE 1978 Helminthosporium and Stagonospora leafspot resistance are unrelated to indole alkaloid content in reed canarygrass. Phytopathology 68:803-807

Table I. Alkaloid content in first leaves of paired isogenic (cv. Manchuria) barley lines carrying alleles conditioning resistance or susceptibility to E. graminis.

Source of Resistance	Resistance Locus	Alkaloid Level
		ug gramine equivs/ml sap[c]
Algerian S[a]	Ml-a-	<30
Algerian R[b]	Ml-a+	<30
Long Glumes S	Ml-a7(L6)-	<30
Long Glumes R	Ml-a7(L6)+	<30
Frangor S	Ml-a6-	<30
Frangor R	Ml-a6+	<30
Rupee S	Ml-a13-	801
Rupee R	Ml-a13+	732
Ml-a10 S	Ml-a10-	<30
Ml-a10 R	Ml-a10+	<30
Proctor		<30
Arimar		756

[a]S = susceptible

[b]R = resistant

[c]Detection limit for indole alkaloids was 30 ug/g fresh weight.

Table II. Effect of E. graminis infection of shoot alkaloid contents of Arimar.

Treatment	Alkaloid Level	
	Sample	
	1	2
	mg gramine equivs/g dry wt	
Infected	2.25	2.38
Non-infected	2.67	2.78

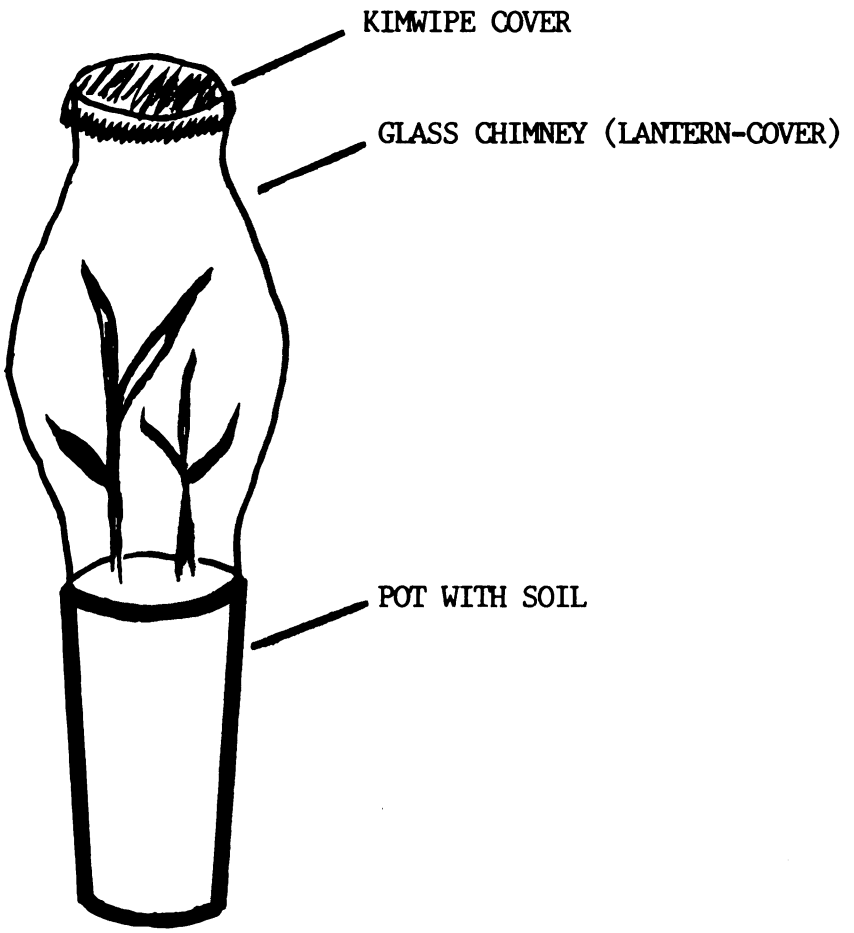


Fig. 1. Apparatus for inoculation of 14d Arimar plants by E. graminis.

CONCLUSIONS

1. The level of NMT activity and NMT cross-reacting protein is increased by growth at high temperatures, suggesting that higher NMT activity is the result of heat induced de novo NMT protein synthesis.

2. High temperature induces NMT activity only in expanding leaves implying that this ability to respond to heat stress is relegated to a rather narrow "window" early in cell-tissue development and differentiation.

3. The heat induced increase in the abundance of NMT protein differs significantly from the heat shock response:

    a) Heat shock proteins are induced by brief exposure to high temperature with levels declining during prolonged exposure; whereas maximal NMT protein levels are observed only after prolonged heat stress.

    b) The heat shock response occurs in almost all tissues while NMT induction is restricted to growing leaves.

    c) NMT protein is highly specific to barley, unlike heat shock proteins which show close homologies among all living organisms.

    d) Heat shock protein induction generally has a sharper temperature threshold than does induction of NMT activity.

4. Several lines of evidence suggest that both N-methylation steps in gramine biosynthesis are catalyzed by a single enzyme:

    a) NMT activity purified >200-fold ran as a single band on SDS-PAGE (Mol. Wt. = 43-45 kD).

    b) The ratio of NMT activity towards AMI and MAMI remained constant throughout purification; in crude extracts this ratio was relatively unaffected by tissue age, position or stress history.

    c) The pH activity profiles for AMI and MAMI NMT activities

were nearly identical.

d) The stability of purified AMI and MAMI NMT activities were the same.

5. Three classes of phenotypes exist for the gramine pathway: Arimar is a gramine accumulator; Proctor lacks indole alkaloids but has NMT activity; Morex lacks indole alkaloids and contains no NMT activity or cross-reacting protein. Results of crosses are consistent with the ideas (a) that the gramine biosynthesis pathway is under the control of at least two genes, one governing the conversion of tryptophan to AMI, the other specifying an NMT that catalyzes methylation of AMI to MAMI and MAMI to gramine, (b) that these genes are coordinately regulated, and maybe genetically linked.

6. The biological stress caused by powdery mildew (Erysiphe graminis) did not replace high temperature in provoking gramine accumulation. Gramine accumulation is not a factor in resistance to powdery mildew.

Drawing together the various threads of investigation on gramine in barley affords a rather unique cross-sectional view of plant biology. At the molecular level, an environmental stimulus is perceived and translated into a metabolic response - gramine biosynthesis. At the physiological level, the induction of gramine biosynthesis in developing tissue suggests that a good deal of plant adaptation to stress consists of and results from the metabolic decisions made in relatively undifferentiated, environmentally-sensitive tissue. Finally, at the ecological level there are the questions of the role of secondary end-products like gramine, in plants, and the origins and evolutionary significance

of the genetic diversity for gramine. Such movement between levels is rare in biology.