Phytochemistry, 1978, Vol 17, pp 963–966. © Pergamon Press Ltd Printed in England 0031-9422/78/0501-0963 $02.00/0

ENDOGENOUS GIBBERELLINS AND INHIBITORS IN CARYOPSES OF RYE*

WILFRIED DATHE, GERNOT SCHNEIDER and GÜNTHER SEMBDNER
Institute of Plant Biochemistry, Research Centre for Molecular Biology and Medicine, Academy of Sciences of the GDR, 401 Halle/Saale, German Democratic Republic

(*Revised received* 24 *November* 1977)

Key Word Index—*Secale cereale*; Gramineae; caryopses; identification; gibberellins; ABA; 4′-dihydrophaseic acid, GC–MS.

Abstract—Gibberellins A_8, A_{16}, A_{24}, and abscisic acid were identified by GC–MS of derivatized extracts from immature fruits of *Secale cereale*. Mature caryopses contained ABA and *trans*-ABA in a ratio 1:1 as well as 4′-dihydrophaseic acid. During milk ripeness a neutral GA conjugate was detected. Free GA, afforded by enzymatic hydrolysis of the conjugate, was chromatographically identified as GA_{16}.

INTRODUCTION

Many publications deal with the identification of gibberellins in dicotyledonous plants; fewer investigations have been done on the endogenous hormones in monocotyledons, and only a few in fruits of cereals. So far gibberellins have been detected and identified in germinating caryopses of *Hordeum distichon* [1], in barley [2] and wheat seedlings [3], in shoots of wheat, rye [4] and bamboo [5], in developing ears of oat [6] as well as in immature barley caryopses [7] ABA content has been studied in developing fruits of some cereals, but most intensively in wheat [8–10].

Our studies were directed to the identification of endogenous gibberellins and inhibitors in immature and mature rye caryopses. Furthermore, we are investigating the alterations of the single hormone components during fruit development as well as their distribution in different parts of the caryopsis, which will be published later.

RESULTS AND DISCUSSION

Identification of endogenous gibberellins

Immature ears (5 kg) harvested about 4 weeks after peak anthesis (milk ripeness) were extracted with MeOH and the remaining aq. phase was partitioned at pH 2.5 with EtOAc and aq. satd *n*-BuOH. The EtOAc fraction was purified on a Si gel column (partition chromatography) and bioassayed [11] using dwarf pea, dwarf rice and wheat seedlings (Fig. 1). According to the activity profile in the dwarf rice bioassay 3 GA-like fractions (GA-A, B, C) were collected. GA-A (0.8 μg GA_3 equiv./kg fr. wt) was separated using DEAE-Sephadex A-25 into a GA-like (1.5 μg GA_3 equiv./kg fr. wt) and an ABA-like fraction (Fig. 2). With further purification of the GA fraction on Sephadex LH-20 (Fig. 3) the GA activity increased dramatically (55.5 μg GA_3 equiv./kg fr. wt)

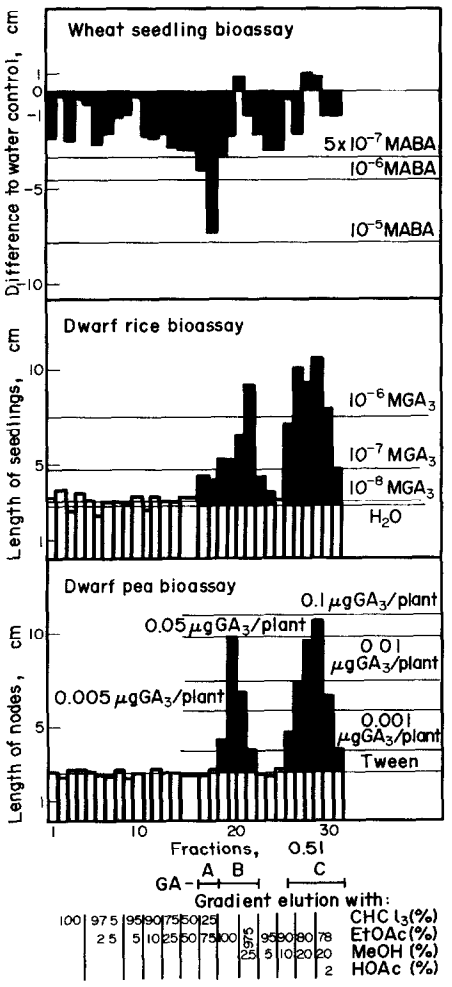

Fig. 1. Chromatography of the EtOAc extract from immature fruits (5 kg) on Si gel partition column and subsequent bioassay by wheat seedlings (1/100/ml) dwarf rice (1/100/ml), and dwarf peas (1/600/plant).

* Gibberellins 63. For Part 62 see Lischewski, M. and Adam, G. (1977) *Tetrahedron* in press.
Abbreviations: GA_x = gibberellin A_x.

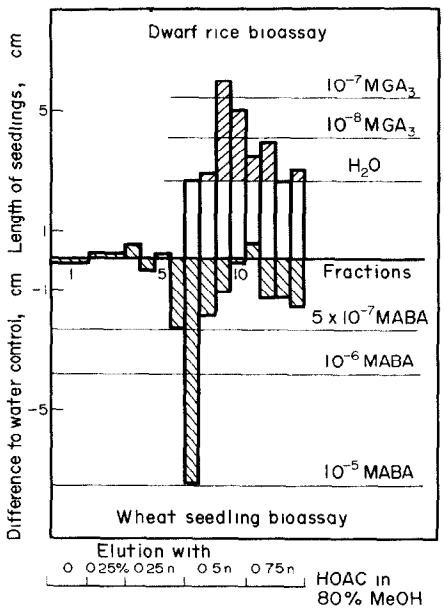

Fig. 2. Chromatography of GA-A (Fig. 1) on DEAE-Sephadex A-25 and subsequent dwarf rice (1/100/ml) and wheat seedling (1/100/ml) bioassay.

apparently due to removal of inhibitors (fractions 5, 6). The GA in fraction 3 was examined by GC–MS as the TMSi-GA-TMSi derivative. The GC behaviour (RR_t = 1.3, referred to 5α-cholestane = 1.0) as well as the MS were identical with authentic TMSi-GA_{24}-TMSi derivative. This is the first identification of GA_{24} in Gramineae. After its isolation from the fungus *Gibberella* [12] GA_{24} has been reported only for Cucurbitaceae [13, 14].

Fraction GA-B (Fig. 1) was purified by subsequent chromatography on DEAE-Sephadex A-25 and Sephadex LH-20 columns. Additional purification of the biologically active fractions 5–7 was accomplished by TLC (system 1: R_f = 0.19–0.27, and system 2: R_f = 0.40–0.46). The GA obtained was examined by GC–MS as the TMSi-GA-Me and TMSi-GA-TMSi derivatives. The TMSi-GA-Me showed a GC peak at RR_t = 2.2 and a molecular ion at *m/e* 506 (M^+) characteristic for TMSi-GA_{16}-Me [15]. The *m/e* values of all fragments were identical with those for authentic TMSi-GA_{16}-Me. Only slight differences in some ion intensities were observed (*m/e* 359—10%, *m/e* 390—50%; authentic TMSi-GA_{16}-Me: *m/e* 359—30%, *m/e* 390—80%). Also the TMSi-GA-TMSi derivative gave a GC peak (RR_t = 2.7) and MS (M^+ at *m/e* 564) identical with authentic TMSi-GA_{16}-TMSi except for small differences in some ion intensities (*m/e* 217—100%, *m/e* 240—80%; authentic TMSi-GA_{16}-TMSi: *m/e* 217—47% *m/e* 240—100%). These data prove the identity of GA-B to be GA_{16}. This evidence is supported by the high biological activity for GA_{16} [16]. Previously, GA_{16} has been isolated only from the fungus *Gibberella fujikuroi* [17].

The fraction GA-C (Fig. 1) was subjected to DEAE-Sephadex A-25 and subsequently to Sephadex LH-20 chromatography followed by TLC (system 2: R_f = 0.42–0.55, and system 3: R_f = 0.32–0.45, both corresponding to GA_8). The GA obtained was examined by GC–MS as TMSi-GA-Me and TMSi-GA-TMSi derivative. Both the GC values (RR_t = 3.1 and RR_t = 4.5) and MS corresponded to those of TMSi-GA_8-Me and TMSi-GA_8-TMSi, respectively. Thus, GA-C is identical with GA_8, known as a natural GA in several plant species [18–20].

Additionally, GA_8 was found to be the main GA component in the *n*-BuOH extract from immature caryopses. The *n*-BuOH extract was purified on Si gel (Fig. 4) and the biologically active fractions 11 and 12 were subjected to DEAE-Sephadex A-25 chromatography and subsequently to TLC (systems 2 and 3). Identification was performed by GC–MS using the TMSi-GA-Me and TMSi-GA-TMSi derivatives. The data mentioned indicate that in rye caryopses, as in dicotyledonous fruits, GA_8 is an important product of GA metabolism.

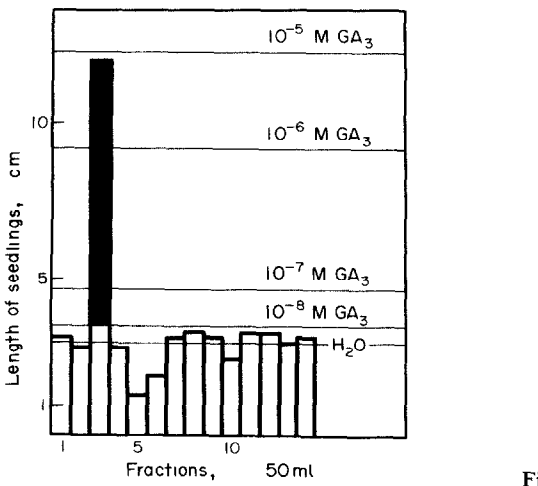

Fig. 3. Chromatography of GA fractions (9 and 10, Fig. 2) on Sephadex LH-20 and subsequent dwarf rice bioassay (1/100/ml).

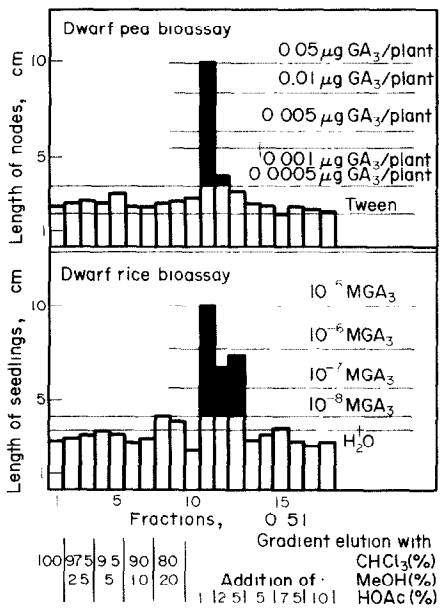

Fig. 4. Chromatography of the *n*-BuOH extract from immature fruits (5 kg) on Si gel partition column and subsequent dwarf pea (1/600/plant) and dwarf rice (1/100/ml) bioassay.

The EtOAc extract of immature caryopses (about 5 weeks after anthesis) contained in addition to the free acidic GAs described a more polar GA-like compound eluted from Si gel column (cf. Fig. 1) by continued elution with 2% acetic acid (fractions 32, 33...). The collected fraction showed a low biological activity in the dwarf pea (3.4 μg GA_3 equiv./kg fr. wt) and a higher activity (11.9 μg GA_3 equiv./kg fr. wt) in the dwarf rice bioassay. Chromatography on DEAE-Sephadex A-25 indicated the GA to be a neutral compound. After enzymatic hydrolysis using helicase and subsequent chromatography on DEAE-Sephadex A-25 GA activity disappeared in the neutral fraction and could be detected in fractions corresponding to free acidic GAs. According to partition on Sephadex LH-20 this GA proved to be chromatographically identical with GA_{16}. Therefore, the neutral GA can be considered to be a conjugate of GA_{16}, in which the carboxyl group is esterified.

In mature caryopses, endogenous GAs have been investigated using 1.5 kg of stored (2 yr) dry grains. The acidic EtOAc fraction obtained from a MeOH extract in the usual manner was purified on DEAE-Sephadex A-25 and bioassayed using dwarf peas and wheat seedlings. The free GAs detected (6.5 μg GA_3 equiv./kg fr. wt) were subjected to Sephadex LH-20 chromatography and subsequently to TLC (system 4, R_f = 0.38–0.52). The chromatographic behaviour of the main GA (about 99% of the total activity) was like $GA_{1/3}$. Conjugated GAs could not be found.

Identification of endogenous inhibitors

In the EtOAc fraction of immature caryopses (about 4 weeks after anthesis) inhibitory activity (35.8 μg ABA equiv./kg fr. wt) was found in fractions 17 and 18 (GA-A) from a Si gel column (Fig. 1). Subsequent chromatography on DEAE-Sephadex A-25 gave an increased inhibitory activity (111 μg ABA equiv./kg fr. wt) due to separation from GA present in fraction GA-A (Fig. 2). After TLC separation (system 5: R_f = 0.10) and subsequent methylation with diazomethane the inhibitor could be identified by GC–MS to be ABA. ORD measurement [21] confirmed its identity.

In mature caryopses endogenous inhibitors have been investigated using 1.5 kg dry stored grains, extracted and fractionated in the usual manner. After chromatography of the EtOAc extract on DEAE-Sephadex A-25 an ABA fraction has been collected showing high biological activity (174 μg ABA equiv./kg fr. wt). TLC yielded two fluorescence quenching zones: (1) corresponding to ABA; R_f = 0.37–0.57; (2) corresponding to 4′-dihydrophaseic acid [22]; R_f = 0.17–0.30. Zone (1) was further purified by TLC (system 5, R_f = 0.04–0.13), methylated and examined by GC–MS. Two peaks were observed with RR_t-values 7.8 and 11.6, identical with ABA-Me and *trans*-ABA-Me in a ratio 1:1. The MS confirmed the assumed identity with ABA. The substance eluted from zone (2), which showed no biological activity, was methylated and identified by GC–MS to be 4′-dihydrophaseic acid. This compound has not previously been detected in Gramineae, but is known as a main product of ABA metabolism [cf. 22] present in mature seeds [23].

EXPERIMENTAL

Plant material. Seed material of *Secale cereale* L., breeding strain No. 1713 was kindly supplied by the Institute of Plant Breeding Gülzow-Güstrow, Academy of Agricultural Sciences of the GDR. Plants were cultivated under field conditions in 1974 and 1975. For studying immature fruits, 5 kg whole ears were extracted about 4 weeks after anthesis (milk ripeness). Material was put in MeOH immediately after collecting in order to prevent wilting and stored at −20° until extraction.

Extraction and purification procedures. Material was extracted 3 × with 80% MeOH. The aq. residue after evaporating the MeOH was frozen at −20° and after thawing at 4° filtered. The aq. filtrate was partitioned with petrol (bp 30–50°) at pH 7.5 (extract discarded), with EtOAc at pH 2.5 and finally with aq. satd *n*-BuOH. The EtOAc as well as *n*-BuOH extracts were evapd *in vacuo* and subsequently purified by chromatography.

DEAE-Sephadex A-25. Prepn of the columns and elution with a discontinuous gradient of HOAc were performed as described [24]. Columns (100 × 2 cm) were used for extracts of mature caryopses (1.5 kg), as well as for fractions GA-A, GA-B, GA-C (Fig. 1), and fraction 11 and 12 (Fig. 4).

Sephadex LH-20. Partition chromatography was as described [25]. Columns (50 × 2 cm) were used subsequent to purification on DEAE-Sephadex A-25 taking 15 fractions (50 ml) with the organic phase and 2 further ones with the H_2O phase.

Si gel. First purification of EtOAc and *n*-BuOH extracts from immature fruits (5 kg) was by Si gel partition CC (400 g Si gel, 1 m × 4 cm). Gradient elution and fractionation is shown in Figs. 1 and 4.

TLC. Si gel GF_{254} (for ABA and 4′-dihydrophaseic acid) and G (for GAs) were used in layers of 0.3 mm (analytical) and 1.0 mm (prep) with the following systems: (1) $CHCl_3$–EtOAc–HOAc (18:2:1) [26]; (2) PrOH–NH_3 (5:1) [27]; (3) EtOAc–HOAc (100:1) [28]; (4) $CHCl_3$–EtOAc–HOAc (5:4:1) [26]; (5) C_6H_6–EtOAc–HOAc (10:1:1) [29]; (6) $CHCl_3$–MeOH–Me_2CO (75:22:3) [22]. Scraped zones were eluted 3 × with MeOH and adhering Si gel removed by DEAE-Sephadex A-25.

Enzymatic hydrolysis. Neutral GA fractions obtained by DEAE-Sephadex A-25 were incubated with helicase (crude enzyme prepn of the digestive tract from *Helix pomatia*) in Pi buffer (Sörensen, pH 6.5) at 36° for 48 hr. The hydrolysates were taken to dryness *in vacuo* and purified on DEAE-Sephadex A-25.

GC–MS. Residues from GA fractions were derivatized to TMSi-GA-TMSi and TMSi-GA-Me as described [30] and examined by GC–MS. Silanized glass columns (180 × 0.4 cm) were packed with 3% QF 1 on 125–250 mesh Gaschrom Q, He flow rate 20 ml/min, and column temp. 210°. The MS were determined at 80 eV (separator temp. 230°).

MS of TMSi-GA_{24}-TMSi. m/e (rel. int.): 490 (M^+) (10), 475 (16), 463 (15), 400 (12), 385 (7), 372 (42), 344 (30), 329 (16), 317 (15), 310 (21), 282 (38), 254 (36), 237 (32), 226 (100).

MS of TMSi-GA_{16}-TMSi. m/e (rel. int.): 564 (M^+) (8), 549 (18), 474 (17), 448 (25), 405 (13), 384 (9), 358 (30), 330 (23), 287 (20), 240 (100), 217 (47).

MS of TMSi-GA_8-TMSi. m/e (rel. int.): 652 (M^+) (100), 637 (16), 562 (13), 535 (85), 506 (22), 475 (12), 445 (15), 433 (23), 415 (15), 355 (23), 329 (22), 311 (43), 296 (45), 281 (33), 235 (32), 219 (45), 207 (92). The MS of TMSi-GA_{16}-Me and TMSi-GA_8-Me published in [14] correspond with our spectra. ABA and 4′-dihydrophaseic acid fraction were methylated with CH_2N_2 and examined by GC–MS using the same column with modified conditions (He flow rate 15 ml/min, column temp. 180°).

Bioassay. For quantitative determination of GA activity we used the dwarf rice (*Oryza sativa* L. cv Tan ginbozu) and dwarf pea bioassay (*Pisum sativum* L. cv Insignis) as described [11]. Inhibitor activities were determined by measuring growth inhibition of wheat seedlings (*Triticum aestivum* L. cv Carola) grown for 5 days in test soln (1 ml, 5 seedlings) at 20° (16 hr fluorescent light, 8 hr dark) after germination for 3 days at 20° in the dark.

Acknowledgements—The authors are grateful to Prof. J. MacMillan, Department of Organic Chemistry, The University, Bristol, UK, for identification of 4′-dihydrophaseic acid by GC–MS by comparison with authentic material. We thank Prof. Takahashi, Department of Agricultural Chemistry, University

Tokyo, for samples of GA_{16} and GA_{24}, and Prof. Y. Murakami, National Institute of Agricultural Sciences, Tokyo, Japan, for dwarf rice seed. The bioassays were carried out by Mrs S Vorkefeld and the GC–MS spectra by Ms C Kuhnt.

REFERENCES

1. Murphy, G. J. P. and Briggs, D. E. (1973) *Phytochemistry* **12**, 1299.
2. Faull, K. F., Coombe, B. G. and Paleg, L. G. (1974) *Australian J. Plant Physiol.* **1**, 183.
3. Browning, G. and Saunders, P. F. (1977) *Nature* **265**, 375.
4. Eckert, H., Schilling, G., Podlesack, W. and Franke, P. (1977) *Biochem. Physiol. Pflanzen* in press.
5. Murofushi, N., Iriuchijima, S., Takahashi, S., Tamura, S., Kato, J., Wada, Y., Watanabe, E. and Aoyama, T. (1966) *Agric. Biol. Chem.* **30**, 917
6. Kaufman, P. B., Ghoshen, N. S., Nahosteen, L., Pharis, R. P., Durley, R. C. and Morf, W. (1976) *Plant Physiol.* **58**, 131.
7. Jones, D. F., MacMillan, J. and Radley, M. (1963) *Phytochemistry* **2**, 307.
8. McWha, J. A. (1975) *J. Exp. Botany* **26**, 823.
9. King, R. W. (1976) *Planta* **132**, 43.
10. Radley, M. (1976) *J. Exp. Botany* **27**, 1009
11. Sembdner, G., Borgmann, E., Schneider, G., Liebisch, H. W., Miersch, O., Adam, G., Lischewski, M. and Schreiber, K. (1976) *Planta* **132**, 249.
12. Harrison, D. M. and MacMillan, J. (1971) *J. Chem. Soc. C* 631.
13. Graebe, J. E., Hedden, P., Gaskin, P. and MacMillan, J (1974) *Phytochemistry* **13**, 1433.
14. Beeley, L. J., Gaskin, P. and MacMillan, J. (1975) *Phytochemistry* **14**, 779.
15. Binks, R., MacMillan, J. and Pryce, R. J. (1969) *Phytochemistry* **8**, 271.
16. Reeve, D. R. and Crozier, A. (1974) *J. Exp Botany* **25**, 431.
17. Galt, R. H. B. (1968) *Tetrahedron* **24**, 1977.
18. MacMillan, J., Seaton, J. C. and Suter, P. J. (1962) *Tetrahedron* **18**, 349.
19. Durley, R. C., MacMillan, J. and Pryce, R. J. (1971) *Phytochemistry* **10**, 1891.
20. Yokota, T., Takahashi, N., Murofushi, N. and Tamura, S (1969) *Planta* **87**, 180.
21. Milborrow, B. V. (1967) *Planta* **76**, 93.
22. Sondheimer, E., Galson, E. C., Tinelli, E. and Walton, D. C (1974) *Plant Physiol.* **54**, 803.
23. Martin, G. C., Dennis, F. G., Gaskin, P. and MacMillan, J. (1977) *Phytochemistry* **16**, 605.
24. Gräbner, R., Schneider, G. and Sembdner, G. (1975) *J. Chromatogr.* **121**, 110.
25. MacMillan, J. and Wels, C. M. (1973) *J. Chromatogr.* **87**, 27.
26. Sembdner, G., Groß, R. and Schreiber, K. (1962) *Experientia* **18**, 584.
27. Sembdner, G., Schneider, G., Weiland, J. and Schreiber, K. (1964) *Experientia* **20**, 89.
28. Graebe, J. E., Hedden, P., Gaskin, P. and MacMillan, J. (1974) *Planta* **120**, 307.
29. Dörffling, K., Sonka, B and Tietz, D. (1974) *Planta* **121**m 57.
30 Schneider, G., Jänicke, S. and Sembdner, G. (1975) *J. Chromatogr.* **109**, 409.