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GC-MS QUANTIFICATION OF GIBBERELLIN A_{20}-13-*O*-GLUCOSIDE AND GIBBERELLIN A_8-2-*O*-GLUCOSIDE IN DEVELOPING BARLEY CARYOPSES†

BIANCA SENNS, PETRA FUCHS and GERNOT SCHNEIDER*

Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; GC-MS quantification; gibberellin-*O*-glucosides.

Abstract—Gibberellin A_{20}-13-*O*-glucoside (GA_{20}-13-*O*-Glc) and GA_8-2-*O*-Glc were identified by GC-MS as permethylated derivatives in mature grains of both barley varieties *Hordeum vulgare* L. cv. Himalaya and cv. Salome.

Changes of the pool size of these GA conjugates together with those of the parent GA_{20} and GA_8 were estimated during seed ripening by internal standard quantification. The pools of GA_{20} and GA_8 were found to reach a maximum and then to decrease to small amounts at the stage of full ripeness. The amounts of GA_{20}-13-*O*-Glc were higher at the beginning of ripeness and then declined until full ripeness but these changes were not as pronounced as for free GA_{20}. Thus, in mature caryopses of both varieties cv. Himalaya and cv. Salome the content of GA_{20}-13-*O*-Glc was three to four times higher than that of GA_{20}. The pool of GA_8-2-*O*-Glc increased dramatically during the final stages of ripening, in cv. Himalaya earlier than in cv. Salome, and reflected the intensive GA_{20} metabolism during ripening. This is the first report on the simultaneous quantification of the pools of GA-*O*-glucosides and their parent GAs during seed development on the basis of a GC-MS/internal standard technique.

INTRODUCTION

Gibberellins (GAs) are known to play an important role in flowering, seed development and germination. The induction of enzymes in cereal caryopses during germination has been studied, but mostly by applying GAs exogenously [1, 2]. As an endogenous source of GAs is required to initiate α-amylase induction it is assumed that the biosynthesis of GA occurs in the scutellum/embryo [3].

But, the biosynthesis of GA does not precede the induction of α-amylase [4] so that other models or sources of GAs are under consideration. Thus, different compartmentalization of the α-amylase isoenzymes and their different GA requirements for induction have been discussed to explain the initial steps of germination [5, 6]. However, GA conjugates, which were found in mature seeds e.g. of leguminous plants [7, 8] could also be potential candidates to provide free GAs during the early stage of germination by hydrolytic release. Some indications are reported in the literature for this conversion of GA conjugates during germination. Thus, after feeding radioactive GA_{20} to maturing maize cobs metabolically formed, but unidentified polar fractions were found, which release free GA_{20} in germinating caryopses [9, 10].

In order to approach the question regarding the physiological role of endogenous GA conjugates during seed development, we have searched for GA-*O*-glucoside pools in mature barley grains and to subsequently follow the build-up of these conjugate pools during seed ripening. So far, in immature seeds of barley cv. Trumpf GA_1-3-*O*-Glc and GA_{20}-13-*O*-Glc in addition to GA_8-2-*O*-Glc and GA_{29}-2-*O*-Glc have been identified [11]. For our experiments we have selected two other barley varieties: cv. Himalaya, which is widely used in GA bioassays and α-amylase induction research, and cv. Salome, another summer barley.

RESULTS AND DISCUSSION

Endogenous occurrence of GA-O-glucosides in mature grains of cv. Himalaya and cv. Salome

In order to select relevant GA-*O*-Glc pools of mature caryopses of barley we extracted mature grains

* Author to whom correspondence should be addressed.

† Dedicated to Professor Dr G. Adam on the occasion of his 65th birthday

Table 1. Chromatographic retention time (R_I), as well as characteristic ions (m/z) from full scan spectra and their abundances (%) of permethylated gibberellin-*O*-glucosides from mature caryopses of *Hordeum vulgare* L. cv. Himalaya and cv. Salome in comparison with permethylated standard compounds

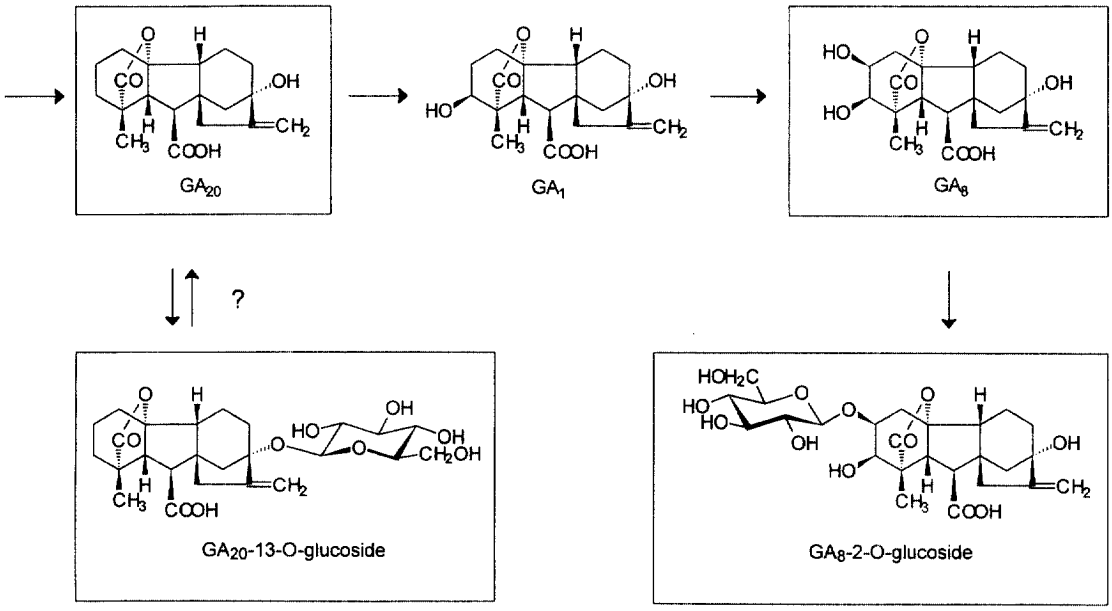
Compound	R_I	Characteristic ions							
GA_{20}-13-*O*-glucoside		88	101	187	209	297	329	389	*m/z*
Standard	3426	100	48	9	8	10	41	9	%
cv. Himalaya	3423	100	43	8	6	8	34	7	%
cv. Salome	3426	100	39	7	5	6	35	7	%
GA_8-2-*O*-glucoside		88	101	187	313	345	389	449	*m/z*
Standard	3656	100	79	10	8	9	11	14	%
cv. Himalaya	3651	100	72	10	7	8	12	12	%
cv. Salome	3650	100	70	8	7	8	9	11	%

of cv. Himalaya and cv. Salome. The extracts were purified, derivatized and analyzed by GC-MS as permethylated derivatives [12]. On the basis of synthetic standards, GA_{20}-13-*O*-Glc and GA_8-2-*O*-Glc could be identified in both varieties by full scan mass spectra at the appropriate retention (R_I) as shown in Table 1. Neither GA_1-3-*O*-Glc nor GA_1-13-*O*-Glc could be detected in mature grains of both varieties. Thus, only GA_{20}-13-*O*-Glc may represent a source for providing active GAs after hydrolysis to GA_{20} and its subsequent transformation to GA_1 and GA_3 [13]. However, GA_8-2-*O*-Glc also deserves attention, because the pool of this compound reflects the metabolic flux of GA_{20} through GA_1/GA_8, especially as any 3-hydroxylation after 2β-hydroxylation of GA_{20} to GA_{29} has not been reported yet.

Accordingly, GA_{20}-13-*O*-Glc and GA_8-2-*O*-Glc together with their parent GAs were subjected to GC-MS quantification in caryopses during ripening (Scheme 1).

Quantification of GA_{20} and GA_8 as well as of GA_{20}-13-O-Glc and GA_8-2-O-Glc during seed development of barley caryopses

Caryopses of cv. Himalayas (1994) and cv. Salome (1996) were harvested every week beginning at the stage of milk ripeness until full ripeness. The extracts of the seeds were spiked with [17-$^2H_2$]-labelled GA_{20}, GA_8, GA_{20}-13-*O*-Glc and GA_8-2-*O*-Glc and worked-up according to a modified procedure reported previously [14]. After confirmation of the identity by R_I



Scheme 1. The biosynthetic pools of the early 13-hydroxylation pathway of GA metabolites through GA_1 in barley caryopses.

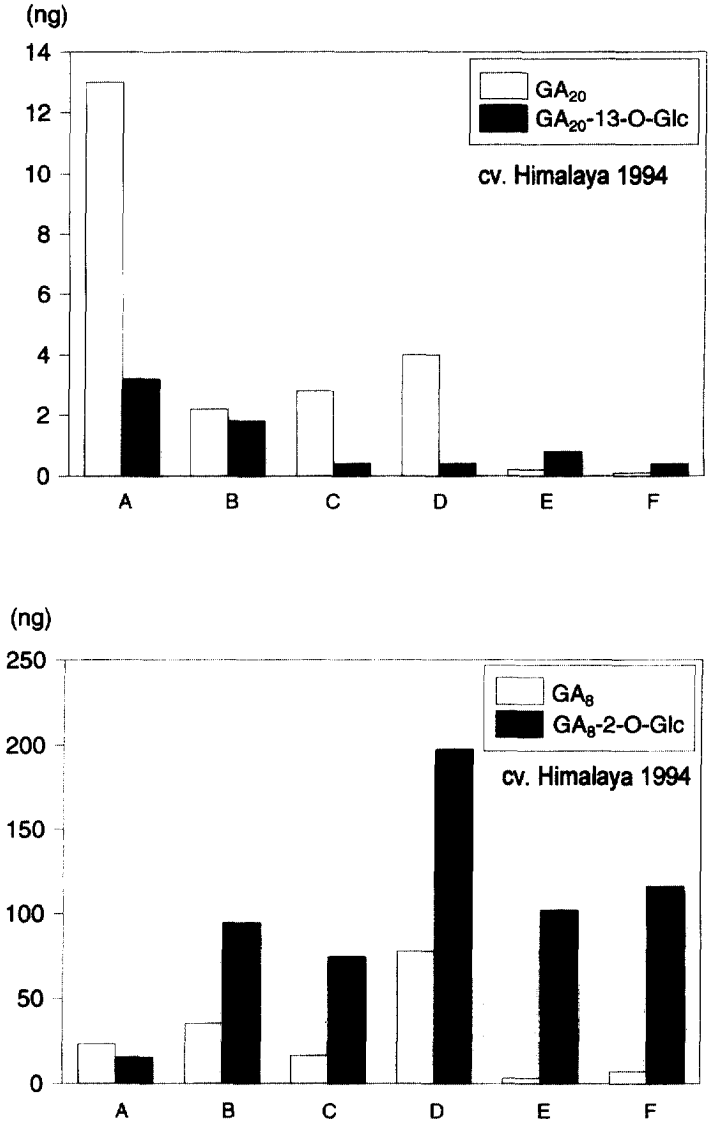


Fig. 1. Pool sizes (ng per g fr. wt) of GA_{20}, GA_8, GA_{20}-13-*O*-Glc and GA_8-2-*O*-Glc of cv. Himalaya estimated by GC-MS of permethylated samples using [17-$^2H_2$]-labelled standards during seed ripening (A: milk ripeness, B: early yellow ripeness, C: late yellow ripeness, D: early ripeness, E: late ripeness and F: dormant seed).

and mass spectra, selected ion monitoring (SIM) was used to estimate the isotope ratios for quantification. Appropriate calibration curves of the diagnostic ions were set up using seven calibration mixtures of authentic standards. The estimated absolute amounts of free GAs and their *O*-glucosides per g fr. wt of the harvests (A ··· F) of cv. Himalaya and cv. Salome are summarized in Figs 1 and 2, respectively.

In cv. Himalaya (Fig. 1) the pool of GA_{20} exhibited the maximum during milk ripeness followed by the physiologically expected decrease. For GA_8 the maximum appeared 1–2 weeks later. The content of GA_{20}-13-*O*-Glc was also higher at the younger stage and declined during full ripeness. The final amount of GA_{20}-13-*O*-Glc (0.4 ng per g fr. wt) exceeded that of GA_{20} (0.1 ng per g fr. wt) in the mature grains. In contrast to GA_{20}-13-*O*-Glc, the pool of GA_8-2-*O*-Glc was characterized by a steady increase during ripening and therefore gives an impression of GA_{20} metabolism via GA_1 (compare Scheme 1).

In the case of cv. Salome (Fig. 2) the amounts of GA_{20} (1.1 ng per g fr. wt in mature grains) were in general higher than in cv. Himalaya and the maximum appeared two weeks later. The amounts of GA_{20}-13-*O*-Glc were comparable to those of cv. Himalaya in the course of the ripening process (see Figs 1 and 2). The contents of free GA_8 were similar to those of cv. Himalaya, whereas the GA_8-2-*O*-Glc could not be detected at the early stages of ripening; it only appeared in mature grains. This probably indicates

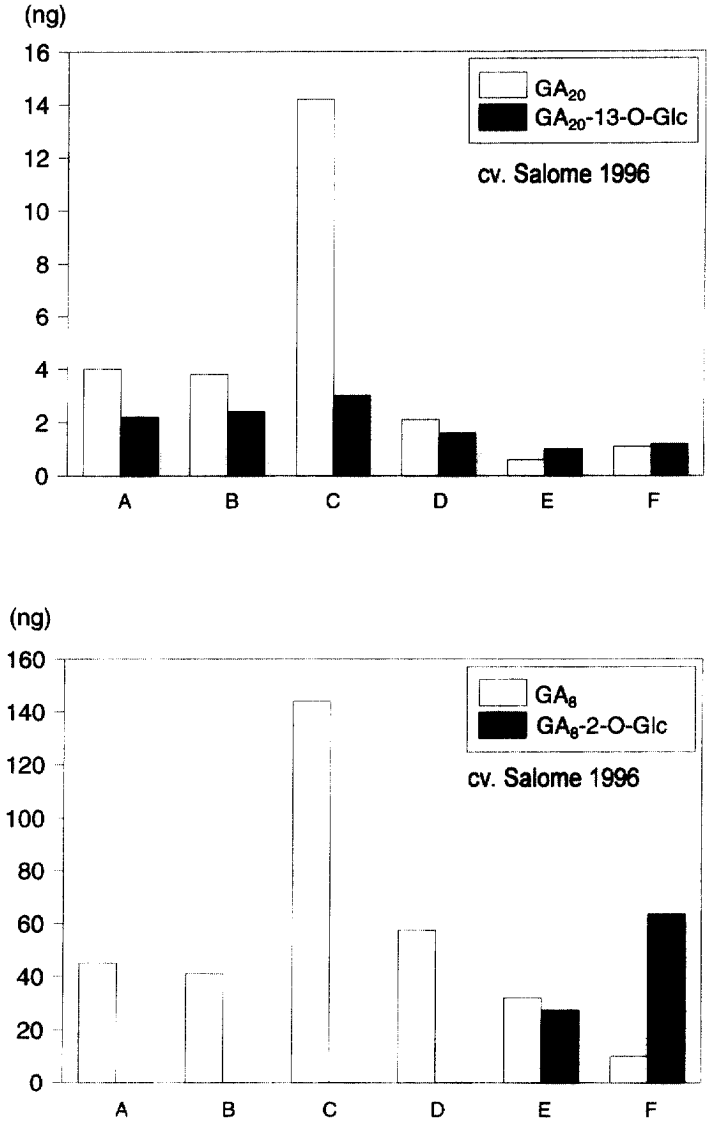


Fig. 2. Pool sizes (ng per g fr. wt) of GA_{20}, GA_8, GA_{20}-13-*O*-Glc and GA_8-2-*O*-Glc of cv. Salome estimated by GC-MS of permethylated samples using [17-$^2H_2$]-labelled standards during seed ripening (A: milk ripeness, B: early yellow ripeness, C: late yellow ripeness, D: early ripeness, E: late ripeness and F: dormant seed).

that in cv. Salome, glucosylation of GA_8 is the major metabolic step only during the late ripening, whereas the oxidation of the 2-keto-catabolite [15] may dominate before.

From the data it can be concluded that GA_{20}-13-*O*-Glc is a steadily associated metabolite of GA_{20} during seed ripening. The changes in the content of GA_{20}-13-*O*-Glc during seed development provide evidence that the pool of this conjugate undergoes further metabolism as rapid as the free GAs.

Although the content of GA_{20}-13-*O*-Glc declined until full ripeness, the remaining amounts were three to four times higher than those of the free GA_{20}. Consequently, the observed amounts of GA_{20}-13-*O*-Glc (0.4 ng or 1.1 ng per g fr. wt), which correspond to those found in mature wheat grains [16], should not be neglected in terms of providing GAs during germination, if hydrolysis occurs and if the conjugate may be efficiently distributed in the grain.

EXPERIMENTAL

Instrumentation

A Fisons quadrupole mass spectrometer MD 800 combined with a gas chromatograph GC 8000, which was equipped with a DB 5 MS fused silica capillary column (15 m × 0.32 mm, film thickness 0.25 μm, phase ratio 250), was used. The He carrier gas flow rate was ca 1.3 ml min^{-1} with 28 kPa head pressure. Injections were made in splitless mode. The injector temp. was 270° and the temp. programme was 60° for

1 min, ramped at 30° min^{-1} to 270° and finally at 20° min^{-1} to 290° with a 5 min hold at the end of the programme. The direct inlet interface temp. was 300° and the ion source temp. was 200°. The electron impact energy was 70 eV. The mass range of the monitored ions was 50–650 *m/z* with dwell times of 80 ms. The Kovats retention indices (R_I) were estimated on the basis of the retention times of parafilm hydrocarbons [17].

Standard substances

[17-$^2H_2$]-labelled GA_{20} and GA_8 (1.9 atoms deuterium per molecule) were synthesized by using the corresponding 17-norketones and Lombardo's reagent [18]. The GA-*O*-glucosides were obtained according to the lit. method [19]. For the quantification of the endogenous free GAs and the GA-*O*-glucosides, 50 μg of [17-$^2H_2$]-labelled GA_{20} and 50 μg GA_{20}-13-*O*-Glc (standard soln 1) and of 50 μg of [17-$^2H_2$]-labelled GA_8 and 50 μg GA_8-2-*O*-Glc (standard soln 2) were dissolved in 100 ml EtOH. These standard solns were added to the extract as internal standard as described below.

Plant material

Ears of field grown barley *Hordeum vulgare* L. cv. Himalaya (1994) and cv. Salome (1996) were harvested every week ($\times 5 = A \cdots E$) starting at the stage of milk ripeness (A = 20 days after anthesis) and until the stage of dormant seeds (F = 10 weeks after the first harvest). The harvest dates of cv. Himalaya and cv. Salome, respectively, were at A: 27.06, 19.07 (milk ripeness), B: 04.07, 26.07 (early yellow ripeness), C: 11.07, 01.08 (late yellow ripeness), D: 18.07, 09.08 (early ripeness), E: 25.07, 16.08 (late ripeness) and F: 05.09, 20.09 (dormant seed).

Caryopses (50 g) of the harvests were frozen with liquid N_2 and stored at −20° until extraction. From the caryopses of cv. Salome the seed testa was removed before storage.

Extraction and purification

Caryopses (50 g) were macerated and extracted with 150 ml 80% MeOH overnight at 4°. The extraction was repeated twice. The combined MeOH extracts were evaporated *in vacuo* and the aq. phase (ca 60 ml) partitioned with *n*-hexane. Thereafter 2 ml of standard soln 1 (20 ng [17-$^2H_2$]-labelled GA_{20} and GA_{20}-13-*O*-Glc per g fr. wt) and 5 ml of standard soln 2 (50 ng [17-$^2H_2$]-labelled GA_8 and GA_8-2-*O*-Glc per g fr. wt) were added to the aq. extract. The aq. phases were adsorbed onto celite, dried *in vacuo* and loaded onto DEAE-Sephadex A-25. After elution with an acetic acid gradient in MeOH the pooled acidic fractions (0.25, 0.5, 1, 2, 4 and 6 M HOAc) were evaporated and taken to dryness. Of these acidic fractions of the DEAE-Sephadex A-25 column, 50 mg were resuspended in MeOH, were loaded on a silica column and the free GAs and GA-*O*-glucosides were eluted with a MeOH gradient in $CHCl_3$ with 1% HOAc. The pooled eluates with 10 to 30% MeOH were evaporated, treated with ethereal CH_2N_2 and subsequently permethylated with CH_3J/NaH in DMF under standard conditions [12, 14]. The crude products were purified on a silica column eluted with an EtOAc gradient in toluene. The fractions, containing permethylated GAs and GA-*O*-glucosides were ready for GC-MS after evaporation [14].

The recoveries of the chosen procedure for both free GAs and GA-*O*-glucosides were proved with [^{3}H]-labelled standards to be 73% for [^{3}H] GA_{20} PME, 79% for [^{3}H] GA_{20}-13-*O*-Glc PME and 34% for [^{3}H] GA_8-2-*O*-Glc PME.

Calibration curves

For calibration purposes standard soln of 1 mg of each unlabelled and the corresponding [17-$^2H_2$]-labelled standards were dissolved in 1 ml EtOH. From these stock soln mixtures of GA_{20}/[17-$^2H_2$]GA_{20}, GA_8/[17-$^2H_2$]GA_8 or GA_{20}-13-*O*-Glc/[17-$^2H_2$]GA_{20}-13-*O*-Glc with ratios of 1:25, 1:20, 1:15, 1:10, 1:5, 1:1 and 5:1 were prepared. The mixtures were evaporated to dryness, permethylated as described above and then subjected to GC-SIM. The following pairs of ions were monitored and integrated: *m/z* 360/362 for GA_{20} PME/[17-$^2H_2$]GA_{20} PME, *m/z* 420/422 for GA_8 PME/[17-$^2H_2$]GA_8 PME, *m/z* 389/391 and *m/z* 329/331 for GA_{20}-13-*O*-Glc PME/[17-$^2H_2$]GA_{20}-13-*O*-Glc PME. The ratios of the peak areas of these ions of unlabelled and labelled standards (threefold injection SD% = 5%) were plotted against the amount ratios using seven different calibration mixtures. These curves were fitted by linear regression: $y = 0.1606 + 1.06846x$ ($r = 0.98431$, SD = 0.18277, $n = 7$) for the curve of GA_{20} PME/[17-$^2H_2$]GA_{20} PME, $y = 0.11699 + 0.2136x$ ($r = 0.9827$, SD = 1.02523, $n = 7$) for the curve of GA_8 PME/[17-$^2H_2$]GA_8 PME or $y = 0.131178 + 1.26181x$ ($r = 0.99903$, SD = 0.11148, $n = 7$) for the curve of GA_{20}-13-*O*-GlcPME/[17-$^2H_2$]GA_{20}-13-*O*-GlcPME.

For the [17-$^2H_2$]GA_{20}-13-*O*-Glc PME two diagnostic pairs of fragment ions at *m/z* 389/391 and *m/z* 329/331 were used [12]. The calibration curves of both ions were found to be parallel showing the absence of deuterium loss for these fragments.

These results were also applied for the estimation of GA_8-2-*O*-Glc PME/[17-$^2H_2$]GA_8-2-*O*-Glc PME at *m/z* 449/451 and at *m/z* 389/391.

The amounts of endogenous free GAs and GA-*O*-glucosides were determined from the ratio of peak areas on the basis of the corresponding calibration curves. For the conclusive definitive identification of the endogenous compounds mass spectra at appropriate R_I were recorded

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