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Cytokinins and gibberellins in sap exudate of the oil palm

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Abstract

Exudates were collected from stumps of pre-anthesis inflorescences of oil palm and analysed for cytokinin and gibberellin content using combined HPLC-ELISA techniques. Three antisera, for zeatin-type, dihydrozeatin-type and *iso*pentenyladenine-type cytokinins, were used in ELISAs to identify members of these three groups of cytokinins. Ribotides, 9-glucosides, free bases and ribosides were detected for each of the groups with zeatin riboside the most abundant cytokinin identified in the exudate. *Iso*pentenyladenine-type and dihydrozeatin-type cytokinins were also identified but at lower levels. In addition, two monoclonal antibodies were used in the development of novel ELISAs for members of the 13-hydroxylated and non-13-hydroxylated families of gibberellins. The new ELISAs allow the determination of gibberellins in smaller amounts of tissue than are required for GC–MS. The most abundant gibberellins identified in exudates were GA_{19} and GA_{44}, as well as other members of the early 13-hydroxylation pathway. Gibberellins were confirmed by GC–MS. The presence of these types of growth regulators in exudate supplying immature inflorescences suggest they have a role in growth and development of these structures.

Keywords: *Elaeis guineensis* Jacq.; Palmae; Oil palm; Cytokinins; Gibberellins

1. Introduction

The oil palm is a very important commercial crop in southeast Asia and equatorial America since it has the highest yield of oil of all oil bearing plants, including the coconut palm (Hartley, 1977). The oil is extracted from the oil palm fruit, it is therefore important to understand the mechanism of growth and development of inflorescences, this inevitably involves studying the growth regulators within the palm.

The presence of cytokinins and gibberellins in plant exudates has been previously reported in a number of species. Cytokinins have been identified in both xylem and phloem exudates (Wagner and Beck, 1990; Lejeune et al., 1994; Singh et al., 1992). It has been suggested that zeatin riboside is the major translocatable form of cytokinin in the xylem (van Staden and Davey, 1979) whereas *iso*pentenyladenosine is translocated in the phloem (Grayling and Hanke, 1992).

A number of studies have reported the presence of cytokinins and gibberellins in oil palm. Jones et al. (1995, 1996) have identified and quantified, by HPLC-ELISA and GC–MS, both isoprenoid and aromatic cytokinins in oil palm seedlings, inflorescences, crown tissue and cultured embryos. They also measured cytokinins in young immature inflorescences of oil palm clone 90a, one of those used in the present study, and found the most abundant cytokinins were zeatin riboside and zeatin-9-glucoside but zeatin free-base was not detected. There is less evidence for gibberellins in plant exudates, but gibberellins of the 13-hydroxylated and 13-non-hydroxylated pathways have been found in a number of species (Hoad et al., 1993; Oyama et al., 1999; Stephan et al., 1999). Otusanya and Adebona (1985) identified gibberellin-like activity in phloem sap from male inflorescences of oil palm by a gibberellin bioassay to detect activity ranging in concentration from 1.5 to 45 μg l^{-1} GA_3 equivalents. The present study is the first to report detailed identification and quantification of both cytokinins and gibberellins in exudate from oil palm.

Three antisera were used which are specific for the three families of isoprenoid cytokinins (*iso*pentenyladenine-, zeatin- and dihydrozeatin-types), and two

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monoclonal antibodies which can identify 13-hydroxylated or 13-non-hydroxylated gibberellins. The use of these immunoreagents in combined HPLC-ELISA has enabled identification and measurement of individual members of the cytokinin and gibberellin families naturally occurring in oil palm exudate. Determination of the tissue content of cytokinins by ELISA has been widely used, but here we present a new, sensitive ELISA method for the identification and quantitation of gibberellins, with the advantage over standard GC–MS methods that much smaller amounts of tissue are required. The identification of GAs by the ELISA method has been confirmed by GC–MS here.

2. Results and discussion

2.1. Cytokinins

2.1.1. Separation by HPLC

Fig. 1 shows the separation of standard cytokinins by reverse-phase HPLC using the methanol gradient described in Section 3. The gradient gave good resolution of a mixture of different members of the three isoprenoid families (Z-, *i*PA-, and DHZ-types), i.e. ribotides [9R-MP], ribosides [9R], free bases and 9-glucosides [9G]. Structures of the cytokinins identified in this study are shown in Fig. 2.

2.1.2. ELISAs

Three cytokinin ELISAs were used to quantify individual compounds within three families of isoprenoid cytokinins naturally occurring in oil palm sap. Each ELISA used an antiserum specific for each family, i.e. Z-types, *i*PA-types and DHZ-types. Cross-reactivity between the three families was remarkably low, i.e. no more than 3% but typically $<1.5\%$ (full details in Huntley, 1995; Yeates, 1995), indicating that the individual antibodies were highly selective for cytokinins with the same side-chain at N^6.

The three cytokinin antisera used in the present study were each raised to a cytokinin riboside conjugated to BSA via the periodate-oxidised ribosyl moiety. They therefore cross-react strongly with N^9-substituted cytokinins, i.e. ribosides, ribotides and 9-glucosides, with ca. 50% of this affinity for unsubstituted cytokinin free bases. They cross-react very weakly with the following: (1) purines without the N^6-side chain, (2) cytokinins with any other side chain, (3) cytokinins with a substituted side chain, e.g. *O*-glucosides, and (4) cytokinins substituted at any other position on the purine nucleus, e.g. 7-glucosides. Therefore any *O*-glucosides and 7-glucosides, which may have been present in the sap samples, will not be detected by an immunoassay based on these antisera. However, *O*- and 7-glucosides are almost certainly non-mobile (Fusseder and Ziegler, 1988) and biologically inactive (Letham et al., 1983; Letham, 1978), their presence in sap exudates is not expected except possibly as a result of tissue damage. HPLC separation of the cytokinins combined with the three antisera specific for the three isoprenoid families allowed us to identify the individual cytokinins with some confidence as we were able to detect any non-specific interference and, by reference to estimates of cross-

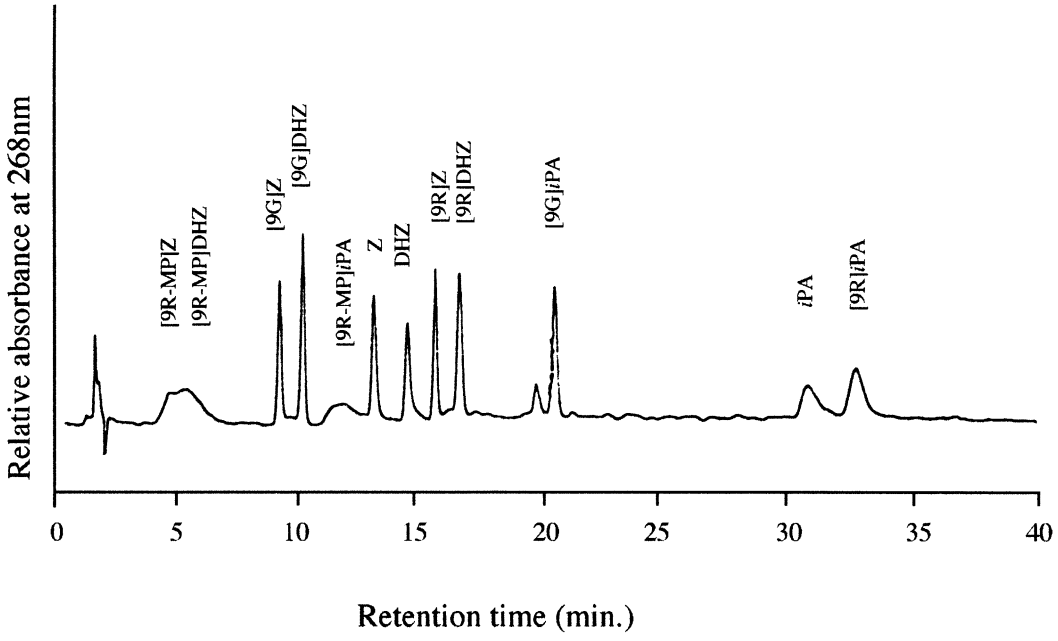

Fig. 1. Separation of authentic isoprenoid cytokinins on reverse-phase HPLC. Mixtures of Z-type, *i*PA-type and DHZ-type cytokinins (0.5 nmol of each compound) were injected onto the HPLC column and separated on a methanol/water gradient of 15–37% MeOH/0.2 mM TEAA in 15 min then isocratic at 37% for a further 25 min.

reactivity, prevented mis-identification of individual cytokinin peaks. Each of the cytokinin ELISAs, then, acts as a control for the other two, providing low values for HPLC fractions containing cytokinins belonging to the other families. These low values establish the absence of non-specific interference and validate the positive values in another cytokinin ELISA as real, not false, positives, confirming both identification and quantitation.

A typical ELISA analysis of zeatin-type cytokinins in oil palm sap is shown in Fig. 3. Each HPLC fraction was assayed for cytokinin content using the Z-type specific antiserum. As in most of the cytokinin ELISAs, the most abundant compound was zeatin riboside followed by the free base zeatin. Table 1 shows the cytokinin content of oil palm exudates. The values are averages of 34 HPLC-ELISA analyses on exudates from individual palms. It is clear that the predominant cytokinins were the Z-types with *i*PA-types also present but in lower concentrations and also small amounts of DHZ-types. Most of the different metabolic forms of cytokinin were represented in the exudate, i.e. free bases, ribosides and 9-glucosides, but ribotides were only represented significantly in the *iso*pentenyladenine group. Zeatin riboside was by far the most abundant cytokinin in the exudate being more than four times greater at 13 pmol ml^{-1} than the next highest, the free base zeatin at 3 pmol [9R]Z eq ml^{-1}. The two major *i*PA-types present were the riboside and free base at 1 pmol ml^{-1} and 0.8pmol [9R]*i*PA eq ml^{-1}, respectively. None of the values for individual compounds have been corrected for cross-reactivity with the appropriate antibody and so are expressed as cytokinin riboside equivalents. Correction for cross-reactivity is invalid because values of cross-reactivity are only correct for one concentration of the standard reference compound, in this case the cytokinin riboside, and the use of equivalents is standard practice. Generally the free base zeatin has about 45% cross-reactivity and both [9G]Z and [9R-MP]Z have 66% cross-reactivity with the anti-[9R]Z antibody relative to the riboside at 100%. For the *i*PA-types relative to the riboside, *i*PA has 38%, [9G]*i*PA has 46% and [9R-MP]*i*PA has 61% cross-reactivity with the anti-[9R]*i*PA antibody, and for the DHZ-types, DHZ has 67%, [9G]DHZ has 84% and [9R-MP]DHZ has 62% cross-reactivity with the anti-[9R]DHZ antibody compared with [9R]DHZ (Huntley, 1995; Yeates, 1995).

[9R]Z was identified as being the most abundant cytokinin in oil palm sap. This provides more evidence that [9R]Z is the major translocatable form of cytokinin in various plant exudates (Lejeune et al., 1994; van Staden and Davey, 1979). Jones et al. (1996) reported higher concentrations of *i*PA-type cytokinins, especially [9R]*i*PA, in oil palm seedlings compared with Z-types.

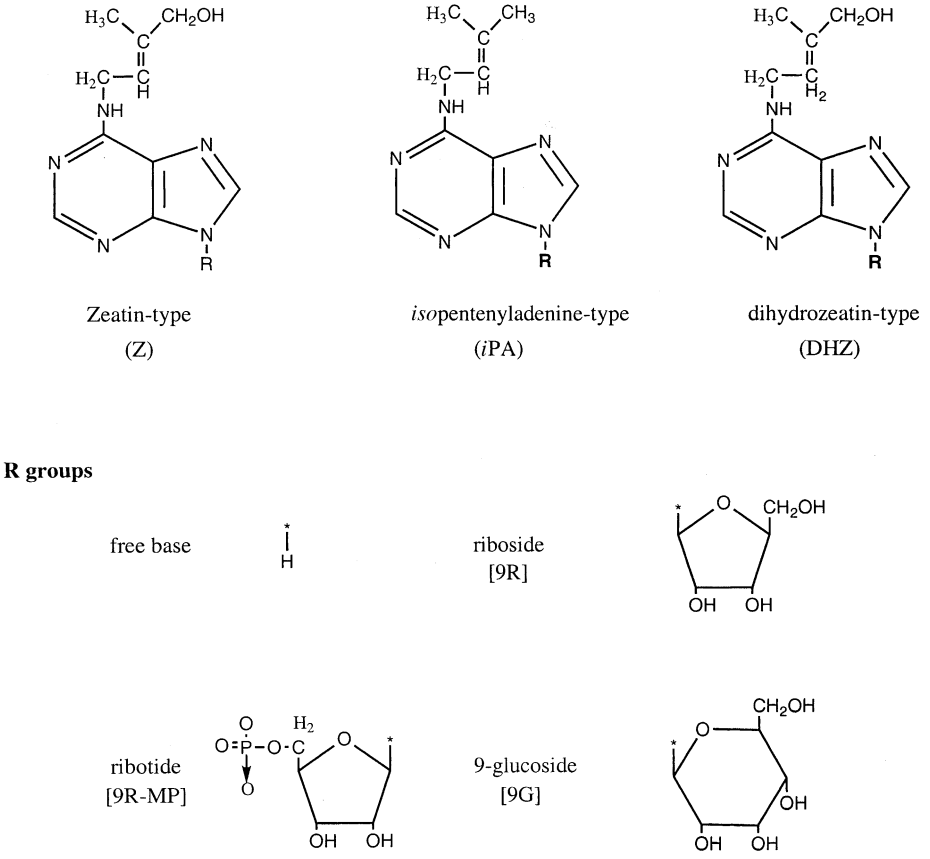

Fig. 2. Structures of the three cytokinin families and their members identified in this study.

In the present study there were only low concentrations of DHZ-type cytokinins in sap in agreement with measurements taken by Jones et al. (1996) on oil palm seedlings. In 1995, Jones et al. measured the concentration of cytokinins in young immature oil palm inflorescences by HPLC-ELISA and found in general that the Z-types, especially [9R]Z and [9G]Z were the most abundant cytokinins, ranging from 0 to 19 pmol g^{-1} fresh weight, with concentrations of *i*PA- and DHZ-types at approximately one tenth. The content of [9R]Z in inflorescence tissue (Jones et al., 1995) was slightly greater than that in inflorescence sap. Contents of *i*PA- and DHZ-types were also greater in tissue compared with sap.

Table 1
Cytokinin content of oil palm exudates (values are the mean of individual analyses on exudate from 34 palms)

CK identity from HPLC retention time	[CK] (pmol eq ml^{-1})
[9R-MP]Z	<0.1
[9G]Z	1.0±0.3
Z	3.0±0.8
[9R]Z	13.0±2.6
[9R-MP]*i*PA	0.5±0.1
[9G]*i*PA	0.2±0.06
*i*PA	0.8±0.2
[9R]*i*PA	1.0±0.3
[9R-MP]DHZ	<0.1
[9G]DHZ	0.8±0.3
DHZ	0.2±0.06
[9R]DHZ	0.2±0.04

Cytokinins have been detected in xylem and phloem exudates from a number of species including *Perilla* (Grayling and Hanke, 1992), *Xanthium strumarium* (Kinet et al., 1994) and *Ricinus communis* (Komor et al., 1993; Kamboj et al., 1999). In *Perilla* and *Xanthium*, the concentrations of cytokinins as measured by HPLC-RIA were in the same range as ours (0–3 and 1.5–25 pmol ml^{-1}, respectively). In *R. communis*, Komor et al. (1993) measured, by HPLC-ELISA, higher concentrations of *i*PA-types than Z-types; the concentration of *i*PA-types in phloem sap was greater than in xylem sap (70 and 9 pmol ml^{-1}, respectively) with Z-types at ca. 0.2 pmol ml^{-1} in both phloem and xylem saps whereas Kamboj et al. (1999) measured high levels of Z-types by GC-SIM in *Ricinus* phloem sap (up to ca. 700 pmol ml^{-1}) and xylem sap (up to ca. 70 pmol ml^{-1}).

The results of Grayling and Hanke (1992) suggest that in *Perilla* *i*PA-type cytokinins are made in leaves and are transported in phloem sap to the roots where they are converted to Z-types, which are in turn transported back to the shoot in xylem sap. van Staden and Davey (1979) have shown that Z-type cytokinins are translocated in xylem. It may be that sap collected by the inflorescence tapping method in the present study mainly consists of xylem sap due to the higher concentration of Z-types and the low concentrations of *i*PA-types and carbohydrates. Carbohydrate levels

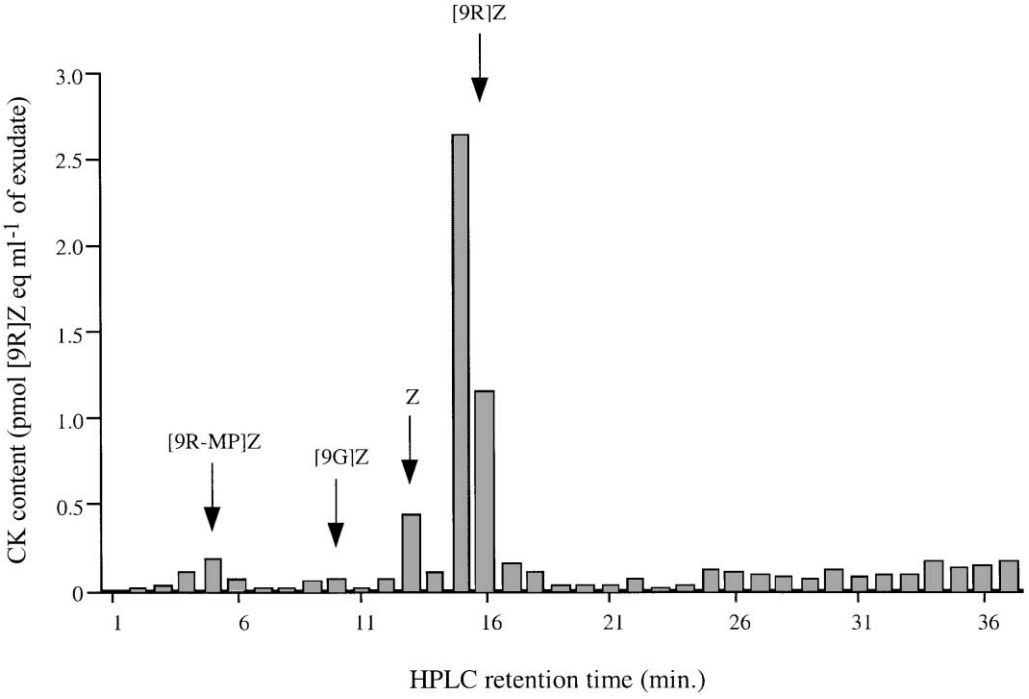

Fig. 3. A typical HPLC-ELISA analysis of a single sample of oil palm exudate to quantitate Z-type cytokinins. Individual Z-type cytokinins in a sample of exudate were separated by reverse-phase HPLC and each HPLC fraction analysed by ELISA using a Z-type specific antibody. Quantification was by comparison to standard concentrations of authentic [9R]Z, the values are not corrected for cross-reactivity, thus sample concentrations are expressed as [9R]Z equivalents. Each arrow marks the retention time of a cytokinin standard.

(measured according to the method of Outlaw and Tarczynski, 1984) were low, typically <1 g l^{-1} in this study compared with 120–140 g l^{-1} reported in oil palm sap by Eze and Uzoechi-Ogan (1988) and these are known to be transported in phloem (Lejeune et al., 1994; van Die and Tammes, 1975). The presence of carbohydrates, therefore, suggests that the sap in this study is a mixture of xylem and phloem exudate.

The presence of the 9-glucosides was unexpected since they are reported to be sequestered in the vacuole and are non-mobile (Palmer et al., 1981; Letham et al., 1983), however, their presence is most likely due to the tissue damage incurred in removal of the inflorescence.

Three sap exudate samples were also tested for the presence of aromatic cytokinins using antisera to benzyladenine, *ortho*-hydroxy-benzyladenine and *meta*-hydroxy-benzyladenine. With the exception of an unidentified compound eluting at five minutes, possibly a ribotide as it was sensitive to alkaline phosphatase hydrolysis, the concentrations were extremely low, suggesting these cytokinins are not transported in the xylem or phloem. No further analyses of aromatic cytokinins were carried out (data not shown).

2.2. Gibberellins

2.2.1. GC–MS analysis

Gibberellins have not yet been definitively identified in oil palm, therefore exudate was analysed by full-scan GC–MS to identify the more abundant GAs present (Table 2). The majority of GAs identified were members of the early 13-hydroxylation (13-OH) pathway, but some novel GAs were also putatively identified including 2β-OH GA_{12} (whose structure has since been confirmed by synthesis; Owen et al., 1998); 16,17-dihydro-GA_{44}-17-ol, 16,17-dihydro-GA_{53}-17-ol and 2β,7β-$(OH)_2$-kaurenoic acid. Subsequent quantitative analysis by GC-SIM was then performed, the results of which are shown in Table 3. This analysis confirmed that the 13-OH gibberellins were the most abundant, specifically the precursor GA_{19}. Structures of GAs from the 13-OH pathway identified in this study are shown in Fig. 4.

2.2.2. ELISAs

Two gibberellin ELISAs using two different monoclonal antibodies were developed for this study, which in conjunction with HPLC, could be used to quantify members of the 13-OH family of gibberellins. The antibodies used in the two ELISAs were raised against GA-KLH conjugates for work described in Knox et al. (1987). MAC 136 was raised against a GA_1-3-KLH conjugate, the KLH being linked via the 3β-hydroxy group of GA_1. This enables MAC 136 to recognise features of the GA molecule most distant from the coupling site, i.e. the 7-oic acid and 13-hydroxyl groups. MAC 136 was therefore used in assays to detect the 13-hydroxylated gibberellins. MAC 182 was raised against a GA_4-17-KLH conjugate, in which the KLH was linked via carbon 17 of GA_4. MAC 182 therefore recognises the 7-oic acid and the 3β-hydroxyl groups of a gibberellin molecule.

An ELISA using the monoclonal antibody MAC 182 was used as a control assay for the MAC 136 assay since positive 13-hydroxylated gibberellin activity present in the MAC 136 assay should not be detected in the MAC 182 assay. This is because MAC 182 was raised against a GA not hydroxylated in the 13C position, therefore it does not recognise GAs substituted in the 13C position. GA_1 was detected in both ELISAs. As in the case of the cytokinin ELISAs, the control ELISA using MAC 182 is indispensable for GA analysis because the low values for HPLC fractions containing 13-hydroxylated gibberellins establish the absence of non-specific interference and validate the positive values in the MAC 136-based

Table 2
Identification of endogenous GAs by full-scan GC–MS of the methylesters or MeTMSi[a] derivatives in exudate from oil palm inflorescences

GA	HPLC fraction	KRI[b]	Ion m/z[c] (relative abundance)
GA_8	10–13	2822	594(100),579(7),535(8),448(20),379(9),238(13),207(47),194(15)
GA_{29}	10–13	2690	506(100),491(9),477(3),447(8),389(11),375(16),303(37),235(22),207(46),193(16)
GA_{97}	14–16; 17–19	2701	536(18),521(6),504(5),477(9),446(4),387(6),371(5),239(52),208(76),207(100)
GA_1	17–19	2676	506(100),448(49),416(62)
16,17-dihydro-GA_{44}-17-ol (putative)	23–24	3083	522(7),507(54),475(62),447(38),432(49),391(86),359(100),297(39),237(23),207(37)
GA_{20}	25–26	2496	418(100),403(18),389(7),375(59),359(19),301(20),235(10),207(42),193(9)
16,17-dihydro-GA_{12}-16,17-diol	25–26; 27–30	2791	538(1),523(1),463(2),435(80),403(8),375(100),344(10),315(10),299(9),143(13)
GA_{19}	27–30	2608	462(6),434(100),402(34),374(62),345(24),285(29),239(40),208(40),193(34),167(32)
GA_{44}	27–30	2807	432(52),417(9),404(3),373(16),259(5),238(37),219(5),207(100),180(13),167(7)
16,17-dihydro-GA_{53}-17-ol (putative)	27–30	2763	538(8),523(3),448(14),407(51),389(10),375(100),347(30),297(20),241(17),207(15),181(23)
2β-OH-GA_{12} (GA_{110})	31–32	2552	448(4),433(4),416(20),388(50),298(100),283(65),272(39),258(22),239(89),223(33),145(28)
2β, 7β-$(OH)_2$-KA (putative)	31–32	2606	492(14),477(2),432(4),417(2),402(21),362(10),343(12),312(73),272(28),253(100),223(32)
GA_{53}	34–36	2507	448(54),433(9),416(16),389(32),373(15),251(34),235(33),208(93),207(100),193(35),181(77)

[a] MeTMSi, methyl ester trimethylsilylether.
[b] KRI, Kovat's Retention Index.
[c] m/z, mass/charge.

Table 3
Endogenous GA concentrations (pmol ml^{-1}) in exudate from mature oil palm inflorescences

GA	Concentration (pmol ml^{-1})
GA_8	0.23
GA_1	0.03
GA_{20}	0.10
GA_{19}	0.52
GA_{53}	0.03

Analysis was performed by GC–SIM. Recovery was determined by addition of deuterated authentic GAs as internal standards.

ELISA as real, not false, positives, confirming both identification and quantitation.

A typical ELISA analysis of gibberellins in oil palm sap is shown in Fig. 5. Each HPLC fraction was assayed for gibberellin content using the 13-OH-GA (MAC 136) specific antibody. It was not possible, however, to separate GA_{19} and GA_{44}, so they were expressed as a single value. GA_{19} and GA_{44} were among the most abundant gibberellins present in the exudate, and this is confirmed by GC–SIM data. Table 4 shows the average gibberellin content (pmol GA_{20} eq ml^{-1}) of oil palm exudate from a total of 34 palms as analysed by HPLC-ELISA. The

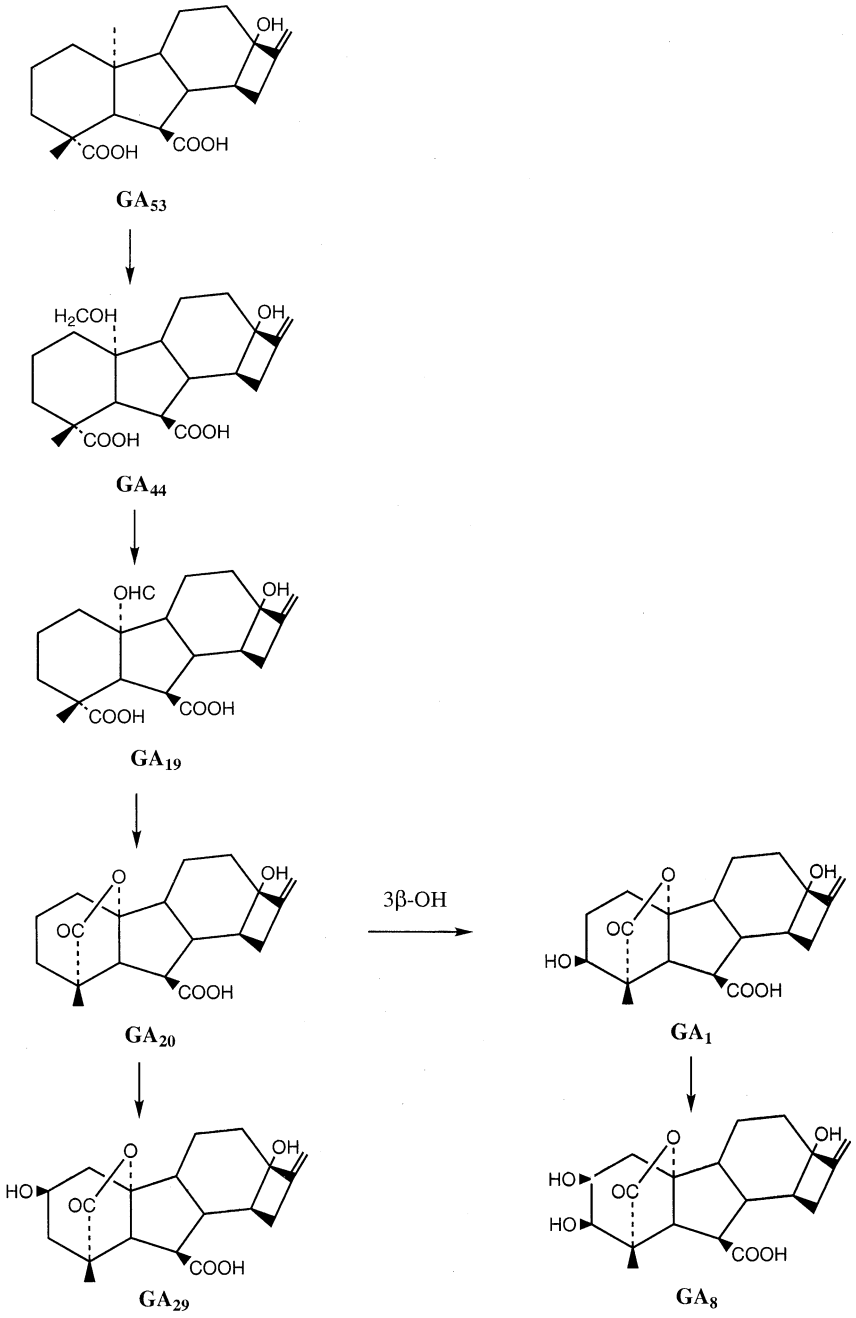

Fig. 4. Structures of gibberellins in the 13-OH pathway identified in this study.

most abundant gibberellins present were the precursors to the active GAs, i.e. GA_{44} and GA_{19} at ca. 23 pmol GA_{20} eq ml^{-1}, the next abundant was another precursor, GA_{53} at 8 pmol GA_{20} eq ml^{-1}. The active GA GA_1 and its precursor, GA_{20}, were present at lower concentrations (3.5 and 2.5 pmol ml^{-1}, respectively). Again, as for cytokinin ELISAs none of these values can be legitimately corrected for cross-reactivity of the individual gibberellins with the antibody and so they are expressed as GA_{20} equivalents. Cross-reactivities of gibberellins with both MAC 136 and MAC 182 in radioimmunoassays have previously been published by Knox et al. (1987). Basically, apart from GA_1 which has high cross-reactivity in both assays, gibberellins with a high cross-reactivity in the MAC 136 assay had a low cross-reactivity in the MAC 182 assay and vice versa.

Table 4
Gibberellin content of oil palm exudates (values are the mean of individual analyses on exudate from 34 palms)

GA identity from HPLC retention time	[GA] (pmol eq ml^{-1})
$GA_{8/29}$	3.0±0.7
GA_1	3.5±0.7
GA_{20}	2.5±0.4
$GA_{19/44}$	23.0±3.5
GA_{53}	8.0±1.3

GC–MS and GC–SIM analyses identified gibberellins of the 13-hydroxylated pathway as the most abundant as well as a number of putative GAs, one of which (2β-OH-GA_{12}) has been confirmed by synthesis and nominated as GA_{110} (Owen et al., 1998). The amounts of gibberellins detected by GC–SIM are up to 33-fold less than by HPLC-ELISA, this may be because the GC–SIM analysis was only performed on one sap sample whereas the concentration of total gibberellin given for HPLC-ELISA analysis was an average of values obtained from 34 sap samples taken from different palms, the amount of variation between sap samples can be quite large so the difference is unsurprising. The agreement between the HPLC-ELISA and GC–MS results on the identity and relative abundance of the major gibberellins in oil palm exudate confirms our findings, and gives us confidence in the validity of using HPLC-ELISA to analyse gibberellins in plant sap samples.

There have been very few studies on the measurement of GAs in exudates of any plant species but there is one such study in oil palm (Otusanya and Adebona, 1985). They reported gibberellin-like activity in phloem sap of the oil palm using a barley half-seed bioassay, the levels fluctuated throughout the year from 1.5 to 45 μg of GA_3 equivalents per litre. Gibberellins from the early 13-OH and non-OH GA pathways have been detected in exudates from other species (Hoad et al., 1993; Oyama et al., 1999; Stephan et al., 1999). Hoad et al. (1993)

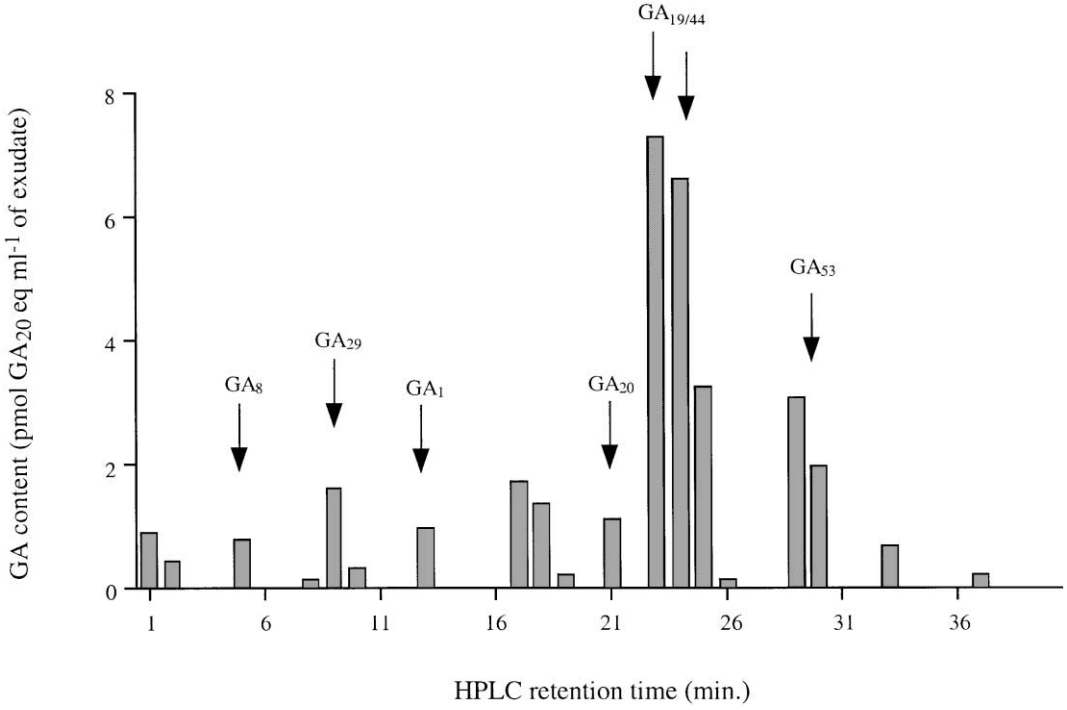

Fig. 5. A typical HPLC-ELISA analysis of a single sample of oil palm exudate to quantitate gibberellins. Individual gibberellins in a sample of exudate were separated by reverse-phase HPLC and each HPLC fraction analysed by ELISA using a 13-OH-GA specific antibody. Quantification was by comparison to standard concentrations of authentic GA_{20}, the values are not corrected for cross-reactivity, thus sample concentrations are expressed as GA_{20} equivalents. Each arrow marks the retention time of a gibberellin standard.

identified GAs in the phloem sap of white lupin at concentrations between 1 and 50 ng ml^{-1}. The concentrations reported by both Hoad et al. (1993) and Otusanya and Adebona (1985) are in the same range as the concentration of GAs in our study (ca. 14 ng ml^{-1}). It would appear from our results that the main translocatable forms of gibberellins in the palms are precursor types, ie. $GA_{53,44,19,20}$, and presumably once they have been transported to the inflorescences and leaves they would be converted to the active forms and so affect growth and development.

Ours is the first study to quantify individual members of the cytokinin and gibberellin families in oil palm sap. These growth regulators were measured in exudate entering pre-anthesis inflorescences, which suggests that they have a role in the growth and development of these structures as speculated by Otusanya and Adebona (1985) for gibberellins. It has been suggested that growth regulators have an effect on sex determination in the oil palm (Heslop-Harrison, 1957, 1964; Corley, 1976).

3. Experimental

3.1. General experimental procedures

Chemicals were from Sigma Chemical Co. Ltd. unless otherwise stated. Solvents were HPLC grade where possible. Water was ''polished'' grade from an Elga installation.

3.2. Antibodies

The isoprenoid cytokinin antisera (anti-[9R]*i*PA, anti-[9R]Z and anti-[9R]DHZ) were raised in New Zealand white rabbits by Dr. R.J.A. Connett for work described in Grayling (1990). They were raised to the relevant cytokinin riboside conjugated to bovine serum albumin (BSA) via the periodate-oxidised ribosyl moiety and then purified to an IgG fraction for use in ELISAs. The aromatic cytokinin antisera were kind gifts from Dr. M. Strnad, Institute of Experimental Botany, Olomouc, The Czech Republic. The monoclonal antibodies MAC 136 and MAC 182 were purchased from Professor J. MacMillan of Bristol University.

3.3. Synthetic standards and alkaline-phosphatase conjugates

Authentic isoprenoid cytokinins were from Apex Organics Ltd., Oxford, UK. Authentic aromatic cytokinins and aromatic cytokinin-alkaline phosphatase conjugates were kind gifts from Dr. M. Strnad. $GA_{8,19,29,44,53}$ were purchased from, and GA_1 was a kind gift from, Professor L. Mander of The Australian National University, Canberra. The GA_1-KLH conjugate was a kind gift from Dr. M. Beale of IARC, Long Ashton. The GA_3-BSA conjugate was prepared according to the method of Knox et al. (1987) as follows; GA_3 hemisuccinate (107 mg, gift from Dr. M. Beale) was dissolved in 500 μl of acetone. Half of this was evaporated under a stream of nitrogen and then re-dissolved in 10 ml of anhydrous dioxan. The GA_3 hemisuccinate was converted to its less polar mixed anhydride by adding tributylamine (39 μl) and *iso*butylchloroformate (21 μl) and incubating at room temperature for 20 min. To check the reaction was complete, the mixture was analysed by TLC. GA_3 was conjugated to BSA by the gradual addition at 4° C of 85 mg of BSA in 30 ml of sodium borate solution (25 mM, pH 9.0) and 20 ml dioxan. After incubation with stirring for 24 h at 4° C, the solution was dialysed against H_2O for 4 days and then freeze-dried.

3.4. Plant material

The oil palms used for this project were situated on a plantation in Kluang, Johor, Malaysia (Pamol Plantations Sdn. Bhd.). Clonal palms were used to simplify the experiment, since variation between palms in a clone will be minimal. At the start of the present study, the palms were 10 years old.

3.5. Exudate collection

Exudate was obtained from pre-anthesis inflorescences. In order to gain access to the inflorescence, six to seven leaves, including the subtending leaf, were removed. The method for collecting exudate from inflorescences is a modified version of the inflorescence tapping described by Tuley (1965). The inflorescence was removed at a point just below the oldest spikelets. The remaining inflorescence stalk was carved into a 'v'-shape. A stainless steel spigot (ca. 3 cm internal diameter by 16 cm length) was pushed into the inflorescence just below the 'v'-shape so that any exudate would flow from the cut surface and downwards through the spigot into a bottle of ethanol attached to the spigot via a piece of plastic tubing. The whole apparatus was covered with plastic to prevent insects and rainwater entering. Once the exudate had been collected the ethanol concentration was adjusted to ca. 80% (v/v), if necessary, to prevent microbial degradation, the samples were then transported back to the UK. Cytokinin and gibberellin analysis was performed on each individual sap sample and the resulting data pooled to give the average concentrations reported in Section 2.

Some degradation of cytokinin ribotides was seen but the fact that we have measured ribotides in our samples shows that this degradation is not complete. The degradation was greater in samples not collected directly into EtOH, i.e. no ribotides were present and the free bases were the most abundant cytokinins (data

not shown). In contrast, when samples were collected directly into EtOH, as was done for this study, ribotides *were* present and the ribosides were the most abundant with free bases present only at low concentrations. The collection method was the best practical solution with the resources available at the oil palm plantation. Despite the degradation, we feel the data are still valid as the only results available for oil palm exudates.

3.6. Cytokinin extraction

The sap samples were received in 80% ethanol. Tritiated [9R]*i*PA dialcohol (50 μl of 20 nM, ca. 170 Bq; synthesised using a method derived from Weiler and Spanier, 1981 and Turnbull and Hanke, 1985) was added as an internal standard to the equivalent of 1 ml of exudate. The sample was centrifuged for 10 min (2500 ***g***, room temperature) to remove suspended matter and reduced to a minimal volume in vacuo at 35 °C. The resultant liquid was made up to 5 ml with 10 mM triethylammonium acetate (TEAA), pH 7.0, and then part-purified by passage through a C_{18}-Sep Pak cartridge which had previously been primed with 10 ml of methanol followed by washing with 10 ml of aqueous TEAA. Cytokinins were eluted in 10 ml of 50% (v/v) aqueous methanol and the methanol removed in vacuo at 35 °C. The cytokinins in the exudate were separated by reverse-phase HPLC.

3.7. Cytokinin HPLC

The samples were analysed on a 5 μm ODS Dynamax Microsorb C_{18} analytical column (150 mm×4.6 mm). Cytokinins were separated using a gradient of 15 (v/v) to 37% (v/v) MeOH/0.2 mM TEAA in 15 min, then isocratically at 37% for a further 25 min at a flow rate of 1.5 ml/min. Cytokinins were detected at 268 nm. Fractions were collected at one minute intervals and evaporated to dryness at 35 °C in a centrifugal evaporator, then re-dissolved in H_2O before analysis by ELISA. A mixture of cytokinin standards was run before and after each sample to determine retention times of individual cytokinins.

3.8. Cytokinin ELISA

Each HPLC fraction was analysed by three cytokinin ELISAs (one for each family of isoprenoid cytokinin; *i*PA-types, Z-types and DHZ-types) based on that published by Strnad et al. (1992). For this method the IgG fraction was prepared from the antisera by ammonium sulphate precipitation and cytokinin-alkaline phosphatase conjugates were prepared by periodate oxidation of the ribosyl moiety of the cytokinin. The first 24 wells were used for blank controls and for duplicates of authentic cytokinin standards in a logarithmic concentration series from 0 to 1280fM 50 μl^{-1}. The remaining 72 wells were used for duplicated samples of HPLC fractions. Polystyrene 96-well immunosorb plates were coated with the antiserum appropriate to the assay being performed (150 μl of 1:1000 dilution in 50 mM $NaHCO_3$, pH9.6) and incubated at 4 °C for 16 h. Excess antiserum was removed by washing three times with TBS (0.05 M tris base, 0.15 M NaCl, pH7.5) + 1 ml l^{-1} Tween 20 (T). TBS + BSA (200 μl of 0.2 g l^{-1}) was dispensed into each well to block non-specific binding and the plates incubated for 1 h at room temperature. Plates were washed three times as before. Cytokinin standards (0–1280fM/50 μl) and samples (50 μl) of HPLC fractions were dispensed into the wells. Cytokinin-alkaline phosphatase conjugates ([9R]*i*PA-AP, [9R]Z-AP, [9R]DHZ-AP; synthesised according to the method of Erlanger and Beiser, 1964 as described by Weiler, 1980) were diluted 1:5000 in TBS + BSA (0.2 g l^{-1}) and 50 μl added to each well. Plates were incubated for 1 h at room temperature then washed as before. *para*-Nitrophenylphosphate (*p*NPP, 150 μl of 1 mg ml^{-1} in $NaHCO_3$ pH 9.6), was dispensed into each well and the plates left at room temperature until the colour of the 0 fmol cytokinin standard developed to an OD of 1.0. The colour reaction was stopped with 50 μl of 5 M KOH. Optical densities were measured at 405 nm. Values obtained from ELISAs were corrected for extraction and purification losses according to the percentage recovery of the tritiated [9R]*i*PA-diol included in the sample extraction procedure. Recoveries were consistently between 60 and 80%. No correction was made for differences in cross-reactivity, e.g. between [9R]Z and Z using the antibody raised against [9R]Z. Concentrations of individual compounds were calculated by adding together the values, from the corrected ELISA data, which co-eluted with the standard compound on HPLC after subtracting any background value.

3.9. Gibberellin extraction

Samples for GC–MS and SIM were prepared and analysed according to the method of Appleford and Lenton (1991). Briefly, the following tritiated GA standards were added to the equivalent of 15 ml of exudate; [1,2-$^3H_2$]GA_1 (1.65 Tbq $mmol^{-1}$), [1,2-$^3H_2$]GA_4 (1.11 Tbq $mmol^{-1}$), [1,2,3-$^3H_3$]GA_{20} (1.41 Tbq $mmol^{-1}$), [2,3-$^3H_2$]GA_9 (1.74 Tbq $mmol^{-1}$), the ethanol in the sample was evaporated in vacuo and the sample vacuum filtered. The pH was adjusted to 3.0 with acidified H_2O and the sample partitioned first against ethyl acetate then against 5% sodium bicarbonate and H_2O. The pH was adjusted again to 3.0 and the sample re-partitioned against ethyl acetate, vacuum filtered and evaporated almost to dryness in vacuo before being completely dried by vacuum desiccation. The sample was dissolved

in MeOH, the volume increased with H_2O and pH adjusted to 10 with 0.5 M NaOH. The sample was passed through a QAE sephadex A-25 anion exchange column (Pharmacia LKB Technology, Uppsala, Sweden) pre-equilibrated with 0.5 M sodium formate and washed with H_2O. GAs were eluted with 0.2 M formic acid and immediately applied to a pre-equilibrated C_{18} Sep-Pak cartridge [Millipore (Waters), Watford, UK], which was washed with 2 mM acetic acid and the GAs eluted with MeOH:acetic acid (4:1, v/v) and evaporated to dryness in vacuo. GAs were separated by reverse-phase HPLC (Kontron Instruments Ltd., Herts., UK) using a 4.9 mm×250 mm Spherisorb 5-octadecyl siloxane (ODS) 2 column (Hichrome Ltd., Reading, UK) and a linear gradient of increasing MeOH in 2 mM acetic acid (28 to 100% MeOH in 40 min) at a flow rate of 1 ml min^{-1}. Forty 1 ml fractions were collected and aliquots removed for scintillation counting to locate GAs and check recoveries.

3.10. Gibberellin GC–MS

Grouped fractions, based on the location of tritiated GAs, were combined and reduced to dryness, re-dissolved in MeOH, methylated with diazomethane and reduced to dryness in vacuo. Samples were dissolved in ethyl acetate and H_2O added and mixed, the ethyl acetate phase was passed through an aminopropyl anion exchange column. The sample was partitioned twice more and the ethyl acetate phases passed down the column. The eluates were evaporated to dryness in vacuo. Dried fractions were trimethylsilylated in *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (3 μl) at 100 °C for 30 min. and the derivatised samples analysed using a Kratos MS80 RFA GC-MS system (Kratos Analytical, Manchester, UK).

3.11. Gibberellin GC–SIM

In addition to the tritiated standards, the following deuterated internal standards were added for SIM, [17-^{13}C]GA_8, [17-$^2H_2$]GA_1, [17-$^2H_2$]GA_{19}, [17-$^2H_2$]GA_{20}. Samples were analysed using a Hewlett-Packard 5890 gas chromatograph coupled to a HP5970 mass selective detector (Hewlett-Packard, Winnersh, UK).

3.12. Gibberellin ELISA

Preparation of exudate for ELISA analysis was similar to that for cytokinin extraction except for; the internal recovery marker was approximately 50,000 dpm of [^{3}H]GA_{20} (1.41 Tbq $mmol^{-1}$, gift from Dr. M. Beale of IARC, Long Ashton), the sample was taken up in 2 mM acetic acid prior to C_{18}-Sep Pak purification, GA detection during reverse-phase HPLC (using the same column as detailed for cytokinins) was at 254 nm and the gradient used for HPLC separation was linear from 28 to 100% MeOH in 2 mM acetic acid in 40 min with a flow rate of 1.5 ml/min.

Ninety-six-well immunoassay plates were coated with 100 μl of 0.1 μg ml^{-1} GA_3-BSA conjugate (for the assay using the monoclonal antibody MAC 136) or 100 μl of 0.1 μg ml^{-1} GA_1-keyhole limpet haemocyanin (KLH) conjugate (for the assay using the monoclonal antibody MAC 182) in 50 mM $NaHCO_3$, pH 9.6, and incubated at 4 °C for 16 h. The conjugates were removed by washing three times with TBS + T, pH 7.5. Duplicate samples from HPLC fractions (50 μl) were dispensed into the wells and duplicate authentic gibberellins included on each plate in concentrations ranging from 0.098 to 100 pmol 50 μl^{-1}; GA_{20} for the MAC 136 assay and GA_1 for the MAC 182 assay. Fifty microlitres of gibberellin antibody MAC 136 (1:10) or MAC 182 (1:10,000) diluted in 2× (TBS + T + 10 g l^{-1} BSA) was dispensed into each well, initiating the competition between gibberellin bound to the wall and free gibberellin in the sample or standard for the gibberellin antibody. Plates were incubated at room temperature for 2 h. Samples, standards and antibodies were removed by washing as before. Anti-rat IgG–alkaline phosphatase conjugate (100 μl) diluted 1:500 in TBS + T was dispensed into each well. Plates were incubated at room temperature for 1 h. The conjugate was removed by washing as before. *p*NPP (150 μl at 1 mg ml^{-1} in 50 mM $NaHCO_3$, pH 9.6) was dispensed into each well. The colour reaction was stopped by adding 50 μl of 5 M KOH. Optical densities were measured at 405 nm. Concentrations of gibberellin in individual HPLC fractions obtained by ELISA were corrected according to the recovery of [^{3}H]GA_{20} (generally 60–80%) and concentrations of individual gibberellins were calculated as for cytokinins.

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