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Identification of Cytokinins in Young Wheat Spikes (*Triticum aestivum* cv. Chinese Spring)

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Abstract. Cytokinins in young wheat (*Triticum aestivum* cv. Chinese spring) spikes (2–15 mm) were isolated by immunoaffinity chromatography. High-performance liquid chromatography–radioimmunoassay and gas chromatography–mass spectrometry indicated that major cytokinins present were *trans*-zeatin, ribosyl-*trans*-zeatin, ribosyldihydrozeatin *trans*-zeatin-9-glucoside, and the glucosides of *trans*-zeatin, ribosyl-*trans*-zeatin, and *trans*-zeatin-9-glucoside. Dihydrozeatin, *iso*-pentenyladenosine, and *iso*-pentenyladenine were also present but at lower concentrations.

Cytokinins are believed to mediate some of the processes that occur during inflorescence and floret development in a number of plants (Skoog and Armstrong 1970, Andonova et al. 1982). Presumably they also play a role in the development of the wheat spike, but as yet no detailed information is available as to the nature of the cytokinins present or of the dynamics of cytokinin change. Factors such as day length, temperature, and vernalization are known to affect differentiation of the spike and to influence its subsequent growth (Ahrens and Loomis 1963, Levy and Peterson 1972, Wall and Cartwright 1974). It seems likely that terminal spikelet differentiation, determination of spikelet number, determination of floret number, and seed development could all be influenced by endogenous cytokinin levels (Michaels and Beringer 1980). In-

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deed, external application of cytokinins (Lyubarskaya et al. 1982) may enhance grain yield but by still undefined mechanisms.

Gardner et al. (1985) recently described the early morphological changes associated with growth and differentiation of spikelets and florets in wheat. With the availability of this information and with recent advances in the technology of cytokinin analysis (Weiler 1980, MacDonald et al. 1981, Badenoch-Jones et al. 1984, MacDonald and Morris 1985), it is now possible to make meaningful measurements of these hormones in small amounts (50–100 mg) of spike tissue and determine whether there are correlations between type and level of cytokinin present and stage of spike differentiation.

The presence of the cytokinins *trans*-zeatin (Z), ribosyl-*trans*-zeatin (ZR), ribosyldihydrozeatin (DZR), *trans*-zeatin-9-glucoside (Z9G), dihydrozeatin (DZ), *iso*-pentenyladenosine (iPA), *iso*-pentenyl adenine (iP), and the 4′-O-glucosides of Z, ZR, and Z9G in young wheat spikes has now been confirmed by immunoaffinity chromatography, HPLC-RIA, and GC-MS.

Materials and Methods

Plant Materials

Wheat (*Triticum aestivum* cv. Chinese spring) seeds were surface-sterilized (5% calcium hypochlorite for 5 min), washed thoroughly, and germinated in aerated Hoagland's solution (24 h at 25°C). They were then vernalized for 21 days at 8°C with 8-h light periods in silica sand moistened with Hoagland's solution. Uniform, vigorous seedlings (5–8 cm) were selected, washed briefly with tap water, and grown in vermiculite (14-h photoperiod at 15°C for the first 2 weeks, 20°C for the third week, and thereafter 25°C until harvest). Spikes (the inflorescence above the peduncle, size range 2–15 mm long) were dissected and frozen immediately in liquid nitrogen for analysis.

Cytokinin Extraction and Purification on Octadecylsilica

The extraction protocol was modified from that of MacDonald et al. (1985) with all operations being conducted in silanized glassware or in plasticware. In all cases, recoveries (which ranged between 60% and 70%) were estimated by the addition of [^{3}H]cytokinin trialcohols.

Frozen tissue (0.1–1.0 g) was homogenized for 30 sec (Polytron, Brinkmann Instruments, Westbury, NY) in ice-cold methanol (4–6 ml) containing 100 mg sodium diethyldithiocarbamate as antioxidant and [^{3}H]iPA trialcohol (5 nCi, 2.7 Ci/mmol; MacDonald et al., 1981) as internal standard. After centrifugation (20,000*g*, 15 min, 2°C), the supernatant was concentrated to approximately 1.0 ml under nitrogen and was diluted with nine volumes of ammonium acetate buffer (40 mM, pH 6.5) containing sodium diethyldithiocarbamate (10 mM) and 2-mercaptoethanol (30 mM). Samples were then incubated (20 min) with wheat germ acid phosphatase (0.04 U/ml, Sigma, P3627) to dephosphorylate cyto-

kinin-5′-phosphates. This phosphatase has no detectable nucleoside activity and so will not convert ZR to Z.

The bulk of impurities in the extract were removed by passage through a DEAE-cellulose column (Whatman, DE-52, 1.5 × 10 cm, preconditioned as described in MacDonald and Morris (1985)). Cytokinins were then adsorbed onto a column of octadecylsilica (0.5 × 1.5 cm preconditioned as described in MacDonald and Morris (1985)), which was washed with ammonium acetate buffer (40 mM, pH 6.5; 10 vol), and eluted with methanol (4 vol). The eluates were dried *in vacuo* and dissolved in 0.1 ml of a mixture of methanol and triethylamine acetate buffer (40 mM, pH 3.5) (1:1, v/v) for HPLC.

Cytokinin Fractionation and Quantification via HPLC-RIA

High-performance liquid chromatography was as described previously (MacDonald et al. 1981) with minor modifications. Samples were applied to an analytical octadecylsilica column (250 × 4.6 mm, 5 μm particle size, Beckman Ultrasphere). Two elution programs were employed, at a flow rate of 1 ml/min. In the first (gradient I), the initial eluant was triethylammonium acetate (40 mM, pH 3.35) containing 10% acetonitrile:methanol (1:1, v/v). After 1 min, the concentration of organic phase was raised to 18% over 19 min and then to 70% over 23 min. Gradient II employed the same aqueous phase but substituted acetonitrile as the organic phase. The acetonitrile concentration was increased from 10% initially to 15% over 15 min and then to 35% over a further 15 min. Absorbance of the eluate was monitored at 254 nm, and fractions (0.5 ml) were collected for RIA. Radioimmunoassays were performed as described in MacDonald et al. (1981).

Immunoaffinity Purification

Three monoclonal antibodies (clones 16, 10, and 12) having high affinities for ZR, DZR, and iPA, respectively (Trione et al. 1985, 1987), were immobilized to CNBr-activated cellulose (Cuatracasas 1970), giving immunoaffinity matrices capable of binding at least 250 ng/ml each of iPA and ZR (MacDonald and Morris 1985). Excess reactive sites were deactivated by treatment with ethanolamine hydrochloride (pH 8.0). Individual antibody-cellulose conjugates were mixed in suitable proportions to prepare immunoaffinity columns.

Cytokinin-containing plant extracts were processed through DEAE-cellulose and octadecylsilica as described above, dissolved in methanol (0.1 ml), diluted with phosphate-buffered saline (10 ml; 0.14 M NaCl, 0.02 M K_2HPO_4, pH 7.2), and passed through an anticytokinin immunoaffinity column at 37°C (MacDonald and Morris 1985). Columns were washed with PBS (4 vol), PBS containing 0.5 M NaCl and 2% v/v DMSO (3 vol), and water (4 vol). Cytokinins were then eluted with methanol.

Cytokinin side-chain glucosides have little affinity for these immunoaffinity columns and were not retained. After passage of the crude extract through an

immunoaffinity column to remove free cytokinins, the 4′-O-glucosides that were not retained were absorbed onto octadecylsilica, eluted with methanol, and hydrolyzed with β-glucosidase (30 units, Sigma Chemical Co.) for 16 h at 37°C. The resulting free cytokinin bases and nucleosides were collected by immunoaffinity chromatography and analyzed by HPLC-RIA.

Gas Chromatography–Mass Spectrometry

Cytokinins in purified fractions were converted into their permethyl derivatives by on-column methylation with trimethylanilinium hydroxide in methanol (1 μl sample plus 1 μl Methelute, Pierce Chemical Co.) and admitted to the mass spectrometer via a fused silica capillary column (0.25 mm i.d. × 15 m) coated with DB-1 (0.25 μm, J&W Scientific Co.) and employing helium (40 cm/sec) as carrier gas. The injector had a quartz injection liner and was split at a 30:1 ratio. Column parameters were: inlet, 275°C; column program, 70°C for 2 min, to 200°C over 25 min, to 300°C over 10 min. Spectra were acquired at 70 eV on a Finnegan model 4023 quadrupole spectrometer.

Results

Cytokinin Concentration by Solid-Phase Absorption on Octadecylsilica

A pooled sample of wheat spikes ranging in length from 2 to 15 mm was extracted, and the extract was treated with phosphatase. It was passed over DEAE-cellulose, and the cytokinins were collected by solid-phase adsorption onto octadecylsilica (Morris et al. 1976) and then fractionated by HPLC. Individual HPLC fractions were assayed by RIA using polyclonal antibodies raised against iPA, DZR, and ZR protein conjugates. The results are illustrated in Fig. 1.

Large amounts of noncytokinin UV-absorbing impurities were present (Fig. 1A). Extremely large amounts of impurities can alter cytokinin retention times on HPLC (Morris 1985) but did not do so here. Assay with anti-ZR antibody (Fig. 1B) revealed cytokinin cross-reactive compounds having retention times identical to those of authentic Z9G (18.3 min), ZR (30.3 min), and DZR (32.0 min). Radioimmunoassays using anti-iPA antibody revealed peaks corresponding to iPA (39.8 min) and iP (42.2 min). In addition, the anti-iPA antibody (but not anti-ZR antibody) cross-reacted with a compound that had a retention time of 35.5 min, corresponding to no known cytokinin standard. The biological activity of this compound was not determined, and its identity remains to be established. Assay with anti-DZR antibody (Fig. 1C) confirmed that the material at 32.0 min had immunological properties very similar to that of authentic DZR. Smaller amounts of putative DZ were also present (26.3 min).

Purification of Cytokinins by Immunoaffinity Chromatography

To be able to confirm cytokinin identities by mass spectrometry, a large sample

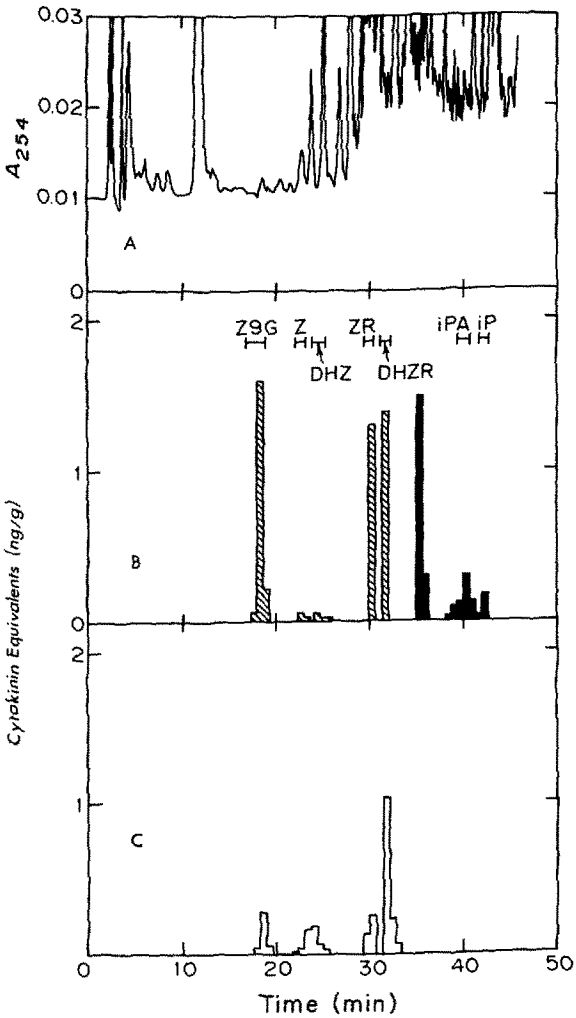

Fig. 1. HPLC-RIA of wheat spike cytokinins purified on octadecylsilica. Wheat spikes (330 mg f.w.) were processed as described over octadecylsilica and fractionated by HPLC using gradient I. Individual fractions were subjected to RIA using polyclonal antibodies directed either against ZR, DZR, or iPA. (A) Absorbance at 254 nm. (B) RIA using anti-ZR antibody (0–35 min) and anti-iPA antibody (35.5 min to end). (C) RIA using anti-DHZR antibody. Retention times of cytokinin standards are indicated by horizontal bars in Fig. 1B.

(17.4 g, f.w.) of wheat spikes having a different size distribution from those used in the first experiments was extracted and purified by immunoaffinity chromatography and analyzed by HPLC-RIA. The cytolinin complement was identical to that observed using adsorption on octadecylsilica but was now purified to near-homogeneity (Fig. 2A). Thus, some peaks were sufficiently pure to allow comparison of their UV-absorbing and RIA properties. The ratio of the amount of putative ZR (19.7 min) measured by integration of A_{254} to that determined by RIA was 0.89, indicating that the material was almost homogeneous. It was present at ~1.2 ng/g fresh weight. The major cytokinin was Z9G (1.5 ng/g) together with smaller amounts of Z, DZR, and iPA. Even though the chromatographic conditions and solvents used in the experiments outlined in Fig. 2 were different from those in Fig. 1, the retention times of putative cytokinin peaks agreed exactly with those of standards.

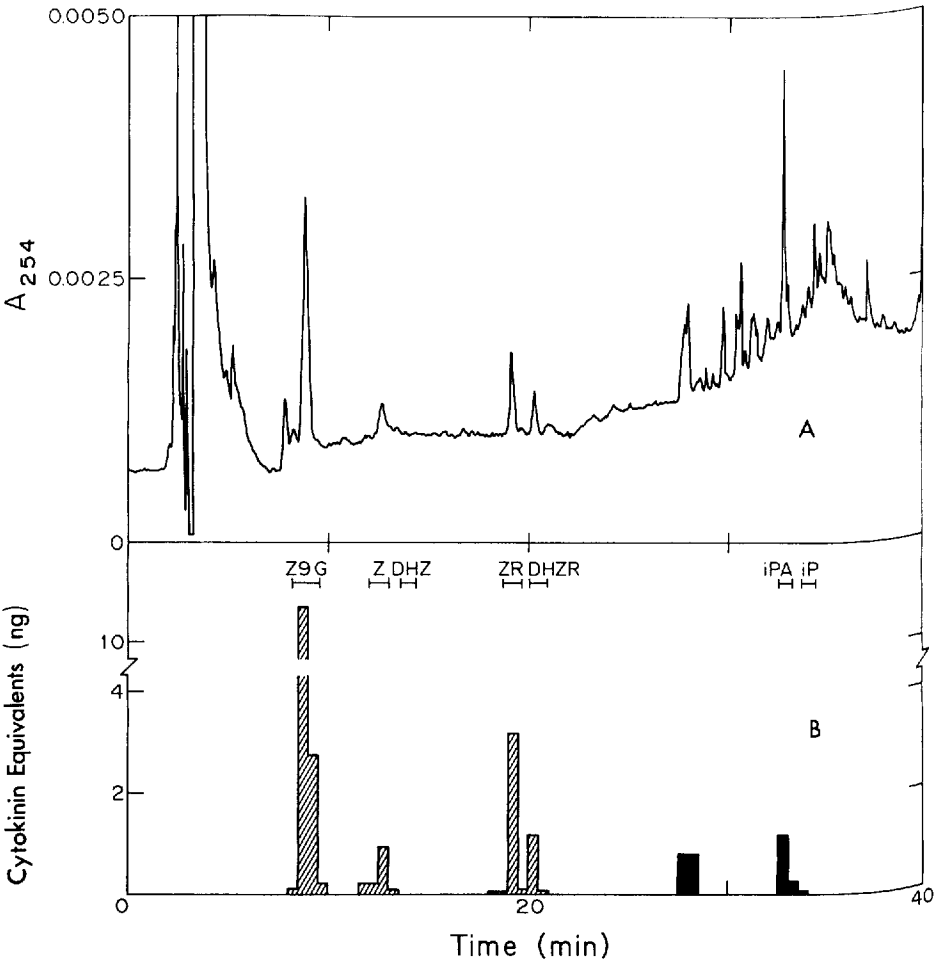

Fig. 2. Preparative scale immunoaffinity-HPLC-RIA of cytokinins from wheat spikes. A large sample of spikes (17.4 g f.w.) was processed by immunoaffinity chromatography and fractionated by HPLC using gradient II. Fractions were subjected to RIA as in Fig. 1. **(A)** Absorbance at 254 nm. **(B)** RIA using anti-ZR antibody (0–28 min) and anti-iPA antibody (28.5 min to end). Retention times of cytokinin standards are indicated by horizontal bars in Fig. 2B.

Chromatography of Cytokinin Glucosides

Cytokinin 4′-O-glucosides, such as ZR 4′-O-glucoside, neither cross-react significantly with antibodies raised against conjugates of free cytokinins nor are retained on immunoaffinity columns constructed from such antibodies. To determine whether side-chain glucosides were present, the nonadsorbed material passing through the immunoaffinity column was collected on octadecylsilica; any cytokinin glucosides were eluted with methanol, and, after β-glucosidase treatment, any free cytokinins that resulted were collected on immunoaffinity columns and fractionated by HPLC. The results of this experiment are illus-

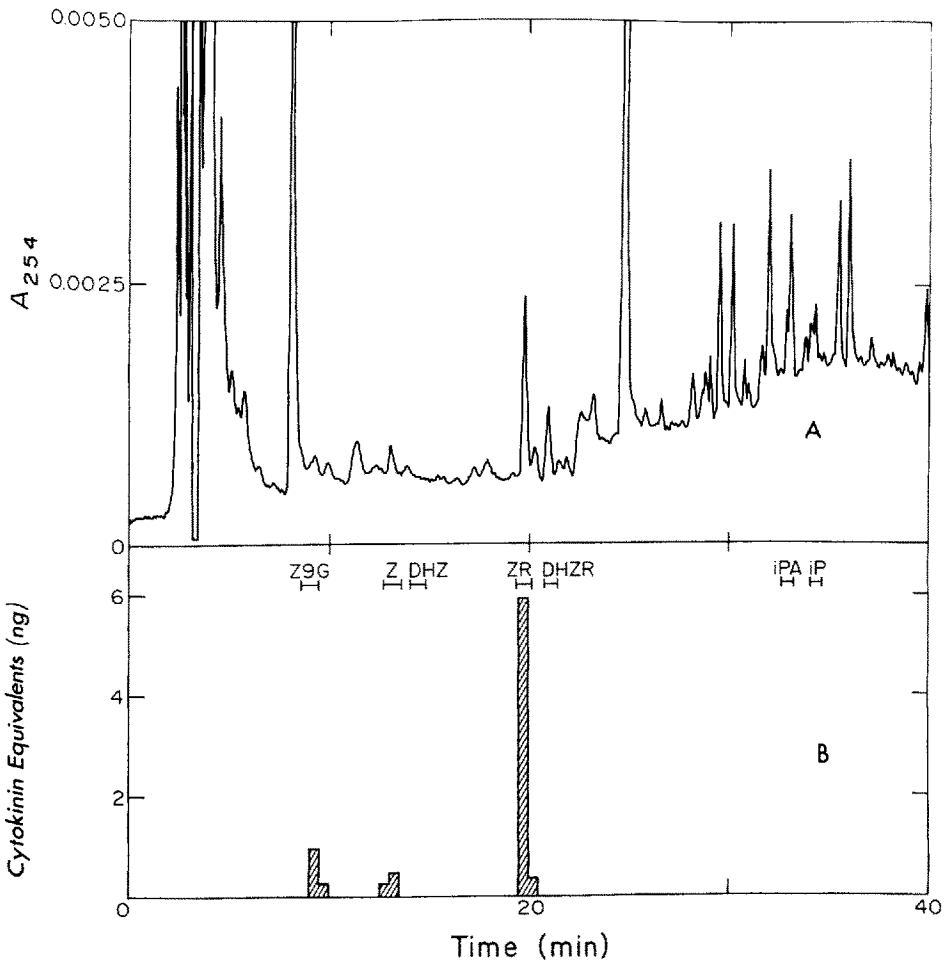

Fig. 3. HPLC of cytokinins derived from wheat spike glucosides. Glucosides that were not retained on the immunoaffinity column (in the experiment described in Fig. 2) were hydrolyzed, subjected to immunoaffinity chromatography, and fractionated by HPLC using gradient II. Individual fractions were then assayed by RIA using anti-ZR antibody. **(A)** Absorbance at 254 nm. **(B)** RIA using anti-ZR antibody (0–28 min) and anti-iPA antibody (28.5 min to end). Retention times of standards indicated by horizontal bars in Fig. 3B.

trated in Fig. 3. Again, almost pure cytokinin fractions were obtained, derived in this case from glucosides present in the original extract. Ribosylzeatin was present together with smaller amounts of Z9G and Z. As expected, neither iPA nor iP was observed.

Confirmation of Cytokinin Identity by Mass Spectrometry

Although the retention times and cross-reactions of cytokinin peaks are all

Table 1. Major fragment ions and relative abundance of permethylated zeatin-9-glucoside, zeatin, ribosylzeatin, and ribosyldihydrozeatin in the wheat spike as determined by mass spectrometry (selective ion current monitoring).

m/z	Percent abundance	Percent abundance (standard)
Zeatin-9-glucoside		
174	12.5	11.6 (from seeds)
216	100.0	100.0
392	0.3	1.0
434	4.2	5.7
465	0.1	0.1
Zeatin		
162	12.3	11.9
188	3.7	2.5
216	0.5	0.1
230	100.0	100.0
231	0.1	0.1
261	0.1	0.1
Ribosylzeatin		
174	8.7	9.6 (from seeds)
202	3.6	3.2
216	100.0	100.0
246	0.5	1.4
272	0.7	0.8
348	0.6	0.8
376	0.1	0.2
390	5.4	6.5
421	0.1	0.1
Ribosyldihydrozeatin		
250	100.0	100.0
278	15.1	43.3
336	8.9	11.9
392	3.5	8.7
408	2.0	3.8
423	1.8	1.0

consistent with the structural assignments given, independent confirmation was sought by mass spectrometry. Individual cytokinin-containing fractions resulting from HPLC were derivatized by on-column methylation, fractionated by GLC, and subjected to mass spectrometry. Table 1 summarizes the fragment ions that originate from putative Z9G, Z, ZR, and DZR isolated from wheat spikes. Fragment ion masses and abundances of the fragment ions corresponded to those of standards. In addition, the GLC retention times of the permethylated derivatives corresponded exactly to those of standards (data not shown).

Discussion

In summary, application of solid-phase enrichment either on octadecyl silica or

on a highly specific immunoaffinity matrix followed by HPLC-RIA has allowed identification of cytokinins in young wheat spikes. Cytokinins present include Z, ZR, Z9G, DZ, DZR, iPA, and iP. Glucosides of ZR, Z, and Z9G were also present, and because they were not atttracted to immunoaffinity columns and did not cross-react with available antibodies, they were likely to be 4'-O-glucosides in which the glucose moiety blocks interaction with the antibody. Confirmation of glucoside structures awaits future mass spectrometry. The identities of the free cytokinins—Z, ZR, Z9G, and DZR—were confirmed by mass spectrometry.

Because young wheat spikes contain at most 5 ng/g of Z9G and lower amounts of other cytokinins, and because the detection limits of bioassay or GC-MS in our hands are approximately 10 ng, collecting sufficient immature wheat spike material for analysis by these methods would have been prohibitively time-consuming. The solid-phase extraction procedure used in association with HPLC-RIA has a detection limit of less than 100 pg and thus allowed analyses to be performed on 100-mg aliquots of tissue which required collection of only 20–30 spikes.

Quantitation was also relatively straightforward by either the conventional solid-phase adsorption followed by HPLC-RIA or by immunoaffinity absorption and HPLC-RIA. Some HPLC peaks (Z and ZR) following immunoaffinity chromatography were sufficiently homogeneous to allow quantitation by direct integration of the A_{254} trace. The results agreed well with parallel RIA quantitation. However, the detection limit by A_{254} measurements was only about 1–5 ng. For small samples, the higher sensitivity of RIA was necessary. The method does not have the inherent precision of isotope dilution mass spectrometry (Summons et al. 1979, Scott and Horgan 1980), but it does allow rapid and moderately accurate analyses to be performed on small plant tissue samples.

There have been several reports of the cytokinins in inflorescences of other plants (Donchev 1982, Leonard and Kinet 1982, Saha et al. 1985). Bioassays indicated that Z, ZR, and cytokinin glucosides were present. To our knowledge, the data here represent the first well-documented profile of the nature, confirmation, and approximate levels of cytokinins in the young inflorescence of a grass.

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