Release of Active Cytokinin by a (β-Glucosidase Localized to the Maize Root Meristem

Bretislav Brzobohaty, Ian Moore, Peter Kristoffersen, Laszlo Bako, Narciso Campos,* Jeff Schell, Klaus Palme†

A β-glucoside encoded by a cloned *Zea* mays complementary DNA *(Zm-p60.* 1) cleaved the biologically inactive hormone conjugates cytokinin-O-glucosides and kinetin-N3-glucoside, releasing active cytokinin. Tobacco protoplasts that transiently expressed *Zm-p60.1* could use the inactive cytokinin glucosides to initiate cell division. The ability of protoplasts to sustain growth inresponse to cytokinin glucosides persisted indefinitely after the likely disappearance of the expression vector. In the roots of maize seedlings, Zm-p60.1 was localized to the meristematic cells and may function in vivo to supply the developing maize embryo with active cytokinin.

Inactive phytohormone conjugates are abundant in plant tissues *(1,* 2), but their normal biological functions remain obscure. The apparent physiological activity of any particular conjugate correlates with its rate of hydrolysis in plant tissues *(1, 2).* The bacterial pathogen *Agrobacterium rhizogenes* may promote abnormal development in plants through the action of its *rolC* oncogene, which encodes a β-glucosidase that can release free cytokinin from inactive conjugates (3). Despite this demonstra-tion that phytohormone conjugates can be exploited to modify plant development, we lack knowledge about hydrolases in normal plants that are capable of releasing phytohormones (4).

In the young seedling, the appearance of free forms of auxin, cytokinin, and gibberellin is correlated with a marked decrease in the abundance of their conjugates in the endosperm (4). In maize seedlings, auxin and cytokinin appear to be transported as conjugates from the endosperm to the embryo, where they are activated by hydrolysis to the free phytohormones (5, 6). Here, it is shown that cytokinins may be released in this system by a β-glucosidase, Zm-p60, recently purified from maize seedlings (7).

A partial amino acid sequence of Zm-p60 facilitated isolation of a complementary DNA (cDNA) clone, *Zm-p60.I* (Fig. 1) (8). The deduced amino acid sequence of the protein encoded by the *Zm-p60.1* cDNA matched 110 of the 116 known amino acid residues of Zm-p60 (Fig. 1). The existence of two or three Zm-p60 isoforms in maize seedlings (7) may account for the differences in amino acid composition between Zm-p60 and Zm-p60.1. Maximum amino acid sequence identity was observed between Zm-p60.1 and a family of glycosidases present in both prokaryotes (39%, *Caldocellum saccharolyticum)* and eukaryotes (46%, *Trifolium repens).* The importance of the similarities between preliminary Zm-p60 peptide sequences and rolC, discussed in (7), remains to be determined as further peptide sequencing and elucidation of the complete Zm-p60.1 sequence revealed no significant overall sequence similarity to the *rolC* product of *A. rhizogenes.* It appears, then, that the Zm-p60 family and rolC have distinct phylogenies despite similarities in their enzymatic activities. Northern (RNA) hybridization analysis of maize seedlings revealed a single mRNA species of approximately the same size as that of the cDNA species. The abundance of this transcript was found to be tightly regulated during the maize life cycle, with the largest amounts found in the developing embryo (Fig. 2).

Recombinant Zm-p60.1 expressed in *Escherichia coli* (9) showed exactly the same restricted substrate specificity reported for Zm-p60 (10). Zm-p60 has no or very low activity on substrates from several groups of naturally occurring glycosides, including disaccharides involved in plant cell wall degradation (cellobiose and laminiaribiose), phenolic glucosides (salicin), and flavonoid glycosides (rutin) (7). Prompted by the presence of Zm-p60 in young seedlings and by its original identification through photoaffinity labeling (7) with an azido derivative of indoleacetic acid (IAA), we tested whether common phytohormone conjugates could be hydrolyzed. We were not able to demonstrate hydrolysis of IAA-glucose-ester by Zm-p60, which is consistent with its relative specificity for the glycosidic bond (7, *11).* However, cytokinin-O-glucosides and kinetin-N3-glucoside, but not cytokinin-N7-glucosides or cytokinin-N9-glucosides, were hydrolyzed by both recombinant Zm-p60.1 (Fig. 3) and Zm-p60 (10). In plant tissues, O-glucosides are the major mobilizable conjugated form of cytokinin from which active cytokinins can be released by endogenous hydrolases (1).

The interesting observation that the protein encoded by the *rolC* gene, but not Zm-p60.1, is able to hydrolyze zeatin-N7-glucosides and zeatin-N9-glucosides can be rationalized with regard to the physiological roles proposed for each enzyme; cytokinin glucosylation on N7 and N9 is considered to be one of the mechanisms of irreversible cytokinin inactivation in plant cells (1), and thus Zm-p60.1, an endogenous plant enzyme, does not attack substances that

Fig. 1. Amino acid sequence of the *Zm-p60.1* cDNA. The deduced sequence is denoted by the single-letter code (*19),* and the sequenced Zm-p60 tryptic peptides are indicated above the sequence. The NH_2-terminal sequence of a β-glucosidase purified from maize by Esen *(21)* is identical to resi-dues 55 to 74 of Zm-p60.1; however, the NH_2-terminus we determined for mature Zm-p60 isolated from coleoptiles corresponds to Ser^{60}. The significance of this is unclear, and as no further sequence data were provided by Esen, it is not possible to decide how similar these two β-glucosidases are. The nucleotide sequence of *Zm-p60.1* cDNA has been submitted to the European Molecular Biology Laboratory (EMBL) Data Library under accession number X74217.

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   1  MAPLLAAAMN HAAAHPGLRS HLVGPNNESF SRHHLPSSSP QSSKRRCNLS
                S QNGVQMLSP
  51  FTTRSARVGS QNGVQMLSPS EIPQRDWFPS DFTFGAATSA YQIEGAWNED
                        ILD GSNSDIGANS YHMYK
 101  GKGESNWDHF CHNHPERILD GSNSDIGANS YHMYKTDVRL LKEMGMDAYR
                   ELGYIQP DGIK
 151  FSISWPRILP KGTKEGGINP DGIKYYRNLI NLLLENGIEP YVTIFHWDVP
           YGGF LDK
 201  QALEEKYGGF LDKSHKSIVE DYTYFAKVCF DNFGDKVKNW LTFNEPQTFT
                   AIPGL?? AYPTGNSLVE PY
 251  SFSYGTGVFA PGRCSPGLDC AYPTGNSLVE PYTAGHNILL AHAEAVDLYN
                         V PYGTSFLDK
 301  KHYKRDDTRI GLAFDVMGRV PYGTSFLDKQ AEERSWDINL GWFLEPWRG
                              LAGSYN MLGLNYYTSR
 351  DYPFSMRSLA RERLPFFKDE QKEKLAGSYN MLGLNYYTSR FSKNIDISPN
 401  YSPVLNTDDA YASQEVNGPD GKPIGPPMGN PWIYMYPEGL KDLLMIMKNK
      YGNPPIYITE NGIGDVDTK
 451  YGNPPIYITE NGIGDVDTKE TPLPMEDALN DYKRLDYIQR HIATLKESID
                                  YGIV YVOR
 501  LGS:NVQGYFA WSLLDNFEWF AGFTERYGIV YVDRNNNCTR YMKESAKWLK

 551  QFNAAKKPSK KILTPA
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B. Brzobohaty, Max-Planck Institut fur ZOchtungsforschung, Carl-von-Linne-Weg 10, D-50829, Kain 30, Federal Republic of Germany, and Institute of Biophysics, Kralovopolska 135, CZ-61265 Brno, Czech Republic.
I. Moore, L. Bako, N. Campos, J. Schell, K. Palme, Max-Planck Institut !Or Zuchtungsforschung, D-50829, Kain 30, Federal Republic of Germany.
P. Kristoffersen, Max-Planck Institut !Or ZOchtungsforschung, Kain 30, Federal Republic of Germany, and DANISCO Biotechnology, 1 Langebrogade, DK-1001 Copenhagen, Denmark.

'Present address: Department de Bioquimica y Fisiologia, Faculdad de Quimica, Universidad de Barcelona, Marti i Franques 1, 08028 Barcelona, Spain. tTo whom correspondence should be addressed.

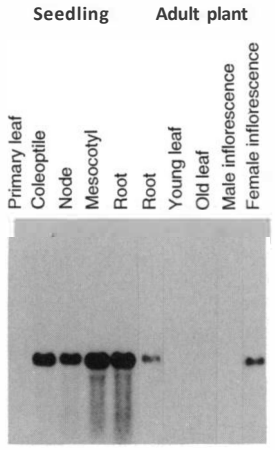

Fig. 2. Northern blot analysis. Total RNA (20 µ.g) was isolated from various tissues (22) and used for Northern blot analysis (23) with a 32P-labelled DNA probe specific for the 3' end (nucleotides 1732 to 1947) at 65ºC.

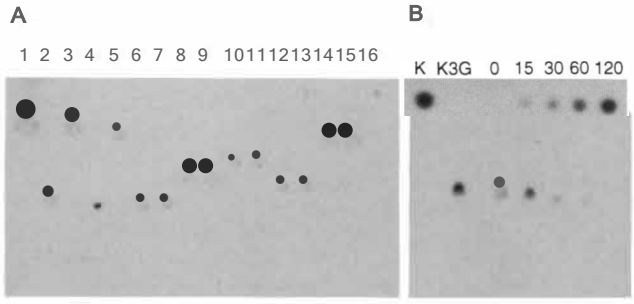

Fig. 3. Enzymatic activity of recombinant Zm-p60.1. Thin-layer chromatography (TLC) of the products of glucosidase assays was performed with recombinant Zm-p60.1 isolated from E *coli* strain BL21 (DE3) *(20)* containing the Zm-p60.1 expression plasmid pET3x::Zm-*p60.1 (9).* (**A**) Lane 1, zeatin; lanes 2 and 3, ZOG; lanes 4 and 5, zeatin-O-β-glucoside riboside; lanes 6 and 7, zeatin-7-β-glucoside; lanes 8 and 9, zeatin-9-β-glucoside; lanes 10 and 11, kinetin-3-α-glucoside; lanes 12 and 13, kinetin-3-β-glucoside; lanes 14 and 15, kinetin-9-β-glucoside; lane 16, kinetin; without (lanes 1, 2, 4, 6, 8, 10, 12, 14, and 16) and with (lanes 3, 5, 7, 9, 11, 13, and 15) 1 mU of enzyme. (**B**) Hydrolysis of K3G is more clearly seen on prolonged incubation. At the times indicated (in minutes), aliquots were withdrawn for TLC analysis. Lane K, kinetin; lane K3G, kinetin-3-β-glucoside.

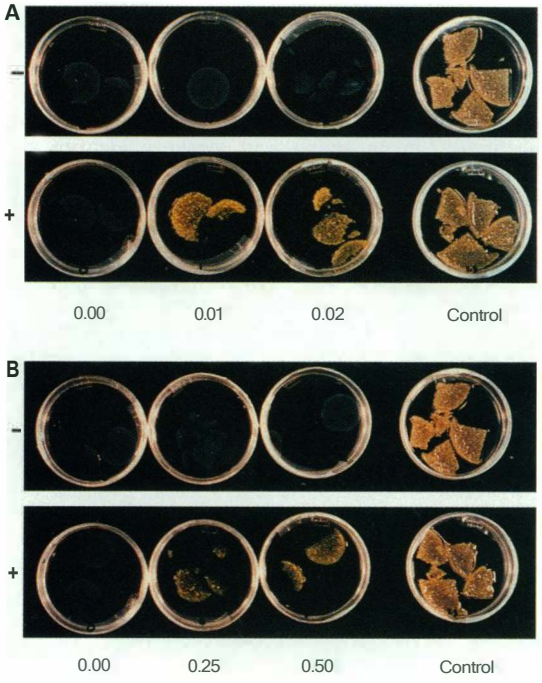

Fig. 4. Transient expression of *Zm-p60.1* in tobacco protoplasts led to the formation of calli in culture media containing cytokinin-glucosides. Protoplasts (0.8 x 10^5) transfected (*15*) with pRT101::*Zm-p60.1* DNA (+) or pRT101 (-) were plated in 1 ml of medium supplemented with ZOG (**A**) or kinetin-N3-glucoside (**B**) at the concentrations (mg/liter) indicated, cultured for 1 week in the dark, and for a further 3 weeks embedded in agarose. The control was protoplasts treated as above, except kinetin was used (0.2 mg/liter) instead of cytokinin-glucosides.

accumulate as inactivated end products under normal physiological conditions. In contrast, the rolC protein is part of the mechanism by which the phytopathogen *A. rhizogenes* subverts normal plant development to its own advantage; there is no obvious reason for the activity of such an enzyme to be restricted to those cytokinin glucosides that are normally mobilizable by the plant host. The ability to hydrolyze N7 and N 9 glucosides, which accumulate in large amounts in some plant species, increases the substrate pool for this enzyme during pathogenesis and may simultaneously remove one of the plant's means of compensating for abnormally increased cytokinin levels.

To assess the biological significance of the β-glucosidase activity observed in vitro on cytokinin-O-glucosides and kinetin-N3-glucoside, we expressed *Zm-p60.1* transiently in tobacco mesophyll protoplasts. The induction of cell division in tobacco proto-plasts is dependent on an exogenous supply of cytokinin (12, 13). Transfection with *Zm-p60.1* expressed from a cauliflower mosaic virus 35S promoter (14) caused a transient increase in the β-glucosidase activity of the protoplasts (from about 0.6 to 25 nmol mg^{-1} min^{-1} assayed on the general β-glucosidase substrate 4-itrophenyl-β-D-glucopyranoside). Protein immunoblot analysis confirmed that the β-glucosidase synthesized in the protoplasts was identical in size to the Zm-p60 isolated from maize (10). This experiment revealed that the endogenous β-glucosidase activity of tobacco protoplasts is relatively low. Figure 4 shows that transfected protoplasts that produced Zm-p60.1 were able to use the exogenously supplied zeatin-O-glucoside (ZOO) or kinetin-N3-glucoside to activate cell division whereas the control protoplasts divided only exceptionally and showed morphological changes typical of cytokinin deficiency. These observations have now been confirmed with protoplasts derived from stable transformants expressing Zm-p60.l, and in further transient expression experiments, concentra-tions as low as 0.005 mg/liter of ZOG and 0.002 mg/liter of free zeatin sufficed to pro-mote cell division (10).

Calli that arose from protoplasts transiently transfected with *Zm-p60.* 1 appeared to grow on ZOO indefinitely [more than 6 months and long after transient expression would normally be expected to have ceased (15)]. Furthermore, the frequency of micro callus formation under optimal conditions was indistinguishable from that of controls supplied with free cytokinin; this far exceeded the frequency of stable transformation expected with the use of supercoiled plasmid DNA with our transfection method [about 0.01 to 0.1% of surviving protoplasts (15, 16), with the prediction of no more than about 10 to 100 stable transformants per plate]. Nonexpressing microcalH may have been supplied with free cytokinin that was liberated into the medium from transformed cells. When tested, only 27% of microcalli appeared to obtain cytokinin in this way, which suggested that the amount of cytokinin in the medium was insufficient to explain the frequency of callus formation and the sustained growth (17).

The high frequency at which calli were formed and their indefinite growth on ZOG may have resulted from the induction of endogenous β-glucosidase activity or from

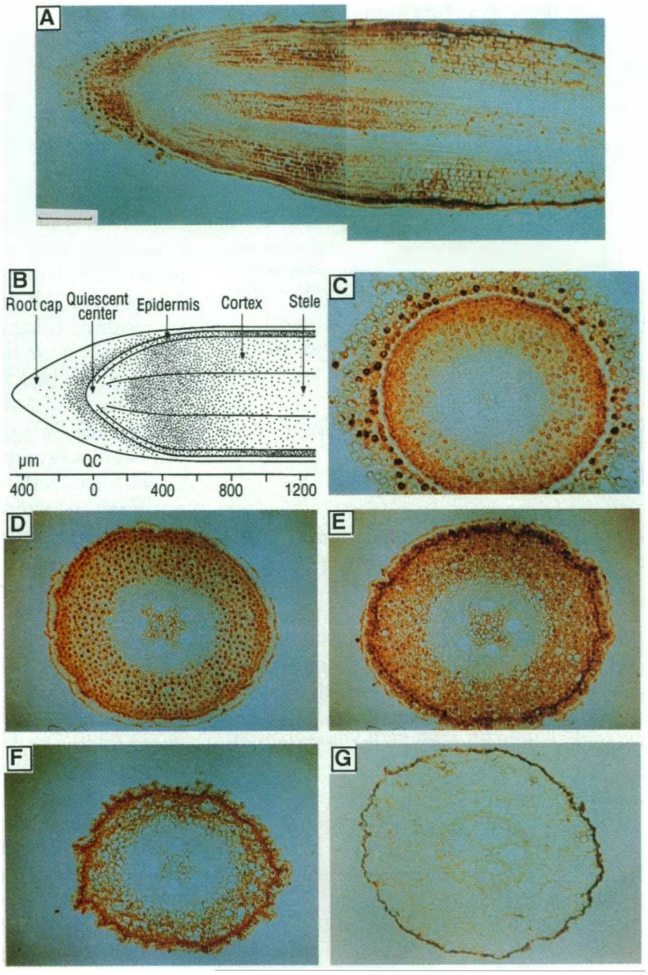

Fig. 5. Zm-p60.1 distribution in the maize seedling root. Shown are longitudinal (A) and cross sections (C to G) at various distances from the root cap boundary (rcb) stained with antisera raised against recombinant Zm-p60.1. Preimmune sera weakly stained similar sections only in the epidermis *(10)*. (A) Staining was seen in the youngest cells of the root cap and in the epidermis, cortex, and stele beyond the immediate periphery of the quiescent center. Maximum staining intensity coincided with the regions of maximum meristematic activity in each tissue [depicted schematically in (B) by stippling; based on information in (18)). The staining of the stele and cortex faded to background levels at the most distal point of the section shown here and in the quiescent center. Both staining and cell division in the epidermis extend further from the rcb than in other tissues (magnification, x 100; bar = 200 μm); (C) 100 μm from rcb (magnification, x200); (D) 500 μm from rcb (x100); (E) 1000 μm from rcb (x100); (F) 1500 μm from rcb (x100); and (G) beyond the tip (approximately 8000 μm from rcb) in the mature, mitotically inactive region of the root (x100).

the acquisition of cytokinin independence (habituation) by the microcalli after transient expression. When tested, untransformed tobacco protoplasts cultured in kinetin-containing media for various lengths of time acquired the ability to grow on ZOO (0.02 mg/liter) within 3 to 7 days (coinciding with an increase in their β-glucosidase activity) but ceased to develop when kinetin was withdrawn. Furthermore, calli regenerated on ZOO after transfection of protoplasts with Zm-p60.1 were similarly dependent on an external cytokinin source for continued growth (10). Thus, it appeared that developing calli acquired the ability to efficiently use ZOO but did not become habituated for cytokinin.

We then analyzed the distribution of Zm-p60.1 in the maize root tip relative to the regions active in cell division, growth, and differentiation. Immunocytochemical analysis of sections of 3-day-old roots (Fig. 5) revealed that Zm-p60.1 was restricted to zones of active cell division in the root tip (18). The slowly dividing quiescent center was barely stained, whereas the actively dividing meristematic cells derived from epidermal, cortical, and stele initials were all heavily stained. This distribution of Zm-p60.1 agrees with observations that cytokinin-O-glucosides are generally not cleaved in all parts of the root during transport from the endosperm (6).

The distribution and activities of Zm-p60.1 support a biochemical model proposed (6) to explain how maize embryo cells are supplied with free cytokinin. We suggest that in maize root meristems, Zm-p60 liberates free cytokinins from the exogenous supply of cytokinin-O-glucosides arriving from the endosperm and consequently maintains meristem activity.

REFERENCES AND NOTES

1. D. S. Latham and L. M. S. Palni, *Annu. Rev. Plant. Physiol.* 34, 163 (1983).
2. J. D. Cohen and R. S. Bandurski, *ibid.* 33, 403 (1982).
3. J. J. Estruch *el al., EMBO J.* 10, 2889 (1991).
4. W. Schliemann, *Naturwissenschaften* 78, 392 (1991).
5. J. Nowacki and R. S. Bandurski, *Plant Physiol.* 65, 422, (1980).
6. A. R. Smith and J. van Staden, *J. Exp. Bot.* 29, 1067 (1978).
7. N. Campos *et al., Plant* J 2, 675 (1992).
8. The Zm-p60 protein was purified (7), and tryptic peptides were sequenced in an Applied Biosystems 477A Sequenator (Darmstadt Welterstadt, Germany). Degenerated oligonucleotides 5'-TARCARATGTARAAA-3' (R: CT) and 5'-ACR,-TAR,ACR:,ATR $_2$CCR$_1$TA-3' (R$_1$: AG; R$_2$: ACGT; R$_3$: AGT) derived from residues 131 to 135 (YHMYK) and 527 to 532 (YGIVYV), respectively, were used to generate a Zm-p60-specific hybridization probe from a maize coleoptile cDNA library by the polymerase chain reaction (PCR) *(19)*. The resulting 1.1-kb DNA fragment was partially sequenced to confirm its identity and used to isolate the full-size *Zm-p60. 1* clone from the same library. Sequencing was carried out on deletion clones generated by exonuclease III digestion [S. Henikoff, *Gene* 28, 351 (1984)] with an Automated Laser Fluorescent. DNA sequencer (Pharmacia). The DNA sequences were analyzed on a VAX computer with the GCG package [J. Devereux, P. Haeberli, 0. Smithies, *Nucleic Acids Res.* 12, 387 (1984)].
9. To construct expression plasmid pET3x::Zm-p60.1, we modified the coding region of *Zm-p60. 1* by PCR and cloned it in pET3x. The plasmid pET3x was derived from pET3a (20) by deletion of the Eco RV fragment. The NH$_2$-terminal sequence of this recombinant Zm-p60.1 protein is Met-Ala-Ser, the third residue of which corresponds to the serine determined at the NH$_2$-terminus of Zm-p60. After induc, lion (with 0.5 mM isopropyl-I3-0-thiogalactopyranoside), soluble recombinant Zm-p60.1 was purified from the bacterial lysate by chromatography on a DEAE-Sepharose coll.ITYI. We analyzed P-glucosidase activity as described (7), except that a different thin-layer chromatography mobile phase was used [n-butanol:acetic acid:water (450:113:188)].
10. B. Brzobohaty *et al.,* unpublished data.
11. We found, however, that compared with IAA and glucose, IAA-glucose-ester was a particularly effective inhibitor of cytokinin glucoside hydrolysis by Zm-p60, which supports the conclusion that it is not a substrate and raises tl]e possibility of the interaction between auxin and cytokinin conjugate metabolism.
12. J. l. Nagy and P. Maliga, *Z. Pflanzenphysiol.* 78, 446 (1976).
13. Y. Meyer and R. Cooke, *Plan/a* 147, 181 (1979).
14. To express *Zm-p60.1* in tobacco protoplasts, we cloned the complete *Zm-p60. 1* coding region (nucleotides 23 to 1902) in pRT101 (15) cut by Xho I-Xba I to produce pRT101 ::Zm-p60. *1*
15. *Nicotiana tabacum* SRI protoplasts (1Cl^6) were transfected with 120 μg of plasmid DNA and diluted to between 5 x 10^4 and 10^5 starting protoplasts per milliliter as described [M. Preis, R. Tepfer, J. Schell, H.-H. Steinbiss, *Plant Cell Rep.* 7, 221 (1988)], except that the culture medium contained glucose instead of sucrose and ZOO (0.005 to 0.02 mg/liter) or kinetin-3-Φ-glucoside (K3G) (0.25 to 0.5 mg/liter) instead of kinetin.
16. l. Moore, thesis, University of Edinburgh (1989); U. Wirtz, J. Schell, A. P. Cernilofsky, *DNA* 6, 245 (1987).
17. Protoplasts were transfected with pRT101::Zm-*p60.1* DNA (p60$^+$), and, in parallel, protoplasts prepared from a kanamycin-resistant transgenic tobacco line were .transfected with pRT101 DNA (Km^r/p60-J. Aliquots of each population were cultured either as 1:1 mixtures or separately, as controls, on media with either ZOO (0.02 mg/liter) or kinetin (0.2 mg/liter). Individual calli derived from these cultures were assayed for kanamycin-resistant growth. Calli derived from the controls behaved as expected. Among the calli derived from the mixtures of Km^r/p60· and p60$^+$ protoplasts, significantly fewer kanamycin-resistant calli developed on ZOG than on kinetin (16 and 57%,

respectively; in a I test I = 7.16, $P < 0.001$), which indicates a bias in favor of $p50^+$ protoplasts on ZOG. Thus, although some protoplasts that lack the ability to use ZOG can obtain cytokinin from protoplasts that express Zm-p60.1, it is unlikely that the majority of $p60^+$ protoplasts also obtained cytokinin from a small fraction of highly expressing or stably transformed protoplasts.

18. F. A. L. Clowes, *New Phytol.* **74**, 263 (1975); K. Esau, *Anatomy of Seed Plants* (Wiley, New York, ed. 2, 1977), pp. 227--232; A. F. Lyndon, *Plant Development* (Unwin Hyman, London, 1990), pp. 23-24 and pp. 170-172.
19. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, lie; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gin; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
20. F. W. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorf, *Methods Enzymel.* 185, 60 (1990).
21. A. Esen, *Plant Physiol.* **98**, 174 (1992).
22. P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987).
23. J. Sambrook, A. Frisch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).
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