Volume 249, number 2, 401–406 FEB 07236 June 1989

Construction of a heat-inducible chimaeric gene to increase the cytokinin content in transgenic plant tissue

T. Schmülling, S. Beinsberger*, J. De Greef*, J. Schell, H. Van Onckelen* and A. Spena

MPI für Züchtungsforschung, 5000 Köln 30, FRG and **Dept of Biology, UIA, B-2610 Wilrijk, Belgium*

Received 3 April 1989; revised version received 20 April 1989

The *ipt* gene of *Agrobacterium tumefaciens* T-DNA encodes an isopentenyltransferase which causes cytokinin overproduction and developmental alterations in transformed plants. A chimaeric gene constructed by positioning the *ipt* coding region under the control of the hsp70 gene from *Drosophila melanogaster* allows heat-regulated expression in transgenic plant tissue. Heat-shock treatment of tobacco calli transgenic for the chimaeric *hsipt* gene increases the endogenous cytokinin concentration and enables these calli to grow on cytokinin-free medium. Transgenic plants regenerated from calli transformed with the *hsipt* gene and grown at normal temperature are phenotypically normal.

Heat shock; Cytokinin; DNA, recombinant; Transgenic plant

1. INTRODUCTION

Cytokinins (CK) are plant growth regulators able to induce cell division in the presence of auxin [1]. In plant tissue culture, an increased ratio of CK to auxin is used to promote shoot induction, while in the whole plant, CK applications can release lateral buds from apical dominance [2]. *A. tumefaciens* can transfer DNA segments (T-DNA), from Ti plasmids to susceptible plant cells [3]. Integration of wild type T-DNAs into the plant genome induces tumorous growth mainly through the expression of two auxin biosynthetic genes (i.e. *iaa*M and *iaa*H) and the *ipt* gene responsible for cytokinin biosynthesis [4]. The isopentenyltransferase encoded by the *ipt* gene converts 5′-AMP and isopentenylpyrophosphate into isopentenyladenosine-5-monophosphate [4]. Isopentenyladenosine-5-monophosphate is then rapidly converted to isopentenyladenosine ([9R]ip) and isopentenyladenine (ip). Hydroxylation of [9R]ip and ip generates, respectively, zeatinriboside and zeatin [4]. We have constructed a heat shock-inducible cytokinin-producing gene by positioning the *ipt* coding region under the control of the hsp70 promoter of *D. melanogaster* and have monitored the effect of its expression in transgenic tobacco calli and transgenic plants.

Correspondence address: A. Spena, MPI für Züchtungsforschung, 5000 Köln 30, FRG

Abbreviations: CK, cytokinin; [9R]ip, $N^6(\Delta^2$-isopentenyl)adenosine; ip, $N^6(\Delta^2$-isopentenyl)adenine; [9R]Z, zeatinriboside; Ab, antibody; PVP, polyvinylpyrrolidone; RIA, radioimmunoassay

2. MATERIALS AND METHODS

2.1. *Bacterial strains and cultures*

Bacterial strains and cultures have been previously described [5].

2.2. *Construction of plasmids*

Standard techniques were used for the construction of recombinant DNA plasmids [6]. Plasmid pIPT was obtained by subcloning the 1348 bp *Rsa*I fragment from bp number 8488 to 9836 from the T-DNA of the octopine Ti plasmid pTi 15955 [7] into the binary vector pPCV002 [8]. The *Rsa*I fragment of 1348 bp spans the entire coding region of the *ipt* gene, 283 bp of the 5′-flanking sequences as well as 373 bp of its 3′-flanking sequences. To obtain the chimaeric *hsipt* gene, the *hsp70* promoter from *D. melanogaster* [9] was positioned upstream of a *Bal*31-derived fragment containing the entire coding region of the *ipt* gene, 16 bp of its untranslated leader region and 373 bp

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

of its 3'-flanking sequences. The vector is designated pHSIPT (see fig.1).

All constructions were transferred to *Escherichia coli* strain SM10, then mobilized to *A. tumefaciens* strain GV3101 (pMP90RK) as previously described [8].

2.3. *Plant tissue culture and transformation*

Leaf protoplasts of *Nicotiana tabacum* cv. Petit Havana SR1 [10] were isolated from sterile shoot cultures grown on MS medium according to [11]. Transformation by protoplast cocultivation with *A. tumefaciens* GV3101 (pMP90RK) strains harbouring the different constructions, and regeneration of transformed plants were performed according to established methods [12–14]. Kanamycin-resistant transformants were selected on MS medium [15] supplemented with 1 mg/l of naphtyl acetic acid (NAA), 0.2 mg/l kinetin, and containing 50 mg/l kanamycin sulphate. Subsequently, calli were grown on solid MS medium containing 0.6 mg/l NAA and 0.2 mg/l kinetin. Plants were regenerated on MS medium containing 0.5 mg/l benzylaminopurine (BAP). Shoots were rooted on hormone-free medium.

2.4. *Heat-shock treatment*

For the analysis of heat-shock induced growth of calli transgenic for the chimaeric *hsipt* gene, approximately 200 mg of callus material was placed on MS medium containing 1 mg/l NAA. No cytokinin was added exogenously to the culture medium. Heat shocks were performed every day for 1 h in a 40°C incubator over a period of 4 weeks. The growth coefficient was calculated as $(w - w_0) \times w_0^{-1}$. Two samples were analysed per clone and the experiment was repeated twice.

2.5. *RNA analysis*

RNA was extracted from transgenic calli treated for 1 h at 40°C as described [16]. After oligo(dT) cellulose chromatography, poly(A)$^+$ RNA was separated on a 1.5% agarose-formaldehyde gel, transferred to nylon membranes and hybridized to radioactive probes. Purified DNA fragments were labelled by nick-translation. Hybridizations of Northern blots were performed in 1 M NaCl, 1% SDS, 10% dextran sulphate, and 100 μg of herring sperm DNA at 65°C. Filters were washed in 2 × SSPE, 1% SDS at room temperature and then in 0.2 × SSPE, 1% SDS at 65°C. In order to be certain that equal amounts of poly(A)$^+$ RNA were loaded per lane, nylon membranes were hybridized to a soybean actin probe. For these hybridizations, washes were performed in 1 × SSPE, 1% SDS at 60°C.

2.6. *Extraction and analysis of cytokinins*

After weighing, calli were frozen in liquid nitrogen and stored at −20°C until analysed. Intact tissue (1–2 g fresh wt) was incubated overnight at −20°C in methanol (9 ml·g^{-1} fresh wt) containing 133 Bq *trans*-[^{3}H]zeatinriboside dialcohol (1.4 TBq mmol^{-1}, Amersham) for recovery measurements. After centrifugation (5 min at 28000 × *g*), the extract was purified on a 2 ml PVP column. The eluate was then diluted to 50% methanol, passed through a Sep-pak C18 cartridge, and washed with 5 ml of 50% methanol. The eluate was vacuum-dried and redissolved in 500 μl of 30% ethanol. 50 μl of this partially purified extract were analysed for radioactivity (dpm ^{3}H, Packard Tri-carb 1500) to determine percent recovery. For cytokinin analyses, 2 × 100 μl were assayed with the [9R]Z-Ab RIA and [9R]ip-Ab RIA systems, respectively (for specifications see [17]). Considering the extraction yield of each sample, original cytokinin concentrations are expressed as pmol [9R]Z equiv.·g^{-1} fresh wt and pmol [9R]ip equiv.·g^{-1} fresh wt.

3. RESULTS

In plant cells, the *hsp70* promoter from *D. melanogaster* is characterised by a very low basal level of expression at normal temperature which is rapidly increased after heat shock [9]. In order to test whether the *hsipt* chimaeric gene can be used to manipulate CK concentration in plants, 99 tobacco calli transgenic for the *hsipt* gene (see fig.1) were split in two parts and tested for their ability to grow on cytokinin-free medium after heat-shock treatment. 62 out of 99 calli tested showed improved growth when being heat-shocked as compared to untreated controls. These 62 calli were greener and firmer than untreated calli (fig.2). The heat-shock regime reduced the growth rate of control calli, as shown in table 1. *Hsipt* gene expression was tested in 9 transgenic calli by Northern blot analysis of poly(A)$^+$ RNA extracted immediately after 1 h of heat shock at 40°C. Seven of them showed an improved growth rate during the heat-shock regime (see corresponding clones in table 1), and also showed a detectable level of *ipt* transcripts after heat shock (fig.3; lanes 2, 4, 6, 7,

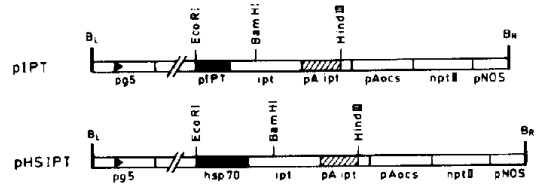

Fig.1. Schematic drawing of the constructions. pIPT contains the *Rsa*I fragment from the T-DNA of the Ti plasmid pTi 15955 corresponding to the *ipt* gene (see section 2). The chimaeric *hsipt* gene contains the coding region of the *ipt* gene under the control of the *hsp70* promoter from *D. melanogaster*. The location of the kanamycin-resistant gene and of the gene 5 promoter of the pPCV002 vector are also indicated. B_L and B_R, left and right border sequences of vector T-DNAs; pg5, truncated promoter of T_L-DNA gene 5; pNOS, pIPT and hsp70, promoters of nopaline synthase, *ipt* and *hsp70* genes; pAocs and pAipt, polyadenylation sequences of octopine synthase and *ipt* genes; nptII, neomycinphosphotransferase II gene of transposon Tn5.

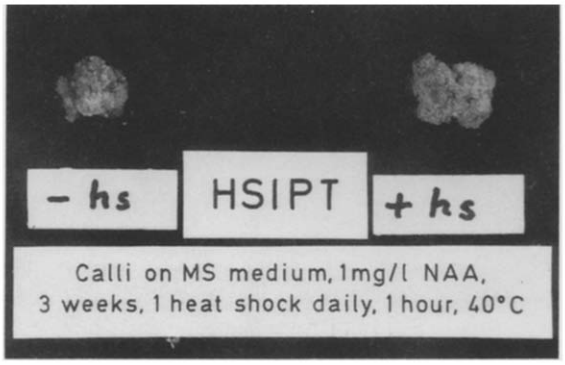

Fig.2. Tobacco calli transgenic for the *hsipt* gene.

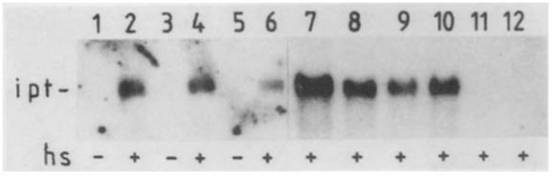

Fig.3. Northern blot analysis of poly(A)$^+$ RNA extracted from tobacco calli transgenic for the *hsipt* gene. Lanes 1, 3, and 5, untreated calli clones HSIPT1, -2, and -3, respectively. Lanes 2, 4, 6 and 7–12, heat-shock treated calli clones HSIPT1, -2, -3, -25, -36, -38, -84, -78, and -85, respectively.

8, 9 and 10). Conversely callus clones HSIPT78 and HSIPT85, which did not show an improved growth rate during the heat-shock regime (see table 1), lack detectable levels of *ipt* specific transcripts even after heat shock (fig.3; lanes 11 and 12).

3.1. *Cytokinin measurements*

Comparison of cytokinin concentrations (expressed as pmol [9R]Z equiv.$\cdot g^{-1}$ fresh wt) extracted before and at various intervals after heat-shock treatment revealed a transient increase of [9R]Z like CK concentration, which was highest 3 h after the end of the heat-shock treatment reaching a level three times higher than basal and then slowly declined (data not shown). Tissue samples were therefore subsequently collected 3 h after the last heat shock. Fig.4 shows that the concentration of [9R]Z equivalents in heat-shock treated *hsipt* calli increases over the total period of the growth assay. Thus, while a single heat-shock treatment results in a transient increase, repeated heat shocks (1 h daily at 40°C for three weeks) cause a considerable increase in the concentration of zeatinriboside-like cytokinins. In contrast, cytokinin levels remain at approximately the same level in control calli (regardless of heat-shock treatment; data not shown) and in untreated *hsipt* transgenic calli (fig.4). Untreated *hsipt* transgenic calli averaged only a two-fold higher concentration than control calli (4.7 pmol [9R]Z equiv.$\cdot g^{-1}$ in HSIPT1, 5.0 pmol [9R]Z equiv.$\cdot g^{-1}$ in HSIPT3 versus 2.6 pmol [9R]Z equiv.$\cdot g^{-1}$ in control calli). The [9R]Z equivalent concentration did not change significantly in heat-shock treated control calli (2.3 pmol$\cdot g^{-1}$ vs 2.6 pmol/g in untreated control calli). As shown in table 2, a similar, but less pronounced increase in [9R]ip equivalents was found in heat-treated *hsipt* calli. The cytokinin concentration in various calli transgenic for the wild-type *ipt* gene (fig.1; see also section 2) was between 47 and 132 pmol/g (data not shown).

Table 1

Growth of untreated and heat-shock treated *hsipt* transgenic calli on cytokinin free medium

Clone	Untreated calli $(w - w_0) \times w_0^{-1}$	Heat-shock treated calli $(w - w_0) \times w_0^{-1}$	Difference of growth in %
HSIPT1	3.2	5.2	+62.5
HSIPT2	4.1	5.7	+39.0
HSIPT3	5.5	7.2	+31.0
HSIPT25	4.5	7.7	+71.1
HSIPT36	4.5	6.1	+35.5
HSIPT38	3.5	5.5	+57.1
HSIPT84	2.3	4.9	+113.0
HSIPT78	4.0	3.3	−17.0
HSIPT85	2.9	1.5	−48.3
pPCV002I[a]	2.8	2.1	−25.0
pPCV002II[a]	3.2	2.7	−15.6

[a] These control calli were transformed with the vector T-DNA only. Approximately 200 mg callus tissue was transferred to solid MS medium containing 1 mg/l NAA. Heat shocks were performed daily during 4 weeks for 1 h at 40°C

3.2. *Phenotypic alterations of transgenic plants*

Several plantlets were regenerated from two calli transgenic for the *ipt* gene under the control of its own regulatory sequences (fig.1). Shoots transgenic for the *ipt* gene were not able to root on hormone-free MS medium. This is most likely due to the inhibitory effect of a high cytokinin concentration on root formation [18]. Consequently these

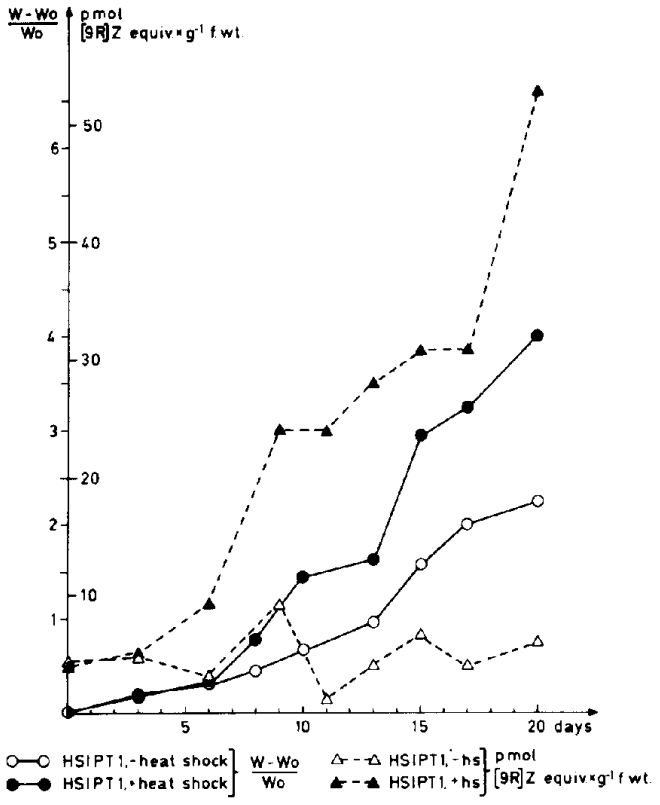

Fig.4. Concentration of [9R]Z equiv.(pmol·g^{-1} fresh wt) and growth rate of callus clone HSIPT1 at normal temperature and under a heat-shock regime (see section 2).

shoots were grafted onto wild-type SR1 tobacco stems. Fig.5a shows that grafts of *ipt* transgenic shoots are stunted, highly branched and with tiny leaves as a result of apical dominance release due most likely to an increased cytokinin content. In leaves and stems of grafted shoot clone IPT1,7 we measured 335 and 524 pmol [9R]Z equiv.·g^{-1} fresh wt, respectively. Stems and leaves of wild type plants contained 13.1 and 0.6 pmol [9R]Z equiv.·g^{-1} fresh wt. In contrast plants regenerated from calli transgenic for the *hsipt* gene are always able to root and show no apparent alterations in their growth habits (fig.5b). Zeatinriboside concentrations in *hsipt* transgenic plants were not significantly different in either roots, stems, or leaves from those measured in wild-type SR1 tobacco plants (data not shown).

Table 2

Concentration of [9R]ip equiv. (pmol·g^{-1} fresh wt) in transformed calli

Callus clone	pPCV002		HSIPT1		HSIPT3	
Heat-shock treatment[a]	−	+	−	+	−	+
Day 1	1.6	0.8	nd	nd	0.1	nd
Day 6	2.3	0.5	nd	2.7	nd	1.8
Day 9	0.6	nd	nd	1.8	nd	0.7
Day 11	1.2	0.1	nd	4.5	nd	14.4
Day 13	nd	0.1	0.3	5.3	nd	2.4
Day 15	0.2	0.2	0.7	12.6	1.8	12.2
Day 17	1.5	nd	nd	17.6	0.5	10.6
Day 20	8.1	1.8	nd	15.5	4.6	14.0

[a] Calli under heat-shock regime were incubated daily for 1 h at 40°C

nd, not detectable

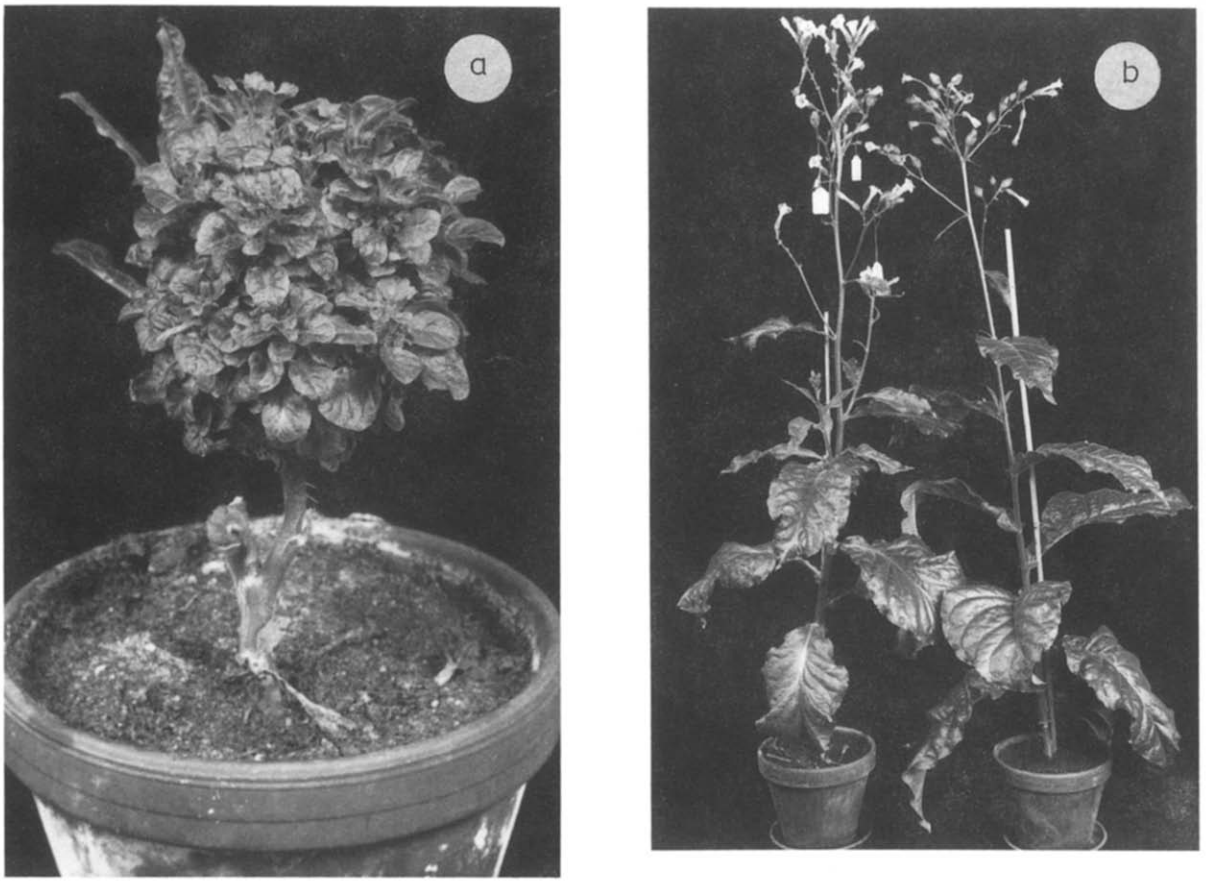

Fig.5. (a) Graft of an *ipt* transgenic shoot on a wild-type SR1 tobacco plant. (b) Tobacco plant regenerated from callus clone HSIPT1 (left) compared with a wild-type tobacco plant.

4. DISCUSSION

The availability of genes affecting cytokinin biosynthesis opens the possibility of generating transgenic plants altered in their hormonal content. Consequently the physiological and developmental effects established by endogenous modification of phytohormones can be monitored [19,20]. In this article, we have shown that by positioning the coding region of the *ipt* gene from *A. tumefaciens* under the control of the *hsp70* promoter from *D. melanogaster* it is possible to increase the CK content by heat treatment, and consequently to achieve callus growth on cytokinin-free medium. Growth on cytokinin-free medium correlates with heat inducibility of expression and an increase in zeatinriboside-like and isopentenyladenosine-like content resulting in a higher chlorophyll content as already observed in soybean calli [21]. The low uninduced level of expression of the *hsp70* promoter does not inhibit root formation and consequently allows the regeneration of fairly normal transgenic plants. Plants and plant tissues transgenic for the *hsipt* chimaeric gene could be used to study the effect of a transient increase of CK concentration on plant development and gene expression.

Acknowledgements: We thank Douglas Furtek for critical reading of this manuscript. This research was supported by a Belgian FKFO grant and a Belgian Research Program no.87-92/119. S.B. is a research assistant and H.V.O. a research director of the Belgian National Science Foundation.

REFERENCES

[1] Krikorian, A.D., Kelly, K. and Smith, D.L. (1987) in: Plant Hormones and their Role in Plant Growth and Development (Davies, P.J. ed.) pp.593–613, Martinus Nijhoff Publishers, Dordrecht.

[2] Tamas, I.A. (1987) in: Plant Hormones and their Role in Plant Growth and Development (Davies, P.J. ed.) pp.393–410, Martinus Nijhoff Publishers, Dordrecht.
[3] Zambryski, P., Tempe, J. and Schell, J. (1989) Cell 56, 193–201.
[4] Morris, R.O. (1986) Annu. Rev. Plant Physiol. 37, 509–538.
[5] Spena, A. and Schell, J. (1987) Mol. Gen. Genet. 206, 436–440.
[6] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[7] Barker, R.F., Idler, K.B., Thompson, D.V. and Kemp, J.D. (1983) Plant Mol. Biol. 2, 335–350.
[8] Koncz, C. and Schell, J. (1986) Mol. Gen. Genet. 204, 383–396.
[9] Spena, A., Hain, R., Ziervogel, U., Saedler, H. and Schell, J. (1985) EMBO J. 4, 2739–2743.
[10] Marton, L., Wullems, G.J., Molendijk, L. and Schilperoort, R.A. (1979) Nature 277, 129–131.
[11] Nagy, J.I. and Maliga, P. (1976) Z. Pflanzenphysiol. 78, 453–455.
[12] Maliga, P., Sz.-Breznovitis, A. and Morton, L. (1973) Nature 244, 29–30.
[13] Horsch, R., Fraley, R., Rogers, S., Sanders, P., Lloyd, A. and Hoffmann, W. (1984) Science 223, 496–499.
[14] Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) Science 227, 1229–1231.
[15] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473–496.
[16] Taylor, B.H. and Powell, A. (1983) BRL Focus 4, 4–6.
[17] Van Onckelen, H., Rüdelsheim, P., Hermans, R., Horemans, S., Messens, E., Hernalsteens, J.-P., Van Montagu, M. and De Greef, J. (1984) Plant Cell Physiol. 25, 1017–1025.
[18] Matthysse, A.G. and Scott, T.K. (1984) in: Hormonal Regulation of Plant Development II (Scott, T.K. ed.) pp.219–243, Springer-Verlag, Berlin.
[19] Ooms, G. and Lenton, J.R. (1985) Plant Mol. Biol. 5, 205–212.
[20] Memelink, J., Hoge, J.H.C. and Schilperoort, R.A. (1987) EMBO J. 6, 3579–3583.
[21] Wyndaele, R., Christiansen, J., Horseele, R., Rüdelsheim, P. and Van Onckelen, H. (1988) Plant Cell Physiol. 29, 1095–1101.