EXPRESSION OF THE *PHSP70(LP19)-GUS* GENE DURING POLLEN EMBRYOGENESIS IN TRANSGENIC PLANTS OF *DATURA INNOXIA*

ELENA BADEA[1], OFELIA GOZIA[2]

Fertile transgenic plants of *Datura innoxia* were obtained through transformation of leaf tissue with disarmed *Agrobacterium tumefaciens* strain LBA 4404, harbouring a binary vector containing a chimeric fusion between the pea *hsp* 70 (LP19) gene promoter and the GUS reporter gene, and *in vitro* regeneration via organogenesis. Molecular analysis of independent transformants confirmed the transgenic nature of plants regenerated by *Agrobacterium* – transformation. Quantitative and qualitative assay of fresh and cultivated anthers revealed a constitutive level of GUS activity in the anthers before cultivation. The enzyme activity decreased in the first 5 days and increased beginning with the 7th day of culture in both fresh and cold treated anthers. The increase of GUS activity could be correlated with the appearance of the first embryogenic structures. Our results suggest the involvement of HSP70 in embryogenesis as a normal part of developmental process, not associated with heat shock.

Key words: Datura innoxia, transgenic plants, hsp70 promoter, *Pisum sativum*, β-glucuronidase, pollen embryogenesis.

INTRODUCTION

In the latest few years it has been clear that the synthesis of at least some heat shock proteins (HSPs), normally induced in response to elevated temperature or to other types of physical or chemical stress (14), is also developmentally regulated under normal growth conditions.

To understand the developmental regulation of plant HSP genes, the promoters and regulatory elements involved should be cloned and analyzed. To date, only a few plant HSP promoters and 5'-flanking sequences have been reported to confer to chimeric genes developmental regulation during zygotic embryogenesis (1, 5, 17, 18).

Members of the HSP70 family have been detected in developing pea cotyledons, axes and seed coats (7). Previously, a protein with homology to members of the *hsp70* gene family was identified as an early-embryogenesis protein in pea (8).

Changes in synthesis and location of members of the 70 kDa class of heat shock proteins were also reported accompanying the induction of embryogenesis in *Brassica napus* microspores (6).

The authors concluded as temperature stress alone is responsible for the induction of embryogenic development, these might be a discrete involvement of HSP70 in this process. In some *Brassica* species, 8–24h of culture at 32°C are sufficient to synchronously and irreversibly induce the embryogenic development (15).

Induction of embryogenic program by high-temperature treatments or "heat-shock" has been observed in many experimental systems (3, 21). A variety of treatments and stress conditions have been found to be effective for the induction of embryogenesis in different species (reviewed by Powell, 1990).

The beneficial effect of low temperature pretreatment in enhancing embryogenic responses in cultured anthers was first demonstrated in *Datura innoxia* (12). If anthers are collected from the donor plants in the optimum stage of pollen development, many plants of *D. innoxia* could be obtained without cold pretreatment and hormones addition in the culture medium (2). In this respect, *D. innoxia* could represent an appropriate model plant for studying the expression of a chimeric gene-*hsp70* promoter GUS- in embryogenesis induction based only on stress conditions generated by *in vitro* culture *per se*. In this report we described the obtaining of *D.innoxia* transgenic plants using pea *hsp70* (LP19) gene promoter-reporter construct expressing a high level of GUS activity. The expression of the chimeric gene under normal growth conditions both in embryonic microspore and during embryogenesis was studied.

MATERIAL AND METHODS

Bacterial strain and vector. The transformation experiments were carried out using the disarmed *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary plasmid pBI 101.2. The plasmid has a 1.8 kb 5' fragment of a pea hsp70 (LP19) gene promoter containing the transcription start site, the translation start codon and the first 18 aminoacids of the HSP70 (LP19) polypeptide, translationally fused with the coding sequence of *uid A* (GUS A) reporter gene encoding the β-glucuronidase (GUS). The neomycin phosphotransferase II (*npt II*) gene driven by nopaline synthase (NOS) promoter and terminator sequences was used as the selectable marker gene. Bacteria were maintained on YEB agar plates containing 100mg-ml kanamycin sulfate.

Plant material. For transformation experiment, *Datura innoxia* Mill (2n = 24) plants, obtained by androgenesis and maintained *in vitro*, were used as a source of explant material. For anther culture, two diploid primary transformants (T_0), grown in the green house of the Institute of Biology, selected by the high level of GUS activity, were used as donor plants. The anther culture experiments were repeated with three diploid homozygous plants (T_1) obtained by androgenesis.

Transformation and regeneration of transformants. *Agrobacterium* strain LBA 4404 was grown 2 days, at 28°C, in YEB liquid medium containing 100mg/ml kanamycin sulfate. This suspension was used for transformation. About 50 leaf – disks (0.5cm diameter) were cut from young leaves collected from the plants grown *in vitro*, using a cork-borer. Explants were immersed in bacterium suspension for 10 minutes, blotted on sterile filter paper and transferred to MS (11) medium. After 2 days of co-cultivation, in the dark, at 25°C, the explants were transferred on MS selection medium with 300 mg/l augmentin + 200 mg/l kanamycin. For shoot regeneration the selection medium was supplemented with 1 mg/L BAP and 0.25 mg/L NAA. After 2 weeks, explants were transferred to fresh selection medium with 200 mg/L augmentin, and 200 mg/L kanamycin. Shoots appeared on the leaf explants near the cut edge and were transferred 8-12 weeks after *A. tumefaciens* infection on MS + 200 mg/L augmentin + 200 mg/L kanamycin, without hormones. All cultures were incubated at 25°C with a 16 hours photoperiod. Plants obtained by rooting of the shoots on the same medium were transplanted into pots and grown to maturity in the green house.

Re-callusing assay. One leaf was removed from regenerated plants and, after cutting in small fragments, was placed on callusing MS medium (1mg/L NAA + 0.25mg/L BA) with 200mg/L kanamycin. Calluses were allowed to develop 4 weeks, afterwards their growth was evaluated. Control assays were also performed with untransformed genotype.

Anther culture. Flower buds, ranging between 30 and 45 mm, which largely correspond to early unicellular and bicellular stage of pollen development, were harvested from two fertile primary transformants, selected on the basis of the level of GUS expression and three T_1 homozygous plants. After surface sterilization in 0.1% $HgCl_2$ solution for 7 minutes, and washing 3–4 times in sterile distilled water, anthers were dissected aseptically and treated either for histochemical assay or were inoculated on 15 x 60 mm Petri dishes (10 anthers/dish), which contained 10ml of Nitsch and Nitsch (1969) medium, 20g/L sucrose and 8g/L agar, pH 5.8. In some experiments the culture medium was supplemented with 1mg/L naphthylacetic acid.

The same protocol was used with buds previously kept in the dark at 4°C, for 48 hours. In order to obtain a homogeneous population of transgenic embryos and homozygous diploid plants and to determine the natural resistance of untransformed microspores to kanamycin, anthers taken both from transformed and untransformed plants were cultivated on medium with 150 and 250 mg/L kanamycin. The dishes, sealed with parafilm, were incubated in the dark at 25°C.

The expression pattern of the chimeric gene during induction of microspore embryogenesis was studied by cytological observations of microspores and GUS assay in samples collected from fresh and cold treated anthers daily, over a 14 day period.

Expression assay for the GUS gene. All primary transformants were analyzed for detection of GUS expression using histochemical staining with 2mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid cyclohexylammonium) as described by Jefferson (1987). In order to study the pattern of transgene expression in microspores, before culture and during androgenesis induction, and in the first stage of embryogenesis, microspores pushed out from fresh, cold treated and cultured anthers were stained with X-Gluc. Blue colour was observed visually or microscopically from assayed tissues and spores after overnight incubation both at room temperature and 37°C.

The expression of β-glucuronidase (*uid* A) gene in the transformed leaves, fresh and cold treated and cultured anthers over a period of 8 days and after 25 days, was conducted fluorometrically in crude protein extracts, using the Bio-Rad VersaFluor Fluorometer and the FluorAce β-glucuronidase reporter Assay Kit.One unit of β-glucuronidase hydrolyzes 1 nanomole of 4-methylumbeliferyl β-D-glucuronide per minute at pH 7.5 at 37°C.The activity was expressed in units/mg protein.

Determination of the protein level. It was performed according to the Lowry (1951) method using the Perkin-Elmer Lambda 12 UV/Vis spectrophotometer with dedicated preprogrammed method for protein analysis.

Polymerase chain reaction analysis. The PCR analysis, performed on T_1 plants obtained by anther culture on selection medium (200mg/L kanamycin), was based on amplification of a fragment of the *uid A* (GUS) gene integrated in the plant genome. Plant DNA for PCR analysis was prepared as described by Offringa and van der Lee (1995). The primers for the *uid A* (GUS) encoding gene amplification were 5'-CTGCGACGCTCACACCGATACC-3' and 5'-GTGCGCCAGGAGAGTTTGTTGATTC-3'. PCR reaction to amplify *uid A* gene was carried out in a 50ml volume containing 10ml of DNA extract, 10ml of dNTP mixture, 1 ml of each primer, and 1 unit of Taq polymerase. The PCR was performed 30 cycles with 1 minute melting at 94°C, 1 minute annealing at 50°C, 1 minute synthesis at 72°C. The experiments have been performed with a Pharmacia kit using a Perkin-Elmer thermocycler. Amplified DNA fragments were electrophoresed on 0.8% agarose gel. Gels were visualized under UV light and photographed.

RESULTS AND DISCUSSION

Transgenic plants production. Explants were co-cultivated with *Agrobacterium* vector containing *phsp70* (LP19)-GUS construct and then transferred to MS medium to select transformed shoots. Co-cultivated explants swelled and developed shoot buds with little callus after 1-3 months on selective shoot regeneration medium. The frequency of shoots regenerated in the presence of kanamycin selection (expressed as shoots/100 explants) was 20. A total number of

19 transformed plants were successfully grown to maturity in the greenhouse. All primary transformants were analyzed using histochemical staining with X-Gluc. A detectable amount of GUS expression in the young leaf tissues was observed in all regenerated plants. When they were transferred in soil, in the greenhouse, most regenerants appeared phenotypically normal, though at the maturity some of them were sterile. Extracts from the leaves of fertile primary transformants (T_0) were fluorimetrically assayed for GUS activity. As expected the level of GUS expression varied between independent transformants carrying the same construct. On the basis of level of GUS expression two of the transformants (numbered 3 and 4) were selected for anther culture.

Segregation of the germinating T_1 seeds on media containing 200 mg/ml kanamycin was used to determine the number of transgene loci present in two primary transformants: 3 and 4. The kanamycin resistant: sensitive ratio observed was approximately 3:1. For each transgenic plant, out of aproximately 400 seeds, 237 plantlets were obtained. The results indicated the presence of the transgene at a single locus.

Analysis of genomic DNA by PCR amplification with primers for GUS gene, from several diploid androgenetic plants obtained by anther culture from transformants number 3 and 4 confirmed their transgenic status (Fig. 1).

Evaluation of chimeric gene expression during microspore embryogenesis. In order to determine the status of hsp70 (LP19) gene promoter activity during pollen embryogenesis, fresh and cold treated anthers from primary transformants (3 and 4) were cultivated on Nitsch and Nitsch (1969) medium, without heat shock. Before culture, GUS activity conferred by the *hsp70* (LP19) gene promoter has been detected in the fresh and cold treated anthers containing microspores in optimal stages for embryogenesis, by both histochemical and fluorometric analysis (Fig. 2). The beneficial effect of cold treatment is mainly attributed to the fact that low temperature retains the pollen viability longer and prevents the abortion (4). In our case no significant differences between fresh and cold treated anthers were detected by GUS assays. This demonstrates that cold treatment did not activate either gene expression or the protein turnover (Fig. 2).

In order to study the time course induction of the *hsp70* (LP19) promoter during the sporophytic program, whole anther collected from culture daily, over a 8 days period, were analyzed for GUS activity. The experiments have been performed on T_0 plants. A constitutive level of GUS activity was recorded in the anthers before cultivation, but never in the microspores. This could mean that the level of enzyme activity is too low to be detected by qualitative assay. The enzyme activity decreased on the first 5 days and increased beginning with the 7th day of culture in both fresh and cold treated anthers (Fig. 2). Since we analyzed whole anthers, we assumed that the value recorded in the first days in culture could reflect the enzyme activity both in the anther wall and microspores. During the first days of culture the anther wall is dying and the large majority of microspores is in

various stages of degeneration or showed no sign of subsequent development. The increase of enzyme activity beginning with the 7th day could reflect the activation of the gene expression in embryogenic microspores. These experiments were repeated with fresh anthers obtained from T_1 plants. The anthers were also cultivated during 10 days on a medium supplemented with NAA in order to study its potential involvement in the regulation of the chimeric gene expression. No significant differences have been observed between the anthers cultivated in the presence or in the absence of NAA (data not shown). Even in the presence of NAA the GUS activity highly increased beginning with the 7th day of culture. The decrease of GUS activity after the first days of culture could be attributed to the browning and death of the anther wall as detected by the diminishing of protein concentration (over 40% after 10 days). By contrast the increase of GUS activity beginning with the 7th day cannot be explained exclusively by the decrease of protein concentration as revealed by fluorometric assay. These analyses were completed with cytological studies of GUS expression in the individual microspores maintained more than 2 weeks in anthers culture. The cytological studies performed with fresh and cold treated anthers before cultivation as well as in the first days of culture localized the GUS expression exclusively in the anther wall. No blue staining was observed in the young microspores or pollen grain. Even after 10 days of culture the blue staining could be visualized in the dead wall of the anther. Therefore, we suspected a potential involvement of HSP70 in anther wall senescence. In this respect, anthers belonging to the same bud were maintained 25 days on culture medium in the presence and absence of NAA. After 25 days of culture some anthers contained young embryos and others were nonresponsive. Even after 25 days the nonresponsive anthers presented a low level of GUS activity.

The GUS activity in stained microspores emphasized a high level of gene expression beginning with the 7th day of culture. The chimeric gene was induced to a high level of expression in some microspores (Fig. 3a) and to a low one in others. Different intensity of staining could be correlated with phase in the cell cycle. Cell cycle progression during embryogenesis induction depends on the initial cell cycle stage (6).

Microspores divide symmetrically or asymmetrically in culture to form typical – a small generative cell and a large vegetative one – or anomalous – two equal, vegetative type cell. Both types showed enzyme activity. Irrespective of the early events in the division, multicellular pollen grains (Fig. 3b-c) and young embryos (Fig. 3d) with high level of gene expression have been visualized beginning with the 10th day of culture. In the anthers maintained more than 10 days in culture were observed 2 types of induced structures: embryogenic and non embryogenic. The expression of chimeric gene was visualized even in non embryogenic structures.

The gene expression was also detected in all stages of embryo development: globular, heart, torpedo, cotyledonal. Different levels of blue staining have been visualized (Fig. 3e). This variability could be explained either by the different ploidy level of the embryos (n, 2n, 3n, 4n), frequently described in androgenetic plants of *Datura* or by rearrangements of the construct induced by *in vitro* culture. This could also be possible due to differential gene regulation at different stages of embryogenesis or in different cell types belonging to tissues which are differentiating.

Many HSP genes are not activated only following thermal stress, but are also subject to developmental control. For example, a plastid localized HSP70 was detected during early stages of pea seed development (8) and the mRNAs for small HSPs were detected during microsporogenesis in soybean (5). Cordwener *et al.* (1995) reported the activation of heat shock proteins during embryogenic induction in *Brassica napus*. Induction of cell division and determination of embryogenesis are considered to be different processes (20). Indeed, we have observed two types of induced structure in the anthers maintained more than 10 days in culture: embryogenic and non-embryogenic. The expression of chimeric gene was high in both types of structure that is rather correlated with high mitotic activity than with the embryogenesis. In pea, HSP71 and HSP70B were expressed from the beginning of seed development till 4 days after seed germination (7). All these results support the hypothesis that the *hsp70* gene is activated during embryogenesis, but the function at this stage is unknown.

HSP70 are binding to a large number of substrates, participating in a variety of protein folding, unfolding, assembly, disassembly and translocation processes. HSPs migh help cell to cope with stress-induced damage polypeptides in two general ways: one consists in degrading damaged polypeptides and another in saving them playing the role of "molecular chaperones" (14). The expression of HSP70 exclusively in the anther wall during the first days of culture, its presence even after wall browning and death illustrated by the decrease of protein concentration, pleads for a potential involvement of HSP70 in anther wall senescence imposed by the *in vitro* culture *per se*. The high level of gene expression even in non embryogenic, abortive structure sustained this hypothesis, moreover as in *Datura innoxia* the androgenesis is a direct process. The complexity of the sporophytic program beginning with its induction (7th day) till its ending (cotyledonary stage) assumes a high level of HSP70 expression, probably as "molecular chaperones".

In conclusion, our results strongly support the involvement of HSP70 in embryogenesis as a normal part of the developmental process, not associated with heat stress.

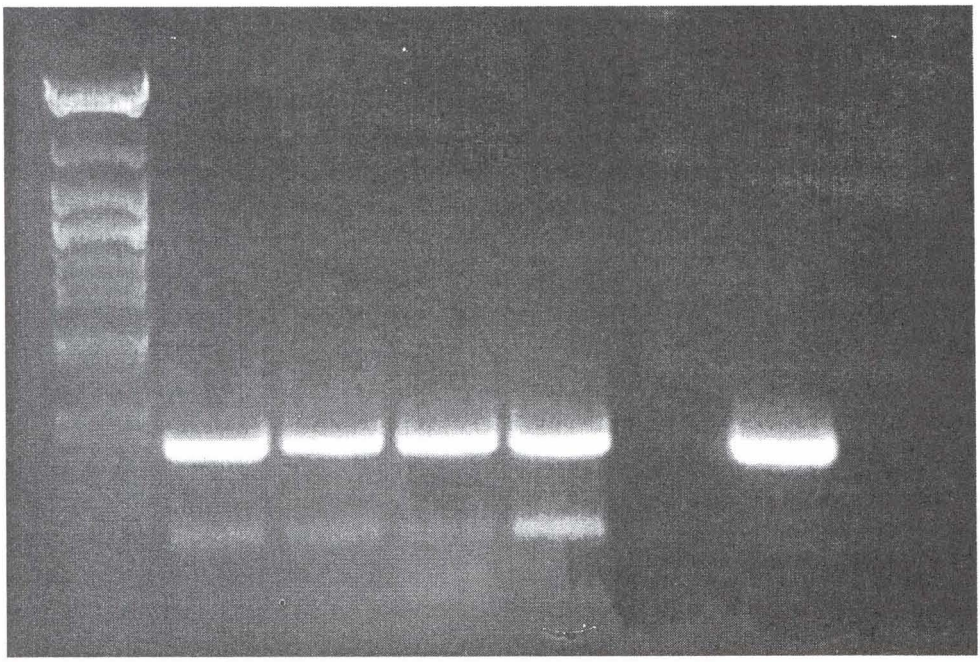

Fig. 1. Results of PCR analysis. 1. DNA marker lambda Eco 471; 2–5. transgenic plants; 6. negative control; 7. positive control – the GUS gene amplified.

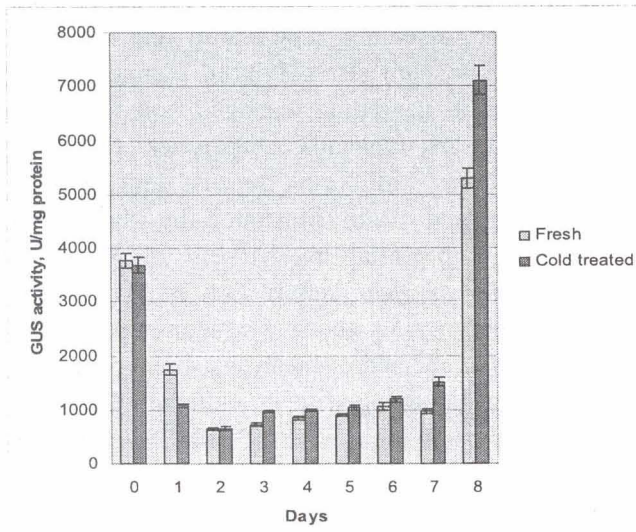

Fig. 2. β-glucuronidase activity evaluation (U/mg protein) of fresh and cold treated anthers.

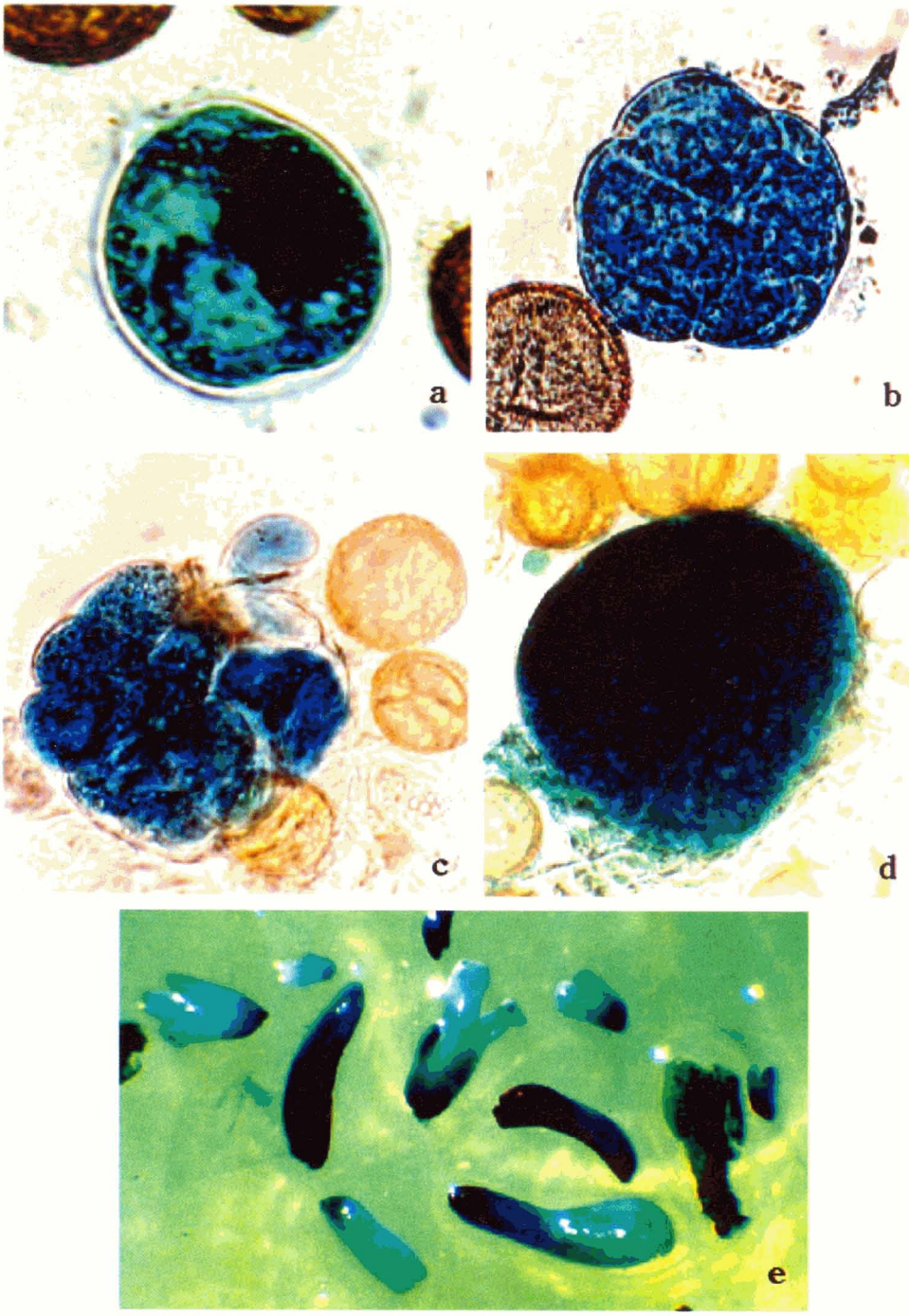

Fig. 3. Light microscopy of *Datura innoxia* anther culture products after GUS assay showing microspore, bi-and multicellular pollen grains and embryos.

Acknowledgements. We want to thank Professor John Gatehouse from Durham University, UK, for permitting us to use his gene construct and Professor Keith Lindsey from Durham University, UK, for the critical review of this article.

REFERENCES

1. Almoguera C., Prieto-Dapena P., Jordano J., 1998, Dual regulation of a heat shock promoter during embryogenesis: stage-dependent role of heat shock elements. The Plant Journal, **13**(4): 437–446.
2. Badea E., Lupuşanski I.,Verzea M., Raicu P., 1994, Studiul unor factori implicaţi în variaţia numărului de cromozomi ai plantelor cu origine polinică. Cercet. Genet. Veget. Anim., **III**: 71–78.
3. Badea E., Săndulescu D., Gozia O., Răduţoiu E.S., Raicu P.,Gorenflot R., 1997, Effets du filtrat de culture de *Fusarium graminearum* et de la temperature sur l'embryogenèse somatique chez *Triticum aestivum*. Rev. Cytol. Biol. Végét., **20**(2): 3–10.
4. Bajaj Y.P.S., 1978, Effect of super-low temperature on excised anthers and pollen embryos of Atropa, Nicotiana and Petunia. Phytomorphology, **28**: 171–176.
5. Coca M.A., Almoguera C., Thomas T.L., Jordano J., 1996, Differential regulation of small heat-shock protein genes in plants: analysis of a water-stress-inducible and developmentally activated sunflower promoter. Plant Mol. Biol., **31**: 863–876.
6. Cordewener H.G., Hause G., Görgen E., Busink R., Hause B., Dons H.J.M., Van Lammeren A.A.M.,Van Lookeren Campagne M.M., Pechan P., 1995, Changes in synthesis and localization of members of the 70 kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta, **196**: 747–755.
7. DeRocher A.,Vierling A., 1995, Cytoplasmic hsp70 homologues of pea: differential expression in vegetative and embryonic organs. Plant Mol. Biol., **27**: 441–456.
8. Domoney C., Ellis N., Turner L., Casey A., 1991, A developmentally regulated early-embryogenesis protein in pea (*Pisum sativum* L.) is related to the heat-shock protein (hsp70) gene family. Planta, **184**: 350–355.
9. Jefferson R.A., 1987, Assaying chimeric gene in plants: the GUS gene fusion system. Plant Mol. Biol. Rep., **5**: 387–405.
10. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J., 1951, Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**: 265.
11. Murashige T., Skoog F., 1962, A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., **15**: 473–497.
12. Nitsch J.P., C. Nitsch, 1969, Haploid plants from pollen grains. Science, **163**: 85–87.
12. Nitsch C., Norreel B.,1973, Effet d'un choc thermique sur le pouvoir embryogene du pollen de *Datura innoxia* cultivé dans l'anthère ou isolé de l'anthère. C.R. Acad. Sc. Paris., **276**: 303–306.
13. Offringa R., van der Lee F., 1995, Isolation and Characterization of Plant Genomic DNA Sequences via (Inverse) PCR Amplification. From: *Methods in Mol. Biol.*, vol. 49: Plant Gene Transfer and Expression Protocols.
14. Parsell D.A., Lindquist S., 1993, The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Ann. Rev. Genet., **27**: 437–496.
15. Pechan P.M., Bartels D., D.C.W. Brown D.C.W., Schell J., 1991, Messenger-RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta, **184**: 161–165.
16. Powell W., 1990, Environmental and genetical aspects of pollen embryogenesis. In: Biotechnology in Agriculture and Forestry 12. Haploids in Crop Improvement I (ed. Y.P.S. Bajaj). Springer-Verlag, 45–65.

17. Prändl R., Schöffl F., 1996, Heat shock elements are involved in heat shock promoter activation during tobacco seed maturation. Plant Mol. Biol., **31**, 157–162.
18. Prändl R., Kloske E., F. Schöffl F., 1995, Developmental regulation and tissue-specific differences of heat shock gene expression in transgenic tobacco and *Arabidopsis* plants. Plant Mol. Biol., **28**, 73–82.
19. Sundeland N., 1974, Anther culture as a means of haploid induction. In: *Haploids in higher plants: advances and potential* (ed.K.J.Kasha). Univ. Guelf, 91–122.
20. Telmer C.A., NewComb W., D.H. Simmonds D.H., 1995, Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas., Protoplasma, **185**: 106–112.
21. Touraev A., Pfosser M., Vicente O., Heberle-Bors E., 1996, Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis, Planta, **200**: 144–152.

[1] *Institute of Biology, 296 Splaiul Independentei Str., Bucharest, Romania*
elenamarcelabadea@yahoo.com
[2] *Institute of Biochemistry, 296 Splaiul Independenței Str., Bucharest, Romania*