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Communication

Heat Inducible Expression of a Chimeric Maize hsp70CAT Gene in Maize Protoplasts[1]

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ABSTRACT

The response of maize (*Zea mays* L.) protoplasts to high temperature stress was investigated. After isolation and electroporation, protoplasts were preincubated for 12 hours at 26°C then incubated for 6 hours at elevated temperatures. The pattern of polypeptides synthesized by these protoplasts during the last hour was monitored by *in vivo* labeling with ^{35}S-methionine. Incubation at 40° and 42°C resulted in the synthesis of polypeptides not detectable at 26°C. Introduction of a chimeric maize heat shock protein 70 promoter-chloramphenicol acetyltransferase coding region gene into protoplasts via electroporation resulted in the temperature-dependent induction of chloramphenicol acetyltransferase activity with maximal activity at 40°C. In the same protoplasts, a second chimeric gene, in which the firefly luciferase coding region was under the control of the 35S promoter from cauliflower mosaic virus, did not show an increase in expression after incubation at higher temperatures. Maize protoplasts provide a system to study molecular responses to high temperature stress.

The advantages of the use of plant protoplasts for biochemical and gene transfer studies have been widely demonstrated. The ability to obtain a homogeneous population of cells without the physical barrier of the cell wall has allowed the measurement of ion channels (23, 29), facilitated the isolation of subcellular components such as vacuoles (33), and led to the development of direct DNA-mediated gene transfer technologies (14, 24).

For physiological studies, the question remains whether protoplasts are a suitable experimental system. There are many cases where isolated protoplasts mimic the behavior of cells in situ. Oat aleurone protoplasts undergo several morphological changes, release acid phosphatase, and induce amylase mRNA (15) in response to applied gibberellic acid as was previously observed in intact aleurone layers. Cucumber cotyledon protoplasts resemble intact cotyledons in their chilling sensitivity (25). Maize protoplasts regulate the expression of the *Adh1* promoter in response to oxygen tension as do whole roots (16, 34).

There are other examples, however, where protoplast behavior does not correspond to the behavior of the organ or tissue from which the protoplasts were isolated. Tobacco mesophyll protoplasts do not synthesize small subunit of ribulose bisphosphate carboxylase (10, 11, 32). Barley leaf protoplasts do not accumulate abscisic acid as a response to decreasing osmotic potential, a phenomenon which is observed in intact leaves (20). Thus, it appears that protoplasts are not universally suitable for experiments in which their behavior must mimic the intact tissue; some responses are adversely affected, possibly because the protoplasts experience a loss of turgor, are incubated in high osmoticum, and/or are exposed to crude digestive enzyme preparations.

The response of plant cells to high temperature stress by the synthesis of a subset of proteins, called heat shock proteins, hsps[4] (17), has been documented in undifferentiated plant cell cultures of soybean, tobacco (3), and maize (8). In addition, tobacco mesophyll protoplasts synthesized hsps (22). We were interested in using a protoplast system to analyze the expression of the maize hsp70 gene (26, 27) during heat shock. The ability to transiently introduce genes into maize protoplasts via electroporation (13) made it possible to analyze the expression of the maize hsp70 gene in a homologous system. We show here that maize protoplasts, even after electroporation, are capable of a heat shock response and show a temperature dependent induction of an introduced maize hsp70-CAT chimeric gene.

MATERIALS AND METHODS

Materials. pCaMVluc was the gift of Jeff DeWet (Stanford University). pMON9508 was the gift of Dilip Shah (Monsanto Company). Maintenance and manipulation of the BMS suspension line and protoplasts were performed as described by Fromm *et al.* (12).

Construction of phsp70cat. pMON9508 was sequentially digested with *Pvu*II and *Bal*31; the ends were filled in with T4 DNA polymerase, and the DNA was digested with *Eco*RI. The hsp70 promoter fragment was then ligated into pUC8 that had been digested with *Sma*I and *Eco*RI. A *Bam*HI fragment containing the hsp70 promoter was ligated into the *Bam*HI site of a derivative pUC8 plasmid containing the CAT coding region (1) and the 260 bp *Sau*3 A fragment containing the poly A addition site of the nopaline synthase gene (4) downstream of the *Bam*HI site. The extent of the *Bal*31 deletion of the promoter fragment was determined by dideoxy sequencing (5).

Electroporation and Enzyme Assays. Protoplasts were electroporated in 1 mL batches in the presence of both phsp70cat and pCaMVluc, pooled together, and then re-aliquoted to minimize between sample variation. Electroporation conditions, prepara-

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[4] Abbreviations: hsp, heat shock protein; CAT, chloramphenicol acetyltransferase; BMS, Black Mexican Sweet; CaMV, cauliflower mosaic virus.

tion of the protoplast extacts, and CAT assays were as described previously (12); the CAT assay included a 10 min incubation at 65°C (28). After separation by TLC, the ^{14}C-chloramphenicol and acetylated products were localized by autoradiography, and the radioactive spots were quantitated individually by scintillation counting. The amount of acetylated ^{14}C-chloramphenicol was divided by the total radioactivity in each lane to determine the percent conversion. Luciferase assays were performed as described in Callis *et al.* (6). Luciferase activity was monitored in a Monolight 2000 photometer; the values given represent integration of the luminosity for a 30 s period after addition of substrate.

***In Vivo* Labeling.** Twelve h after preparation (control) or 12 h after preparation and electroporation, protoplasts were either maintained at 26°C or were incubated at various temperatures for 6 h. During the last hour, ^{35}S-methionine was added to the media to a final concentration of 20 μCi/ml. Protoplasts were then concentrated by centrifugation, washed one time in incubation media with 1 mM methionine, lysed in SDS buffer (18), and sonicated. Total incorporation into acid insoluble material was determined (21). Equal quantities of radioactivity were loaded on a 10% polyacrylamide SDS gel (18). After electrophoresis, the gel was prepared for fluorography by 2,5-diphenyloxazole treatment.

RESULTS

The effect of electroporation on the proteins synthesized by maize (*Zea mays* L.) protoplasts is shown in Figure 1. Protoplasts isolated from BMS suspension cells were electroporated and incubated for 18 h at 26°C, the last hour in the presence of ^{35}S-methionine. The profile of *in vivo* labeled proteins from these electroporated protoplasts (lane 2) is identical to the nonelectroporated control protoplasts (lane 1) which were incubated and labeled identically. The levels of acid precipitable counts were also similar (data not shown). This indicates that after 18 h of incubation, electroporation had not altered the ability of protoplasts to synthesize proteins nor the pattern of proteins synthesized.

Figure 1 also shows the effects of incubation at elevated temperatures on protoplast protein synthesis (lanes 3–6). Electroporated protoplasts were incubated for 12 h at 26°C and then placed at the various temperatures for 6 h. During the last hour of incubation ^{35}S-methionine was added. There was no detectable difference between the proteins synthesized at 26°C (lane 2) and at 30°C (lane 3). At 37°C (lane 4), in addition to the 26°C pattern of protein synthesis, there were barely detectable levels of additional polypeptides. These additional polypeptides were more prominently labeled at 40°C (lane 5) and at 42°C (lane 6) constituted the major polypeptides synthesized. The pattern of polypeptides synthesized at 42°C is very similar to that previously observed in heat shocked BMS cultured cells (8) and in heat shocked maize coleoptiles (27). The rate of protein synthesis was also dramatically affected by incubation at 42°C. On a per cell basis, the amount of acid insoluble counts at 42°C was 2% of that incorporated at 26°C (data not shown). Thus, despite the physical stresses of cell wall removal and electroporation, the protoplasts were still capable of responding to heat shock by increasing the rate of synthesis of a small set of hsps.

Because protoplasts were capable of responding to high temperature stress, we asked whether expression of an extra-chromosomal hsp 70 gene would show regulated expression when introduced into protoplasts via electroporation. To assay for the induction of an hsp70 gene, an hsp70-CAT chimeric gene was constructed (Fig. 2). A DNA fragment from pMON9508 (26) containing approximately 1.1 kb upstream of the transcribed region for an hsp70 gene and all but 24 of the 107 nucleotides of the 5′ transcribed, untranslated leader was placed upstream

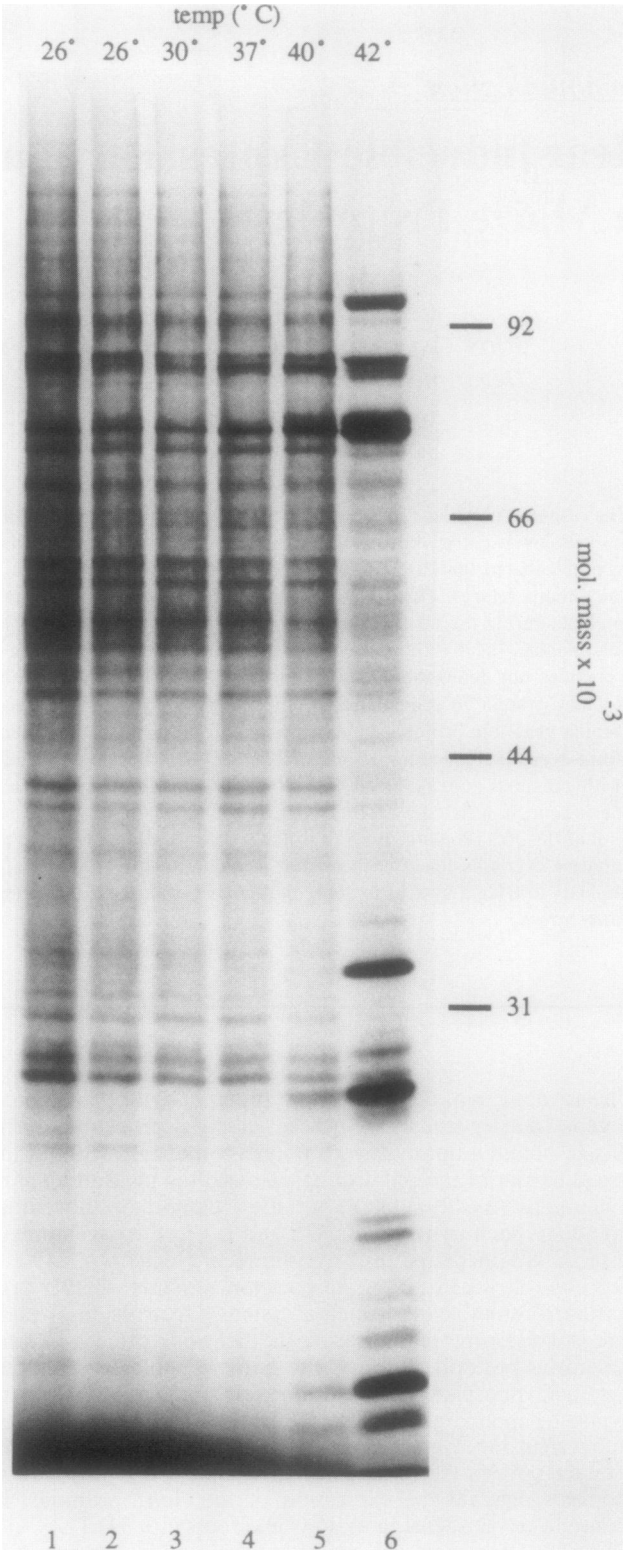
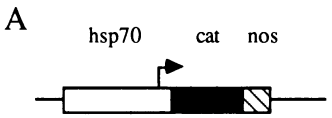


FIG. 1. *In vivo* labeling of BMS protoplasts. Protoplasts were labeled in vivo with ^{35}S-methionine for 1 h at 17 h after either isolation (lane 1) or isolation and electroporation (lanes 2–6). After a 12 h incubation, protoplasts were incubated for 6 h at the temperature indicated above each lane. After 18 h, the protoplasts were lysed, and equal amounts of acid insoluble radioactivity were loaded in each lane of a 10% polyacrylamide SDS gel. Following electrophoresis the polypeptides were visualized by fluorography.



B

+1

hsp70 tctctcgcctgagaaaaaaaatcca
hsp70cat tctctcgcctgagaaaaaaaatcca

cgaaccaatttctcagcaaccagcagcacgacctgtgagggt
cgaaccaatttctcagcaaccagcagcacgacctgtgagggt

+84

tcgaaggaagtagcagTGTTTTTTGTTCCTAGAGG
tcgaaggaagtagcagGGGGATCCGTCGAGATTT

AAGAGATG
TCAGGAGCTAAGGAAGCTAAAATG

FIG. 2. Map of the hsp70cat construction. (A) The hsp70 promoter fragment from pMON9508 (26) represented by the open box was placed in front of the CAT coding region (filled box) and the nopaline synthase poly A addition site (striped box) in the *Bam*HI site of pUC8. The thin line represents plasmid sequences (not to scale). See "Materials and Methods" for details. (B) The nucleotide sequence of the 5′ leader of the endogenous hsp70 gene (top line) compared to the 5′ leader of the chimeric hsp70cat gene (bottom line). They are identical for the first 83 nucleotides, then the chimeric hsp70cat gene contains 36 different nucleotides instead of the 24 nucleotides present in the hsp70 gene. The sequence ends with the translation initiator ATG for the endogenous hsp70 protein and the CAT protein.

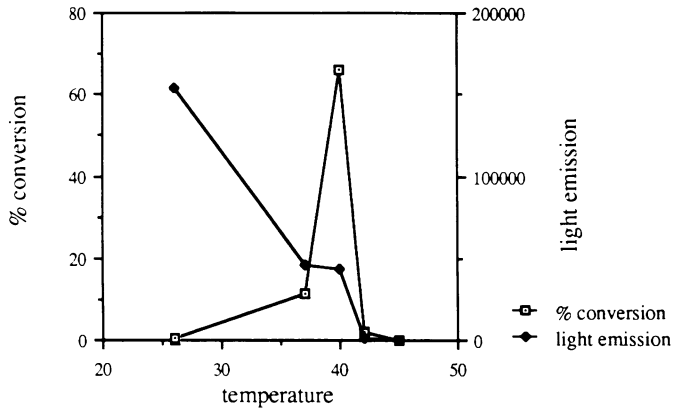


FIG. 3. Graph of CAT (open boxes) and luciferase (closed diamonds) activities in BMS protoplast extracts as a function of the protoplast incubation temperature. Protoplasts were electroporated (field strength 600 V/cm) in the presence of both phsp70cat and pCaMVLuc, incubated for 12 h at 26°C, then transferred to higher temperatures for 6 h. Extracts were prepared and equal quantities of extracts were assayed for CAT activity and luciferase activity as described in "Materials and Methods."

of the bacterial enzyme CAT, a convenient assayable marker, and the nopaline synthase poly A addition site (4). A plasmid containing this chimeric gene, phsp70cat, was introduced into protoplasts via electroporation, and the protoplasts were incubated for 12 h at 26°C. After 12 h, the protoplasts were transferred to higher temperatures for 6 h and then harvested.

Figure 3 shows a graph of the CAT activity (open boxes) present in extracts from maize protoplasts electroporated in the presence of phsp70cat as a function of the temperature during the 6 h incubation period. In extracts from protoplasts incubated continually at 26°C, there was only approximately four-fold more CAT activity than the background level present in the no-DNA control protoplast extracts. However, there was 35-fold more CAT activity in 37°C extracts than in 26°C extracts. The extracts which contained maximal CAT activity were from protoplasts incubated at 40°C, having approximately 200-fold more CAT activity than 26°C extracts. However, the level of CAT activity in extracts from protoplasts incubated at 42°C was only 6-fold over the activity found in 26°C extracts. The reduction of CAT activity in 42°C and 45°C extracts was not the result of in vivo inactivation of CAT during incubation because CAT is not heat-sensitive (see "Material and Methods") (28). The lower level of CAT expression at 42°C can probably be accounted for by the low level of total protein synthesis (see above).

The stimulation of expression with increasing incubation temperature shown here for CAT activity is specific for gene products whose transcription is regulated by the hsp70 promoter. A second plasmid, pCaMVluc, containing the firefly luciferase gene (9) under the control of the 35S promoter from CaMV was co-introduced with phsp70cat into BMS protoplasts. The results of the luciferase assays on the same extracts assayed for CAT activity are also shown in Figure 3 (solid diamonds). In contrast to the observed increase in CAT activity with increasing temperature, luciferase activity from pCaMVLuc did not increase in extracts from protoplasts incubated at 37°C and 40°C. Also, CAT activity in extracts did not increase as a function of protoplast incubation temperature for protoplasts that were electroporated in the presence of pCaMVCN (6), a plasmid which contains the CAT coding region under the control of the 35S promoter (data not shown). Therefore, the increase in CAT activity in protoplasts after incubation at higher temperatures occurred only when the CAT coding region was under the control of the hsp70 promoter.

DISCUSSION

With the exception of pollen, all plant organs investigated so far (2, 7, 8, 17, 35) have been shown to undergo a heat shock response. When plant tissues are incubated at temperatures approximately 10 to 20°C higher than the normal growing temperature, there is transcriptional induction of mRNAs encoding a subset of proteins (reviewed in Ref. 17). Cultured tissues of maize (8), soybean, and tobacco (3) have previously been shown to alter the pattern of polypeptides synthesized in response to high temperature treatment. Tobacco protoplasts maintained at 25°C showed maximal induction of the heat shock proteins at 40°C and protein synthesis was greatly inhibited at 42°C (22). The response observed here of electroporated maize protoplasts to high temperatures is very similar to that previously observed for maize cells and tobacco protoplasts. Thus, electroporated protoplasts preincubated for 12 h are capable of a heat shock response.

The amount of transcript of the endogenous maize hsp70 gene (pMON9508) increased 40- to 60-fold in maize coleoptiles after 2 h of incubation at 42°C (26). This temperature-dependent transcript accumulation is also seen in transgenic petunia leaves for a chimeric maize hsp70 gene containing the promoter from pMON9508 (27). The observations presented here of the temperature-dependent increase in CAT activity in maize protoplasts as a result of expression from phsp70cat are in agreement with these results. Because the mRNAs produced from the hsp70 gene and the hsp70cat gene are not identical, a quantitative comparison may not be meaningful.

Introduction into tobacco of a chimeric construct containing a *Drosophila* hsp70 promoter linked to the neomycin phosphotransferase reporter gene resulted in transcriptional induction of the neo gene after heat shock, but the message was not translated at heat shock temperatures (30, 31). In this work, CAT mRNAs synthesized in maize protoplasts from a maize hsp70 promoter

at heat shock temperatures were translated at heat shock temperatures. Therefore, the 24 nucleotides of the 107 nucleotide 5′ leader of the endogenous hsp70 mRNA not present in phsp70cat are not essential for translation at heat shock temperatures. The synthesis of 25°C polypeptides at heat shock temperatures in maize in addition to hsps (7) contrasts with the repression of normal polypeptide synthesis observed in *Drosophila* incubated at heat shock temperatures (19). Therefore, while the transcriptional induction mechanisms for the heat shock genes are highly conserved across eukaryotic kingdoms, there are other aspects of the heat shock response which do not have a high degree of conservation. The ability to introduce DNA into maize protoplasts and the ability of these protoplasts to respond to incubation at heat shock temperatures provides a useful system for the study of heat stress in maize.

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LITERATURE CITED

1. ALTON NK, D VAPNEK 1979 Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature 282: 864–869
2. ALTSCHULER M, JP MASCARENHAS 1982 Heat shock proteins and effects of heat shock in plants. Plant Mol Biol: 103–115
3. BARNETT T, M ALTSCHULER, CN MCDANIEL, J MASCARENHAS 1980 Heat shock induced proteins in plant cells. Dev Genet 1: 331–340
4. BEVAN M, WM BARNES, M CHILTON 1983 Structure and transcription of the nopaline synthase gene of T-DNA. Nucleic Acids Res 11: 369–385
5. BIGGIN MD, TJ GIBSON, GF HONG 1983 Buffer gradient gels and 35-S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80: 3963–3965
6. CALLIS J, M FROMM, V WALBOT 1987 Introns increase gene expression in cultured maize cells. Genes Dev 1: 1183–1200
7. COOPER P, T-D HO 1983 Heat shock proteins in maize. Plant Physiol 71: 215–222
8. COOPER P, T-D HO, R HAUPTMANN 1984 Tissue specificity of the heat-shock response in maize. Plant Physiol 75: 431–441
9. DE WET JR, KV WOOD, M DELUCA, D HELINSKI, S SUBRAMANI 1987 The firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 7: 725–737
10. FLECK J, A DURR, C FRITSCH, MC LETT, L HIRTH 1980 Comparison of proteins synthesized in vivo and in vitro by mRNA from isolated protoplasts. Planta 148: 453–454
11. FLECK J, A DURR, MC LETT, L HIRTH 1979 Changes in protein synthesis during the initial state of life of tobacco protoplasts. Planta 145: 279–285
12. FROMM M, J CALLIS, L TAYLOR, V WALBOT 1987 Electroporation of DNA and RNA into plant protoplasts. Methods Enzymol 153: 351–366
13. FROMM M, L TAYLOR, V WALBOT 1985 Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82: 5824–5828
14. FROMM M, L TAYLOR, V WALBOT 1986 Stable transformation of maize after gene transfer by electroporation. Nature 319: 791–793
15. HOOLEY R 1982 Protoplasts isolated from aleurone layers of wild oat (*Avena fatua* L.) exhibit the classic response to gibberellic acid. Planta 154: 29–40
16. HOWARD EA, JC WALKER, ES DENNIS, WJ PEACOCK 1987 Regulated expression of an alcohol dehydrogenase 1 chimeric gene introduced into maize protoplasts. Planta 170: 535–540
17. KIMPEL JA, J KEY 1985 Heat shock in plants. Trends Biochem Sci 3: 353–358
18. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
19. LINDQUIST S 1986 The heat shock response. Methods Enzymol 55: 1151–1191
20. LOVEYS BR, SP ROBINSON 1987 Abscisic acid synthesis and metabolism in barley leaves and protoplasts. Plant Sci 49: 23–30
21. MANS RJ, DG NOVELLI 1960 A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochem Biophys Res Commun 3: 540–548
22. MEYER Y, Y CHARTIER 1983 Long-Lived and short-lived heat shock proteins in tobacco mesophyll protoplasts. Plant Physiol 72: 26–32
23. MORAN N, G EHRENSTEIN, K IWASA, C BARE, C MISCHKE 1894 Ion channels in plasmalemma of wheat protoplasts. Science 226: 835–837
24. PASZKOWSKI J, RD SHILLITO, M SAUL, V MANDIK, T HOHN, B HOHN, I POTRYKUS 1984 Direct gene transfer to plants. EMBO J 3: 2717–2722
25. POMEROY MK, JB MUDD 1987 Chilling sensitivity of cucumber cotyledon protoplasts and seedlings. Plant Physiol 84: 677–681
26. ROCHESTER D, JA WINTER, DM SHAH 1986 The structure and expression of maize genes encoding the major heat shock protein hsp70. EMBO J 5: 451–458
27. SHAH DM, DE ROCHESTER, GG KRIVI, CM HIRONAKA, TJ MOZER, RT FRALEY, DC TIEMEIER 1985 Structure and expression of maize hsp70 gene. *In* J Key, T Kosuge, eds, Cellular and Molecular Biology of Plant Stress. Alan R. Liss, New York, pp 181–200
28. SHAW WV 1975 Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol 43: 737–755
29. SHROEDER JI, R HEDRICK, JM FERNANDEZ 1984 K^+ Selective single channels in guard cell protoplasts of *Vicia faba*. Nature 312: 361–362
30. SPENA A, J SCHELL 1987 The expression of a heat-inducible chimeric gene in transgenic tobacco plants. EMBO J 206: 436–440
31. SPENA A, R HAIN, U ZIERVOGEL, H SAEDLER, J SCHELL 1985 Construction of a heat-inducible gene for plants. Demonstration of heat-inducible activity of the Drosophila hsp70 promoter in plants. EMBO J 4: 2739–2743
32. VERNET T, J FLECK, A DURR, C FRITSCH, M PINCK, L HIRTH 1982 Expression of the gene coding for the small subunit of ribulosebisphosphate carboxylase during differentiation of tobacco plant protoplasts. Eur J Biochem 126: 489–494
33. WAGNER GJ, HW SIEGELMAN 1975 Large scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature plant tissues. Science 190: 1298–1299
34. WALKER JC, EA HOWARD, ES DENNIS, WJ PEACOCK 1987 DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene. Proc Natl Acad Sci USA 84: 6624–6628
35. XIAO C, J MASCARENHAS 1985 High temperature induced thermotolerance in pollen tubes of *Tradescantia* and heat shock proteins. Plant Physiol 78: 887–890