The effect of ethylene on MAPKinase-like activity in *Arabidopsis thaliana*

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Abstract Protein kinase activity was studied in cytosolic extracts from leaves of wild type *Arabidopsis thaliana*, the ethylene-insensitive mutant, *etr1*, and the constitutive triple-response mutant, *ctr1*. Treatment of wild type with ethylene resulted in increased myelin basic protein (MBP) phosphorylation. In *etr1*, constitutive protein kinase activity was lower than in wild type, but in *ctr1*, activity was enhanced. A protein of $M_r \sim 47$ kDa associated with MBP-phosphorylating activity was detected using in gel protein kinase assays and phosphorylation of this protein was promoted by ethylene treatment in wild type while activity in the mutants reflected that of MBP phosphorylation. Both MAPKinase (ERK 1) and phosphotyrosine antibodies immunoprecipitated MBP-phosphorylating activity and detected a polypeptide band at $M_r \sim 47$ kDa. Immunoprecipitated MBP-phosphorylating activity was again much lower in *etr1* compared to wild type but much higher in *ctr1*. Antibodies to phosphorylated MAPKinase recognised proteins at ~47 kDa and the signal was upregulated in response to ethylene. The data obtained suggest that the detected protein(s) is a MAPKinase and provide further evidence confirming that a MAPKinase cascade(s) is involved in ethylene signal transduction.

Key words: Ethylene signal transduction; MAPKinase; *Arabidopsis thaliana*

1. Introduction

Genetic and biochemical evidence suggests that ethylene signal transduction may, at least in some responses, be mediated through a MAPKinase cascade [1,2]. The *ctr1* mutant of *Arabidopsis* exhibits constitutive ethylene responses and the gene corresponding to the mutation has been cloned and found to resemble the Raf family of serine/threonine protein kinases [1]. This family encodes the MAPKKKinases which, in animal systems, have been reported to phosphorylate MAPKKinases which then catalyse the activation of MAPKinases [3]. A variety of genes encoding MAPKinases have been identified from plants [4] and data so far obtained suggest that MAPKinases play a role in plant responses to environmental stresses [5] and in the mediation of responses to ABA [6], auxin [7], gibberellin [8] and salicylic acid [9].

The aim of the present work was firstly to determine the effect of ethylene on MAPKinase-like activity in cytosolic extracts from leaves of wild type *Arabidopsis thaliana* and secondly to compare activity in wild type with that of the ethylene-insensitive mutant, *etr1* [10], which appears to be defective in protein phosphorylation mechanisms [11] and the constitutive triple-response mutant, *ctr1*.

2. Materials and methods

2.1. Plant material and treatments

Plants of *A. thaliana* (Columbia wild type, *etr1* and *ctr1* mutants) were grown in compost in a greenhouse under a 16 h photoperiod at 20°C for 6 weeks. Rosette leaves (~10 g FW) were placed in sealed 1 l Kilner jars lined with moist filter paper to which 1 μl l^{-1} ethylene was applied for 1 h in the light at room temperature. After treatment, the leaves were used immediately for protein isolation.

2.2. Protein isolation

All chemicals were purchased from Sigma or Fisher and all procedures were carried out at 4°C. Plant material was homogenised using a Polytron or MSE Atomix homogeniser in buffer (1:1.5, w/v) which contained 50 mM Tris–HCl, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM diethyldithiocarbamic acid sodium salt, 5 mM L-ascorbic acid, 3.6 mM L-cysteine and 250 mM sucrose at pH 7.6. Polyvinylpolypyrrolidone was added to the buffer in a ratio of 1:10 (w/w) of plant tissue. The homogenate was filtered through 200 μm nylon mesh and the filtrate centrifuged at $12\,000 \times g$ for 20 min, the pellet discarded and the supernatant centrifuged at $130\,000 \times g$ for 3 h. The final supernatants were transferred into an appropriate buffer by gel filtration on PD-10 columns (Pharmacia Biotech) and used for biochemical and immunological assays. Protein concentration was determined using the BCA protein assay (Pierce).

2.3. Protein kinase assay

Protein kinase activity was assayed using a modified method of Sontag et al. [12]. Supernatant fractions (5–15 μg of protein) were incubated with myelin basic protein (MBP, 0.25 mg ml^{-1}; Gibco/BRL) for 20 min at 30°C in a final volume of 50 μl of buffer containing 20 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM benzamidine, 10 μM ATP and 74 kBq [γ-^{32}P]ATP (Amersham). The reaction was stopped by the addition of trichloroacetic acid (TCA)–sodium pyrophosphate solution with the final concentration of each component being 5 and 1%, respectively. Proteins were pelleted and washed with stop solution. The pellets were suspended in 0.2 N NaOH, mixed with Emulsifier-Safe (Packard) scintillation cocktail and the radioactivity counted in a Packard TRI-CARB 2500 TR liquid scintillation analyser. Incorporation of ^{32}P into MBP was calculated as the difference between radioactivity of samples incubated in the presence and absence of MBP [13].

Alternatively, the reaction was terminated by adding an equal volume of double concentration Laemmli sodium dodecyl sulfate (SDS) sample buffer [14] and samples were boiled for 3 min. Phosphorylated MBP was resolved using 15% SDS–polyacrylamide gel (PAG) and visualised by autoradiography on Kodak Biomax MR-1 film. Analysis of autoradiographs was carried out by scanning on a GS-690 Imaging Densitometer (Bio-Rad) and quantified using Phoretix 1D (version 4.01) software.

2.4. In gel MAPKinase assay

Supernatant fractions were transferred in 20 mM Tris–HCl, pH 7.6, by gel filtration on PD-10 columns, mixed 1:1 with double concen-

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tration SDS sample buffer containing 10 mM EDTA and 200 mM DTT and boiled for 3 min prior to electrophoresis. Electrophoresis was performed in 10% SDS–PAG to which 0.5 mg ml^{-1} MBP was added before gel polymerisation. All following incubations were performed according to Lee et al. [15]. In order to fix gels and remove SDS, gels were washed twice with 20% 2-propanol in 50 mM Tris–HCl buffer (pH 8.0) and further for 1 h in several gel volumes of 50 mM Tris–HCl (pH 8.0) containing 5 mM 2-mercaptoethanol. Protein kinase was denatured by incubation in the same buffer but containing 6 M guanidine HCl for 1 h and then renatured by five washes each of 10 min in several gel volumes of 50 mM Tris–HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol. The gels were preincubated for 1 h with 40 mM HEPES (pH 8.0) containing 2 mM DTT, 10 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.1 mM EGTA. Phosphorylation of MBP within the gel was carried out by incubation for 1 h in the same buffer but supplemented with 74 kBq ml^{-1} of [γ-^{32}P]ATP and 40 μM ATP. The reaction was terminated by washing the gels several times in the TCA–sodium pyrophosphate stop solution until the radioactivity in the washings reached background levels. Finally, the gels were fixed for 1 h in 20% ethanol:7.5% acetic acid, dried and autoradiographed.

2.5. Immunoprecipitation of MAPKinase

Supernatants were transferred in buffer containing 50 mM Tris–HCl (pH 7.6), 100 mM NaCl, 1 mM PMSF, 1 mM EGTA, 2 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM benzamidine, 1 mM DTT and 0.1% Tween 40 by gel filtration on PD-10 columns. The extracts (1 mg protein) were mixed with 10 μg anti-ERK 1 rabbit polyclonal or 5 μg anti-phosphotyrosine mouse monoclonal antibodies (Santa Cruz Biotechnology) and 25 μl of 50% pre-washed Protein G-Sepharose (Pharmacia Biotech) and incubated with continuous shaking at 4°C overnight. The mixtures were centrifuged for 5 min at 6000×*g* and the pellets washed twice with 0.5 ml of buffer followed by two further washes with reaction buffer (40 mM HEPES, pH 8.0, containing 2 mM DTT, 10 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.1 mM EGTA). The MAPKinase reaction was carried out in 40 μl of reaction buffer to which 0.25 mg ml^{-1} MBP, 40 μM ATP and 185 kBq [γ-^{32}P]ATP were added. The reaction was allowed to proceed for 30 min at room temperature and was terminated by the addition of 40 μl of double SDS sample buffer and boiling for 5 min. After pelleting of Protein G-Sepharose, proteins were electrophoresed and autoradiographed as above.

2.6. Immunodetection of MAPKinase

After electrophoresis (12.5% SDS–PAG), proteins were transferred onto Hybond-C membranes (Amersham) for Western blotting using a Trans-Blot SD Electrophoretic Semi-Dry Transfer Cell (Bio-Rad). Blots were processed using a chemiluminescence kit containing antibodies against phosphorylated and non-phosphorylated MAPKinase according to the manufacturer's instructions (PhosphoPlus MAPK Antibody kit, New England Biolabs Inc.). MAPKinase (p42) protein in the phosphorylated and non-phosphorylated forms served as positive and negative controls, respectively.

3. Results

3.1. Protein kinase assays

Initially, protein kinase activity was assessed in vitro by the incorporation of radioactive phosphate into TCA-precipitated material from cytosolic extracts of ethylene-treated and untreated wild type leaves in the presence and absence of MBP as a substrate. As protein kinases can be dephosphorylated and inactivated by protein phosphatases present in the leaf extracts, the assay mixture therefore contained β-glycerophosphate to inhibit serine/threonine phosphatases and orthovanadate to inhibit protein tyrosine phosphatases. The mixture also contained EGTA to chelate Ca^{2+} and so to inhibit MAPKKinase and divalent cation-dependent proteinases and phosphatases. As MAPKinase activity measured in extracts has been reported to be nearly as high in the presence of Mn^{2+} as in Mg^{2+} [16], a ratio of Mg^{2+}/Mn^{2+} of 10:1 was used. When the leaves were treated with 1 μl l^{-1} ethylene for 1 h prior to extraction, the incorporation of phosphate in wild type extracts was increased from 5535 ± 715 to 17 533 ± 890 cpm per 10 μg protein. In order to show that MBP was phosphorylated, extracts were electrophoresed and autoradiographed. Fig. 1 shows an autoradiograph of SDS–PAG of cytosolic extracts phosphorylated in vitro in the presence of MBP. The phosphorylated bands electrophoresed with a M_r of ~20 kDa which is characteristic of MBP. Phosphorylation of MBP occurs in an MBP concentration-dependent manner in both ethylene-treated and untreated extracts. The pattern of MBP phosphorylation reflects that measured in extracts by scintillation counting hence it appears that ethylene promotes MBP phosphorylation in cytosolic extracts of wild type. There was no effect of ethylene on MBP-phosphorylating activity in *etr1* (data not shown).

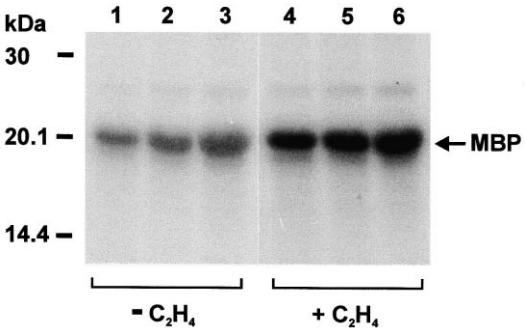

Fig. 1. Autoradiograph of SDS–PAG of MBP phosphorylation in vitro. Protein loading was ~2 μg per lane and the amount of MBP was 1.5 (lanes 1 and 4), 3.0 (lanes 2 and 5) and 6.0 (lanes 3 and 6) μg per lane. Extracts from untreated (lanes 1–3) and ethylene-treated (lanes 4–6) wild type leaves.

3.2. In gel protein kinase assay

In order to characterise further the protein(s) associated with MBP kinase activity, proteins were electrophoresed and renaturated within the gel prior to phosphorylation with radiolabeled ATP and a typical autoradiograph is shown in Fig. 2. MBP-phosphorylating activity is associated with a protein of M_r ~47 kDa in both wild type and mutant extracts and phosphorylation is promoted by ethylene treatment in wild type. The constitutive level of activity in *etr1* is much lower than in wild type but is significantly upregulated in *ctr1* to an even greater extent than ethylene-treated wild type. When ex-

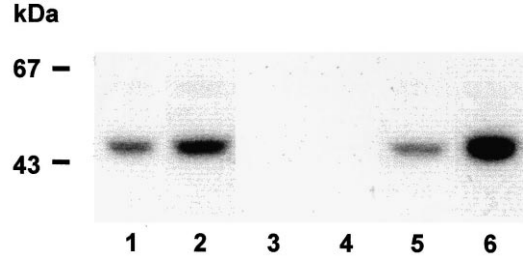

Fig. 2. Autoradiograph of in gel MAPKinase assay. Gel was cast with 0.5 mg ml^{-1} MBP. Extracts were prepared from ethylene-untreated (lanes 1 and 3) and treated (lanes 2 and 4) wild type, *etr1* (lane 5) and *ctr1* (lane 6) leaves. MBP-phosphorylating activity is shown in lanes 1, 2, 5 and 6. This activity was eliminated in the presence of excess ATP (lanes 3 and 4).

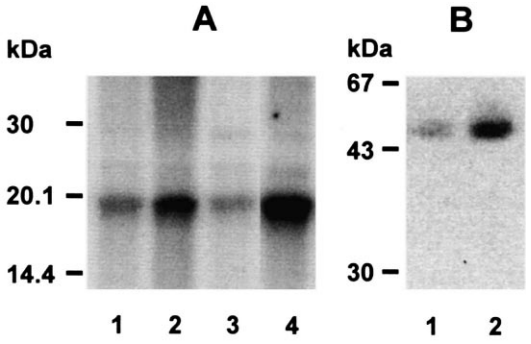

Fig. 3. Autoradiographs of MBP-phosphorylating activity (A) and in gel MAPKinase assay (B) of extracts immunoprecipitated with ERK 1 antibodies. Lane 1, preparation from untreated wild type leaves; lane 2, preparation from ethylene-treated wild type leaves; lane 3, preparation from *etr1* and lane 4, preparation from *ctr1*.

cess ATP was included, the density of bands was reduced to a barely detectable level indicating specific incorporation of radiolabeled phosphate.

3.3. Immunocharacterisation

The in gel MBP-phosphorylating protein(s) was purified further using immunoprecipitation with MAPKinase (ERK 1) antibodies. Protein immunoprecipitated with ERK 1 antibodies electrophoresed to a band of $M_r \sim 47$ kDa and displayed activity in terms of MBP phosphorylation and in gel MAPKinase assays (Fig. 3). This activity was promoted by ethylene in wild type immunoprecipitates (lanes 1 and 2). In ERK 1-immunoprecipitated extracts from *etr1* (lane 3), MBP-phosphorylating activity was reduced by 20% compared to wild type while that of *ctr1* was increased 3.5-fold (lane 4). Similar results were obtained from experiments where protein was immunoprecipitated with monoclonal phosphotyrosine antibodies (data not shown).

As activation of MAPKinase can involve phosphorylation of tyrosine and threonine residues, antibodies raised to both non-phosphorylated and phosphorylated MAPKinase were used to detect proteins in wild type extracts. Fig. 4A shows a Western blot developed with antibodies against non-phosphorylated MAPKinase where the chemiluminescence illustrates that the antibodies cross-react with polypeptides irrespective of phosphorylation status. The amount of protein at $\sim$47 kDa detected by these non-phosphorylated MAPKinase antibodies is not modified by ethylene treatment. When the antibodies to the phosphorylated form were used, the density of the band at $M_r \sim 47$ kDa was increased 3-fold as a result of ethylene treatment compared to the control (Fig. 4B). Cross-reactivity occurred only with the control protein phosphorylated on tyrosine 204 and threonine 202 (lane 2). Thus, a protein of 47 kDa shows the capacity to phosphorylate MBP, the activity of which is upregulated by ethylene, and this protein is recognised by both phosphorylated and non-phosphorylated MAPKinase antibodies.

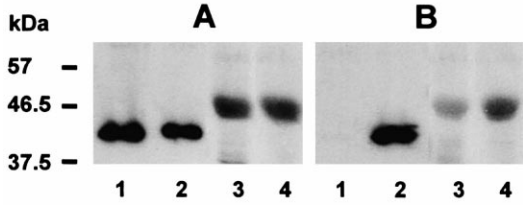

Fig. 4. Western blots of wild type extracts probed with non-phosphorylated MAPKinase (A) and phosphorylated MAPKinase (B) antibodies. Lane 1, non-phosphorylated MAPKinase control protein (PhosphoPlus MAPK Antibody kit); lane 2, phosphorylated MAPKinase control protein (PhosphoPlus MAPK Antibody kit); lane 3, untreated extract; lane 4, ethylene-treated extract.

4. Discussion

Given the effects of ethylene on MBP phosphorylation in cytosolic extracts of wild type and differences in phosphorylation in the mutants *etr1* and *ctr1*, it would appear that modulation of protein kinase activity is involved, at least in part, in ethylene signal transduction. Thus, ethylene treatment in vivo promoted MBP phosphorylation in vitro in wild type extracts. The incubation time of 1 h was selected because previous studies have shown that saturation of fast-associating ethylene-binding sites occurred at that time in leaves of *Arabidopsis* [17]. In addition, the effect of ethylene on MBP phosphorylation in pea over a period of 120 min indicates that the response is biphasic and the second peak of activity reached a maximum after 1 h incubation [18]. The differences in the extent of MBP phosphorylation in wild type, *etr1* and *ctr1* follow similar trends to the constitutive protein phosphorylation capacity [19]. However, as the levels measured represent the steady-state phosphorylation, differences may be brought about not only by altered kinase activity but also by changes in phosphatase activity [20,21], or indeed by the relative balance between these two activities. Nevertheless, it is clear from Fig. 1 that the capacity to phosphorylate MBP is greater in ethylene-treated wild type which is indicative of increased kinase activity especially as phosphatase inhibitors were incorporated into the buffers used throughout.

Estimates of the molecular weight of the enzyme responsible for MBP phosphorylation were provided by in gel protein kinase assays. This activity was localised to a band at $M_r \sim 47$ kDa which falls within the range of MAPKinases reported in plants [6,9,22], yeasts and animal systems [3]. The density of this band was increased in ethylene-treated wild type tissue but interestingly, results suggest that the protein kinase activity was lower in *etr1* but higher in *ctr1* compared to wild type (Fig. 2). However, in vitro phosphorylation estimates comprise a total of activities and even the in gel assays may represent MAPKKinase activity as MBP is not a highly specific substrate and also that MAPKinases are the endogenous substrates for MAPKKinases. Nevertheless, present evidence suggests that the protein kinase activity detected in in gel assays is that of MAPKinase. The molecular weight is comparable to those of other MAPKinases, whereas the molecular weights reported for MAPKKinases are over a much wider range. Additionally, MAPKinase antibodies (ERK 1) immunoprecipitate activity which phosphorylates MBP in vitro and immunoprecipitates display in gel MAPKinase activity (Fig. 3). Both these activities are upregulated in ethylene-treated leaves. The results from immunodetection experiments with phospho-MAPKinase antibodies indicate that the increased phosphorylation of the 47 kDa polypeptide in ethylene-treated extracts is consistent with activation of MAPKinase through phosphorylation of tyrosine and threonine residues (Fig. 4).

As it has been suggested that ethylene inactivates transduc-

tion on binding to ETR1 [23], the relatively low protein kinase activity detected in *etr1* is difficult to interpret until further information on the expression and function of the ethylene receptor family is available. Equally, the upregulation of protein kinase activity in *ctr1* is difficult to reconcile with the proposal that CTR1 acts as a negative regulator of ethylene transduction especially as the constitutive activity is higher than that of ethylene-treated wild type. It is possible that in *ctr1*, the proposed MAPKinase cascade is locked in an activated state. However, this is unlikely since a number of known *CTR1* mutant alleles are null mutations which essentially abolish the CTR1 protein but all show the constitutive ethylene responses [1]. An alternative explanation is that the ethylene signal is transduced through interacting cascades and that the activity detected in *ctr1* is regulated by another Raf-type protein (NCBI Database, [24]).

Future work aims to further characterise and purify the ethylene-modulated MAPKinase(s) to probe for upstream kinases in ethylene signal transduction in wild type, *etr1* and *ctr1* mutants of *Arabidopsis*.

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References

[1] Kieber, K.A., Rothenberg, M., Roman, G., Feldman, K.A. and Ecker, J.R. (1993) Cell 72, 427–441.
[2] Smith, A.R., Berry, A.W., Harpham, N.V.J., Hemsley, R.J., Holland, M.G., Moshkov, I., Novikova, G. and Hall, M.A. (1997) in: Biology and Biotechnology of the Plant Hormone Ethylene (Kanellis, A.K., Chang, C., Kende, H. and Grierson, D., Eds.), pp. 77–86, Kluwer Academic Publishers, Dordrecht.
[3] Marshall, C.J. (1994) Curr. Opin. Genet. Dev. 4, 82–89.
[4] Machida, Y., Nishihama, R. and Kitakura, S. (1997) Crit. Rev. Plant Sci. 16, 481–496.
[5] Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. (1996) Proc. Natl. Acad. Sci. USA 93, 765–769.
[6] Knetsch, M.L., Wang, M., Snaar-Jagalska, B.E. and Heimovaara-Dijkstra, S. (1996) Plant Cell 8, 1061–1067.
[7] Mizoguchi, T., Gotoh, Y., Nishida, E., Yamaguchi-Shinozaki, K., Hayashida, N., Iwasaki, H., Kamada, H. and Shinozaki, K. (1994) Plant J. 5, 111–122.
[8] Huttly, A.K. and Phillips, A.L. (1995) Plant Mol. Biol. 27, 1043–1052.
[9] Zhang, S. and Klessig, D.F. (1997) Plant Cell 9, 809–824.
[10] Bleecker, A.B., Estelle, M.A., Somerville, A.S. and Kende, H. (1988) Science 241, 1086–1089.
[11] Novikova, G., Moshkov, I., Smith, A.R., Kulaeva, O.N. and Hall, M.A. (1999) Planta 208, 239–246.
[12] Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M. and Mumby, M. (1993) Cell 75, 887–897.
[13] Boulton, T.G., Gregory, J.S. and Cobb, M.H. (1991) Biochemistry 30, 278–286.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Lee, H., Ghose-Dastidar, J., Winawer, S. and Friedman, E. (1993) J. Biol. Chem. 268, 5255–5263.
[16] Leevers, S.J. and Marshall, C.J. (1992) EMBO J. 11, 569–574.
[17] Sanders, I.O., Harpham, N.V.J., Raskin, I., Smith, A.R. and Hall, M.A. (1991) Ann. Bot. 68, 97–103.
[18] Hall, M.A., Smith, A.R., Novikova, G.V. and Moshkov, I.E. (1999) Plant Biol. 1, 46–56.
[19] Smith, A.R., Moshkov, I.E., Novikova, G.V. and Hall, M.A. (1999) in: Biology and Biotechnology of the Plant Hormone Ethylene II (Kanellis, A.K., Chang, C., Klee, H., Bleecker, A.B., Pech, J.C. and Grierson, D., Eds.), pp. 77–84, Kluwer Academic Publishers, Dordrecht.
[20] Gupta, R., Huang, Y., Kieber, J. and Luan, S. (1998) Plant J. 16, 581–589.
[21] Meskiene, I., Bögre, L., Glasser, W., Balog, J., Brandstötter, M., Zwerger, K., Ammmerer, G. and Hirt, H. (1998) Proc. Natl. Acad. Sci. USA 95, 1938.
[22] Usami, S., Banno, H., Ito, Yu., Nishihama, R. and Machida, Ya. (1995) Proc. Natl. Acad. Sci. USA 92, 8660–8664.
[23] Bleecker, A.B. (1999) Trends Plant Sci. 4, 269–274.
[24] Jouannic, S., Hamal, A., Leprice, A.-S., Tregear, J.W., Kreis, M. and Henry, Y. (1999) Gene 233, 1–11.