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Short communication

Transcripts of the two NADPH protochlorophyllide oxidereductase genes *PorA* and *PorB* are differentially degraded in etiolated barley seedlings

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Abstract

The light-dependent recduction of protochlorophyllide to chlorophyllide in higher plants is catalyzed by two closely related enzymes, the NADPH-Pchlide oxidoreductases A and B that are encoded by the nuclear genes *PorA* and *PorB*, respectively. The expression of the *PorA* gene is negatively regulated by light. It has formerly been reasoned that, apart from the well-studied transcriptional down-regulation, a post-transcriptional mechanism may exist that contributes markedly to the light-induced decline of *PorA* mRNA steady-state levels. We investigated the degradation kinetics of the *PorA* messenger after inhibiting RNA synthesis with cordycepin. The *PorA* mRNA was found to be inherently unstable. In contrast, the *PorB* mRNA was shown to be stabilized in the presence of cordycepin, suggesting degradation by a mechanism different from that of *PorA* mRNA degradation. The *PorA* messenger instability is postulated to be conferred by a previously described plant-specific DST element in its 3′UTR.

NADPH-protochlorophyllide oxidoreductase (POR; EC 1.6.99.1) catalyzes the only known light-dependent step in chlorophyll synthesis of higher plants, the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) [3, 4]. In barley and *Arabidopsis* two distinct POR proteins, A and B, have been recently identified [1, 12]. Even though the *in vitro* activities of the two enzymes were similar, the expression of their genes during the transformation of etiolated to green seedlings was distinct. While *PorB* mRNA remained at an approximately constant level in dark-grown and green seedlings the *PorA* mRNA rapidly declined during illumination of dark-grown seedlings and soon disappeared [1, 12]. *In vitro* run-on transcription experiments with isolated nuclei had demonstrated that the negative effect of light on *PorA* mRNA concentration correlates with a phytochrome (P_{fr})-induced reduction of *PorA* gene transcription [14, 16]. The reduced transcriptional activity of the *PorA* gene, however, could not fully account for the rapid and drastic decrease in *PorA* mRNA levels. It was, therefore, proposed that POR expression may

also be negatively regulated at the level of mRNA stability [15]. *PorA* is one of the few known plant genes which are negatively regulated by light. Its stringent control is reminiscent of that of other eukaryotic genes involved in the regulation of cell growth and development such as phytochrome (PhyA) in higher plants [19, 22] and lyphokine, cytokine and protooncogenes in mammalians [20]. The transcripts of many of these regulatory genes have been shown to be highly unstable, which facilitates rapid alterations in protein levels [18]. In the present work we have analyzed the stability of *PorA* mRNA by measuring the degradation kinetics of this mRNA *in vivo* in the presence of the inhibitor of RNA synthesis, cordycepin. A similar approach has previously been used to determine the half-lives of Phy A, actin and β-tubulin mRNA in oat [5, 22]. Furthermore, the stability of *PorA* mRNA was compared to that of *PorB* mRNA that is constitutively expressed in the dark and during illumination of etiolated seedlings.

The determination of barley leaf mRNA half-lives

The persistence of *PorA* and *PorB* mRNAs was measured in primary leaves of etiolated seedlings using a method formerly used to investigate the decay of mRNAs in oat leaves [5]. Five-day old etiolated barley seedlings were grown as described [21] and primary leaf blades separated from the coleoptile in complete darkness or under dim green safe light, repectively. About 15 leaves were prepared within 5 min by cutting each leaf transversely into four pieces and placing them into a 50 ml translucent reaction tube containing 25 ml of incubation buffer (1 mM Pipes, pH 6.25, 1 mM sodium citrate, 1 mM KCl, 15 mM sucrose, as described [22]). The incubation buffer contained the desired cordycepin (Sigma, C3394) concentration, or was devoid of cordycepin for control samples, as indicated for each experiment. The submersed leaf tissue was subjected to a 1 min vacuum infiltration and the tubes were subsequently wrapped in aluminum foil. The leaf samples were pre-incubated in the dark for 30 min. During the subsequent incubation the leaf samples were taken at various lengths of time, immediately frozen in liquid nitrogen and stored at -80 °C. RNA was extracted using a small scale procedure described by Wadsworth *et al.* [24]. The guanidinium isothiocyanate extraction buffer used was as described by Chirgwin *et al.* [6]. First, the effect of different concentrations of cordycepin on the P_{fr}-induced expression of *Lhcb1* mRNA was tested to optimize the inhibitor concentration (Fig. 1). Etiolated barley leaves were prepared under dim green safe light and preincubated for 30 min in the dark in the presence of varying concentrations of cordycepin. Leaves were then exposed to a 5 s red light pulse to induce *Lhcb1* gene transcription [16]. Afterwards, samples were incubated for another 4 h in the dark, harvested and the RNAs extracted. As shown in Fig. 1, the maximum inhibition of *Lhcb1* gene transciption was reached at a concentration of 500 μg/ml. A similar optimum concentration of cordycepin had been found previously in studies with oat leaves [5].

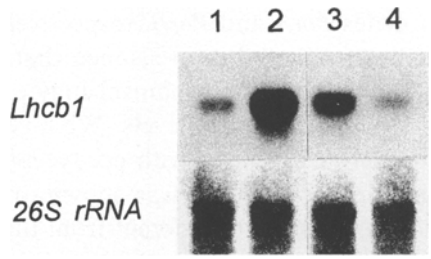

Fig. 1. The effect of different concentrations of cordycepin on the level of *Lhcb1* mRNA in 5-day old etiolated barley leaves which were exposed to a red light pulse. Barley primary leaves of etiolated seedlings were separated from the coleoptile sheath and cut into four pieces. Leaf segments were transferred to incubation buffer, vacuum infiltrated for 1 min and preincubated for 30 min in the dark prior to a 5 s red light pulse treatment. Afterwards samples were incubated at various inhibitor concentrations for 4 h under constant swirling in complete darkness (lanes 2, 3, 4). The control sample in lane 1 was kept in complete darkness without a red light pulse. Cordycepin concentrations were as follows: lane 1, no cordycepin; lane 2, no cordycepin; lane 3, 100 μg/ml cordycepin; lane 4, 500 μg/ml cordycepin. After 4 h incubation the leaf tissue was blotted dry and the RNA was extracted. Equal amounts of total RNA were loaded in each lane (8 μg) and replicate blots hybridized to an antisense RNA probe for *Lhcb1* (pAB96) [12] and *26S rRNA* (pc222) [12].

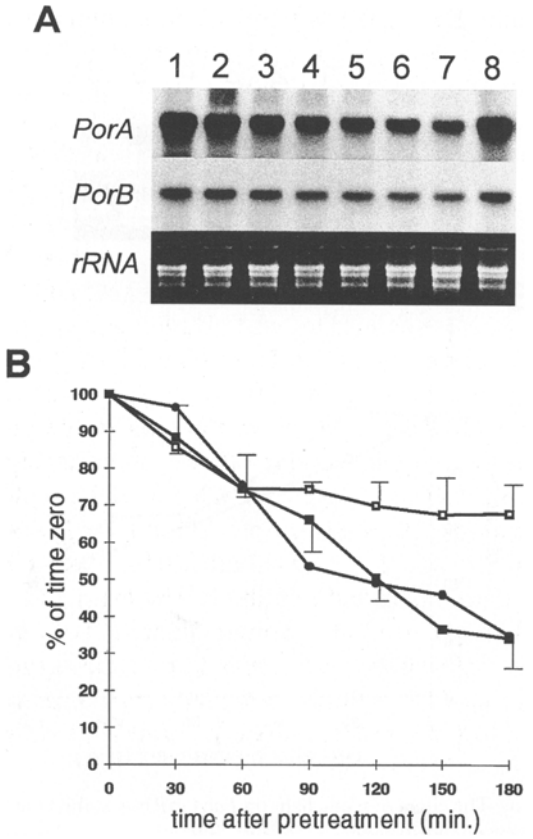

Fig. 2. The effect of cordycepin on mRNA abundances of *PorA*, *PorB* and *PhyA* in dark-grown barley leaves. A. The decay of *PorA* and *PorB* mRNA. Primary leaves of 5-day old etiolated barley seedlings were separated from the coleoptile sheath and leaf blades cut transversely into four pieces. Leaf sections were transferred into incubation buffer containing 500 μg/ml cordycepin and preincubated for 30 min in the dark. The control sample in lane 8 was incubated without cordycepin. Samples were harvested at 0 (lane1, time zero = 100%), 30 (2), 60 (3), 90 (4), 120 (5), 150 (6) and 180 (7) min after pretreatment. The control sample in lane 8 was incubated without cordycepin in the dark for a total of 210 min. RNA (8 μg) was loaded in each lane and replicate blots hybridized to antisense RNA probes for *PorA* (pA) and *PorB* (pB). rRNA was visualized for each independent experiment by ethidium bromide staining of an agarose-formaldehyde gel photographed on a UV-transilluminator prior to blotting. B. A replicate blot was probed with an antisense RNA probe for oat *PhyA* (pAPSX2.7). RNA samples from lane 1 to 7 were the same as described for the blot in panel A. Quantitations of *PorA*, *PorB* and *PhyA* mRNA levels are expressed as a percentage of the signal strength at time zero (lane 1). The intensities of bands were quantified on a PhosphorImager. The data for *PorA* (black squares) and *PorB* (open squares) show the means of 3 independent experiments for timepoint 30, 90, 120 and 150 min, and 4 independent experiments for timepoints 0, 60, and 180 min. Error bars indicate the standard error of the means of the different experiments. The data for *PhyA* (black dots) were obtained from two independent experiments.

Measurement of PorA *and* PorB *mRNA decay rates in the dark*

For all subsequenct experiments a cordycepin concentration of 500 μg/ml was chosen. Leaves of 5-day old etiolated seedlings were incubated with cordycepin as described above. Four independent experiments were performed, two with leaves kept in complete darkness and two with leaves that were isolated under dim green safe light and kept subsequently in the dark. Both sets of experiments yielded comparable results. The mRNA for *PorA* appears to be continuously degraded during the dark incubation. The apparent half-life of *PorA* mRNA was 2 h (Fig. 2B). The time courses of *PorB* mRNA decay were different from those of *PorA*. Both mRNAs were degraded at a similar rate during the first hour of incubation. However, shortly thereafter the decay of *PorB* mRNA stopped and the mRNA remained at a constant level of ca. 70%. The *PorB* mRNA seemed to be stabilized after ca. 1 h of cordycepin treatment, while the *PorA* messenger continued to decay at the initial rate. The stabilization of the *PorB* messenger RNA after only 1 h of the cordycepin treatment suggests that ongoing RNA synthesis is necessary to guarantee continuous *PorB* mRNA decay. Since treatment with inhibitors such as cordycepin have been shown previously in several cases to result artificially in the stabilization of plant transcripts we tried to minimize secondary effects by measuring *PorA* and *PorB* degradation after relatively short time intervals, i.e., less than 3 h after the beginning of inhibitor treatment. The constant decay of *PorA* (and *PhyA*, see below) indicates that the stabilizing effect observed for *PorB* mRNA, in the presence of cordycepin, seems to be specific for this transcript. The control of cut leaves treated without cordycepin shows that during incubation the decay of *PorA* and *PorB* mRNAs is not attributable to unspecific degradation of RNAs.

As a further control replicate blots of the two experiments performed in complete darkness were hybridized with an oat *PhyA* antisense RNA probe generated from plasmid pAPSX2.7 [22]. A representative quantitation of *PhyA* signal strengths is shown in Fig. 2B. The *PhyA* control was included for two reasons. First, *PhyA* in oat seedlings has formerly been shown to be inherently unstable [5, 22], displaying a half-life between 50 min and 2 h. Second, a negative effect of light on the expression of *PhyA* and *PorA* genes occurs in both cases at the level of gene transcription and is induced by active phytochrome (P_{fr}). Fig. 2 demonstrates that *PhyA* mRNA is degraded rapidly in barley leaves in the dark, with an apparent half-life of about 90 min that is similar to that of *PorA* mRNA degradation and that is also consistent with the reported half-life of *PhyA* mRNA in oat coleoptiles [22]. The observed half-life of *PorA* mRNA in leaves of etiolated barley seedlings could account for the rapid down-regulation of *PorA* mRNA levels during illumination [2].

Measurement of PorA *decay rates in the light*

Light has been shown to affect the stability of several plants transcripts, namely the *rbcS* of soybean [23] and the *Fed-1* of pea [7]. We have studied possible changes in the persistence of *PorA* mRNA during illumination of dark-grown barley seedlings. Five-day old etiolated barley primary leaves were prepared and preincubated with or without cordycepin in complete darkness for 30 min before the samples were transferred to continuous white light. Two of such experiments were performed independently and in parallel with two control experiments conducted in complete darkness. As shown in Fig. 3 no pronounced difference in the time courses of *PorA* mRNA decay exists between light- and dark-treated samples. Thus, the stability of *PorA* transcript appears not to be affected by light. The observed short apparent half life of *PorA* mRNA together with the phytochrome-induced reduction of the rate of transcription [14, 16] leads to he rapid light-induced decrease of *PorA* mRNA steady-state levels in etiolated seedlings exposed to light.

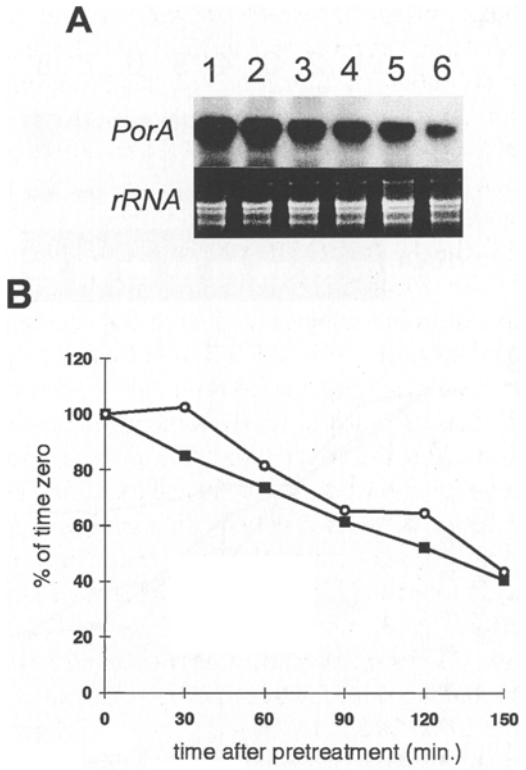

Fig. 3. The effect of white light on *PorA* mRNA stability in the presence of cordycepin. A. Five-day old etiolated barley seedlings were treated as described in the legend to Fig. 2 including a 30 min pretreatment in the dark. Samples in lane2 to 6 were then transferred to continuous white light and incubated for 30 (lane 2), 60 (3), 90 (4), 120 (5) and 150 (6) min in the presence of 500 μg/ml cordycepin. Total RNA (8 μg) was loaded in each lane and two replicate blots were probed with an antisense RNA probe for *PorA* (pA). rRNA was vizualized by ethidium bromide staining of an agarose-formaldehyde gel prior to blotting. B. Quantitation of the relative signal strength of *PorA* mRNA bands. Data of two independent light experiments (open circles) are shown in relation to values obtained from two independent experiments which were performed in complete darkness (black squares). Part of the data for *PorA* derived from the two dark experiments are also included in Fig. 2.

The existence of a plant instability determinant in the PorA *nRNA 3′ UTR*

Several sequence elements that regulate the stability of eukaryotic transcripts have been de-

scribed, many of which reside in the 3′-untranslated region of transcripts [20]. Since the apparent half life of *PorA* mRNA has been found to differ in this respect from that of the *PorB* mRNA we compared the 3′UTRs of both transcripts. The 3′UTRs of the two *Por* mRNA are different in length. The *PorA* 3′ non-coding region is 233, the *PorB* 3′UTR is 96 nucleotides long. Both *Por* downstream sequences when aligned exhibited no homologies. A search for instability determinants of both 3′UTR revealed that the *PorB* sequence did not contain any conserved motif that is known to destabilize eukaryotic mRNAs. On the other hand, the *PorA* 3′ non-coding region harbours a sequence identified as a DST element, which has been demonstrated previously to target reporter transcripts for rapid decay in tobacco [17]. The DST element was first described in 3′ non-coding regions of SAUR transcripts from soybean, mungbean and *Arabidopsis* and was also found in auxin-inducible transcripts from tobacco [13, 17, 25]. SAUR genes are involved in auxin-induced cell elongation [13] and their mRNAs have been demonstrated to be very unstable with an mRNA half-life of 10 to 43 min [8]. The *PorA* sequence element exhibits all known features of a DST element with three highly conserved homology blocks separated by two regions of variable length (Fig. 4). The sequences ATAGAT and GTA in the second and third block are invariant among DST elements of SAUR genes and thus may be important functional determinants [9]. These sequences are conserved also within the putative *PorA* DST element (Fig. 4). All DST sequences described so far reside downstream of the coding region of plant SAUR mRNAs, and it is believed that this element is unique to plants [11].

Conclusions

We have measured half-lives of *PorA* and *PorB* mRNAs in the presence of cordycepin. The degradation kinetics of the *Por* mRNAs are different in the dark. The *PorA* transcript is continuously degraded during an incubation of up to 3 h, whereas the *PorB* mRNA is stabilized after 1 h of inhibitor treatment. Thus, both transcripts are likely to be degraded by two different mechanisms, although the initial rates of degradation appear to be similar during the first hour of treatment. In contrast to *PorA* mRNA, *PorB* mRNA turnover seems to depend on an ongoing RNA synthesis. Previously, synthetic DST elements have been shown to confer instability to reporter transcripts. These data suggest that DST elements found in various unstable plant mRNAs contribute largely to an accelerated degradation. The DST sequence element in the 3′UTRs of *PorA* mRNA is a good candidate for a *cis*-acting instability determinant of this transcript. The inherent instability of *PorA* mRNA argues for a constitutive mechanism that renders the transcript unstable under dark and light conditions. Further experiments, however, are needed to confirm the proposed function of the DST element as a major determinant for *PorA* mRNA instability in barley seedlings.

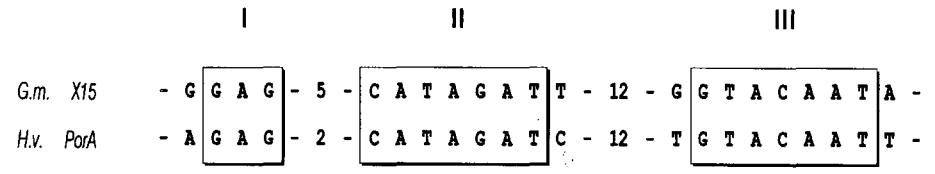

Fig. 4. Alignment of the putative DST sequence motif of the barley *PorA* gene (*H.v. PorA*) with a DST element of a representative *SAUR* gene (*X15*) from soybean (*G.m. X15*). The DST element consists of three conserved homology blocks which are separated by two regions of variable length. The three main regions which are conserved among the DST element of the *PorA* and the *SAUR* transcript are boxed (I, II, III). The distance between the stop codon and the DST element is 148 nucleotides for *PorA*, and 19 nucleotides for *X15*.

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References

1. Armstrong GA, Runge S, Frick G, Sperling U, Apel K: Identification of NADPH: protochlorophyllide oxidoreductases A and B: A branched pathway for light-dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. Plant Physiol 108: 1505–1517 (1995).
2. Batschauer A, Apel K: The inverse control by phytochrome of the expression of two nuclear genes in barley (*Hordeum vulgare* L.). Eur J Biochem 143: 593–597 (1984).
3. Beale SI: Biosynthesis of photosynthetic pigments. In: Baker NR, Barber J, (eds) Chloroplast Biogenesis, pp. 133–205. Elsevier Science Publishers, Amsterdam (1985).
4. Boardman NK, Anderson JM, Goodchild, DJ: Chlorophyll-protein complexes and structure of mature and developing chloroplasts. Curr Top Bioenerg 8: 35–109 (1978).
5. Byrne DH, Seeley KA, Colbert JT: Half-lives of oat mRNAs *in vivo* and in a polysome-based in-vitro system. Planta 189: 249–256 (1993).
6. Chirgwin JM, Przybyla AE, Mac Donald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources rich in ribonuclease. Biochemistry 18: 5294–5299 (1979).
7. Dickey LF, Gallo-Maegher M, Thompson WF: Light regulatory sequences are located within the 5′ portion of the Fed-1 message sequence. EMBO J 11: 2311–2317 (1992).
8. Franco AR, Gee MA, Guilfoyle TJ: Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. J Bio Chem 265: 15845–15849 (1990).
9. Gil P, Liu Y, Orbovic V, Verkamp E, Poff K, Green P: Characterization of the auxin-inducible *SAUR-AC1* gene for use as a molecular genetic tool in *Arabidopsis*. Plant Physiol 104: 777–784 (1994).
10. Gollmer I, Apel K: The phytochrome-controlled accumulation of mRNA sequences encoding the light-harvesting chlorophyll *a/b* protein of barley (*Hordeum vulgare* L.). Eur J Biochem 133: 309–313 (1983).
11. Green PJ: Control of mRNA stability in higher plants. Plant Physiol 102: 1065–1070 (1993).
12. Holtorf H, Reinbothe S, Reinbothe C, Bereza B, Apel K: Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hodeum vulgare* L.). Proc Natl Acad Sci USA 92: 3254–3258 (1995).
13. McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ: Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. Plant Cell 1: 229–239 (1989).
14. Mösinger E, Batschauer A, Vierstra R, Apel K, Schäfer E: Comparison of the effect of exogenous native phytochrome and in-vivo irradiation on in vitro-transcription in isolated nuclei from barley (*Hordeum vulgare* L.). Planta 170: 505–514 (1987).
15. Mösinger E, Batschauer A, Apel K, Schäfer E, Briggs WR: Phytochrome regulation of greening in barley. Effects on mRNA abundance and on transcriptional activity of isolated nuclei. Plant Physiol 86: 706–710 (1988).
16. Mösinger E, Batschauer A, Schäfer E, Apel K: Phytochrome control of in vitro transcription of specific genes in isolated nuclei from barley (*Hordeum vulgare* L.). Eur J Biochem 147: 137–142 (1985).
17. Newman TC, Ohme-Takagi M, Taylor CB, Green PJ: DST sequences, highly conserved among plant SAUR genes, target reporter transcripts for rapid decay in tobacco. Plant Cell 5: 701–714 (1993).
18. Peltz SW, Brewer G, Bernstein P, Hart P, Ross J: Regulation of mRNA turnover in eukaryotic cells. Crit Rev Eukaryote Gene Express 1: 99–126 (1991).
19. Quail PH, Colbert JT, Peters NK, Christensen AH, Sharrock RA, Lissemore JL: Phytochrome and the regulation of expression of its genes. Phil Trans R Soc Lond 314: 469–480 (1986).
20. Sachs AB: Messenger mRNA degradation in eukaryotes. Cell 74: 413–421 (1993).
21. Schulz R, Steinmüller K, Klaas M, Forreiter C, Rasmussen S, Hiller C, Apel K: Nucleotide sequence of a cDNA coding for the NADPH-protochlorophyllide oxidoreductase (PCR) of barley (*Hordeum vulgare* L.) and its expression in *Escherichia coli*. Mol Gen Genet 217: 355–361 (1989).
22. Seeley KA, Byrne DH, Colbert JT: Red-light independent instability of oat phytochrome mRNA in vivo. Plant Cell 4: 29–38 (1992).
23. Thompson DM, Meagher RB: Transcriptional and post-transcriptional processes regulate expression of RNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase differently in petunia and soybean. Nucl Acid Res 18: 3621–3629 (1990).
24. Wadsworth GJ, Redinbaugh MG, Scandalios JG: A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Anal Biochem 172: 279–283 (1988).
25. Yamamoto KT, Mori H, Imaseki H: cDNA cloning of indole-3-acetic acid-regulated genes: *Aux22* and *SAUR* from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol 33: 93–97 (1992).