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A nuclear transcription factor related to plastid ribosome biogenesis is synthesised early during germination and priming

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Abstract Germination is a short developmental process during which many new proteins are synthesised. We have chosen the previously characterised *RPL21* gene encoding plastid-localised ribosomal proteins RPL21, to analyse activation of gene expression during germination. Transcription activation occurs at the P1 promoter during the first hours following imbibition and coincides with the appearance of a *trans*-acting factor that we named AUBE1. AUBE1 binds specifically to a short DNA fragment that encompasses the P1 promoter of the *RPL21* gene. The protein has a size of 28–30 kDa and is transiently expressed during the early phase of germination. Using the properties of primed seeds we show that AUBE1 is maintained after desiccation of primed seeds. We conclude that AUBE1 can be used as a marker in spinach seed priming. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: *RPL21* gene; Imbibition; Spinach seeds; Priming; P1 promoter

1. Introduction

Germination starts with rehydration of dry mature seeds and ends up when the radicle is protruding the seed coat. No cell division occurs during this phase. The uptake of water causes structural changes and the resumption of metabolic activity [1,2]. Mitochondria keep their integrity during the desiccation phase and respiration re-starts rapidly upon rehydration [3]. Several observations have shown that new proteins are synthesised during germination [4,5,6].

While mitochondrial activity and biogenesis have been analysed at some extent during germination only a few studies concern plastid development during this phase. Indeed, chloroplast differentiation starts only after germination with the expression of photosynthesis-related genes that are either encoded in the nucleus or in the plastid genome. On the other hand, nuclear genes encoding components of the plastid translational apparatus are expressed during germination [7,8]. We previously showed that several of these genes, including the *RPL21* and *RPS1* genes, are transcriptionally regulated by the usage of two distinct promoters named P1 and P2 [9,10]. Now, we present results showing that a new protein, that we called AUBE1 (aube is a french word for dawn), binding to the P1 promoter of the *RPL21* gene encoding the plastid L21 ribosomal protein, is transiently accumulated during germination. The appearance of AUBE1 is correlated with the usage of the P1 promoter for transcription. In addition, we show that AUBE1 is stable during a priming treatment that consists of initiation of germination followed by desiccation [11]. We conclude that AUBE1 can be used as a marker for primed spinach seeds.

2. Material and methods

2.1. Priming

Seeds of *Spinacia oleracea* cv. Géant d'hiver were used. All priming treatments were conducted at 24°C, as described by [12]. Seeds were soaked in 100 ml of distilled water for hydro-pre-treatment, or in −0.5 MPa polyethylene glycol (PEG) 8000 solution [13] for osmotic-pre-treatment, in beaker under agitation (100 rpm). After treatment, the seeds were rinsed twice with distilled water for 30 s, briefly blotted on paper W3MM (Schleicher and Schull) and dried at room temperature for 24 h. Verification that seeds recover their initial weight was made. The primed and untreated (control) dry seeds were stored at room temperature in the dark prior to analysis. The concentration of PEG that resulted in optimum germination performance (assessed by percent germination and the time to reach the maximum of germination) was determined from preliminary experiments.

2.2. Germination test

Fifty seeds, either primed or not, were put in a square dish (10×10 cm; inner volume, 100 cm^3) fitted with four layers of paper towels moistened with a volume of water as indicated in Section 3. In standard conditions 4 ml of distilled water was used. The plates were sealed with Parafilm (American National Canada) and placed in the dark at constant temperature between 5 and 40°C (±0.5°C) as indicated. The number of seeds exhibiting radicle emergence was recorded every day until germination was complete. Germination was calculated as percentage of total seeds sown in each plate (four replicates per treatment). The results presented are the means of the germination percentages obtained in four replicates.

2.3. RNA preparation and RT-PCR analysis

Total RNA was extracted from frozen seed material (15–20 seeds) after extensive phenol/chloroform extraction in 2 ml of the extraction medium containing 80 mM Tris–HCl (pH 9.0), 50 mM EDTA, 150 mM LiCl and 5% SDS. DNA was removed by two successive LiCl precipitations.

For RT-PCR reactions, we followed the method described [8]. cDNA synthesis was performed at 42°C for 1 h using 1 μg of RNA. The RT was subsequently inactivated by treatment at 95°C for 5 min and the cDNA was amplified by 33 PCR cycles for *RPL21* or 29 cycles for *RPS1*. The number of cycles has been chosen in order to get a linear signal in relation to the amount of RNA used in the assays. Genomic DNA contamination is detected in the RNA isolated from primed seeds, in the RPL21-P1 assays but not in the RPL21-P1+P2 assays, possibly because the ratio of P1+P2 transcripts

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to contaminating DNA is higher. The following oligonucleotides localised as indicated in Fig. 2a, were used: *RPL21*-A: 5′-AACCGGATAGGATAAAGTG-3′, *RPL21*-B: 5′-CTATCTCTCTCACAGAG-3′, RPL21-C: 5′-CCTTTGAGCCTCTGGGTG-3′, *RPS1*-A′: 5′-CCACTCTACTCATCCCC-3′, *RPS1*-B′: 5′-GTAGCAACAATGGCGTC-3′, *RPS1*-C′: 5′-CTTCACGTACACCTGGG-3′. As a control, a fragment of the *rrn16S* gene was amplified using the following oligonucleotides [14]: 5′-CTCATGGAGAGTTCGAAC-3′, 5′-GGGCAGGTTCTTACGCGT-3′. The expected DNA fragment sizes for cDNA amplification correspond to 504 bp (primers A and C), 428 bp (primers B and C), 449 bp (primers A′ and C′), 411 bp (primers B′ and C′), and 117 bp (*rrn16S*).

2.4. Protein extraction and analysis

Total proteins were prepared from dry control and primed spinach seeds. One hundred seeds (about 1.4 g) were ground with a Retsh ISO-9001 grinder for 1 min at full speed. The powder was resuspended with 7 ml of extraction buffer (15 mM HEPES–KOH, pH 7.5, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol) and mixed for 15 min at 4°C. The sample was centrifuged two times at 9000×*g* during 10 min at 4°C. Glycerol was added to the supernatant to a final concentration of 10%. Total proteins were aliquoted and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay reagents. 20 μg proteins was analysed by 10% SDS–PAGE. The gels were stained with 0.2% Coomassie brillant blue R-250.

2.5. DNA probe and gel-shift analysis

The A3 *Xho*I–*Hpa*II DNA fragment of the *RPL21* promoter (see Fig. 2a), containing the P1 transcription start site [9], was prepared by using two complementary synthetic oligonucleotides synthesised according to the sequence reported in Lagrange et al. [10]. For ^{32}P labelling, 100 ng of double-strand A3 fragment was end-filled with the Klenow enzyme in the presence of α[^{32}P]dCTP.

For gel-shift assays, total seed protein extracts were incubated with ^{32}P-labelled probe in a volume of 20 μl containing 50 mM HEPES–KOH, pH 7.9, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM KCl, 10% glycerol, and 2 μg of poly(dI–dC), at room temperature for 20 min. For competition assays, a 50- to 100-fold molar excess of unlabelled fragment was included in the binding assay and incubated for 10 min before the addition of labelled probe. The non-specific TATA containing fragment was made by two complementary synthetic oligonucleotides corresponding to the sequence of the *S. oleracea RBCS-1* promoter fragment (−38, −18). The following oligonucleotides were used: TATA-top: 5′-GTAGTATTATATATAGAAA-3′, TATA-bottom: 5′-TCTTTCTATATATAATACT-3′. The binding reactions were analysed by electrophoresis on 6% native acrylamide gels in 0.5×TBE buffer, pH 8.3, for 2.5 h at 140 V. After drying, the gel was autoradiographed at −80°C overnight or as indicated.

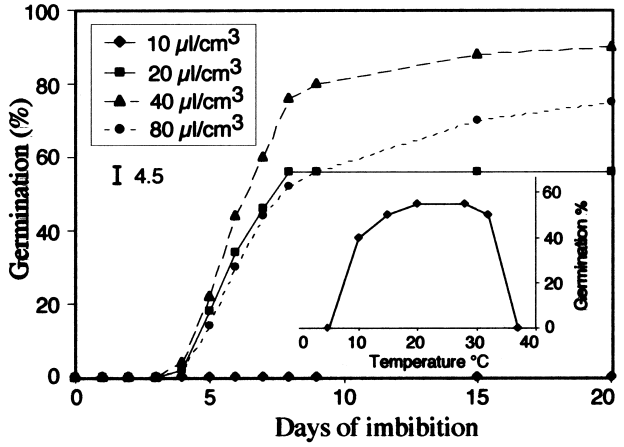

Fig. 1. Optimal conditions for germination. The spinach cultivar Géant d'hiver was used. Time course of germination in the dark using different imbibition conditions as indicated. Insert: Temperature dependence for germination, observed after 7 days, using 40 μl/cm^3 distilled water. The bar reported with 1 value represents the maximum standard deviation observed during experiments.

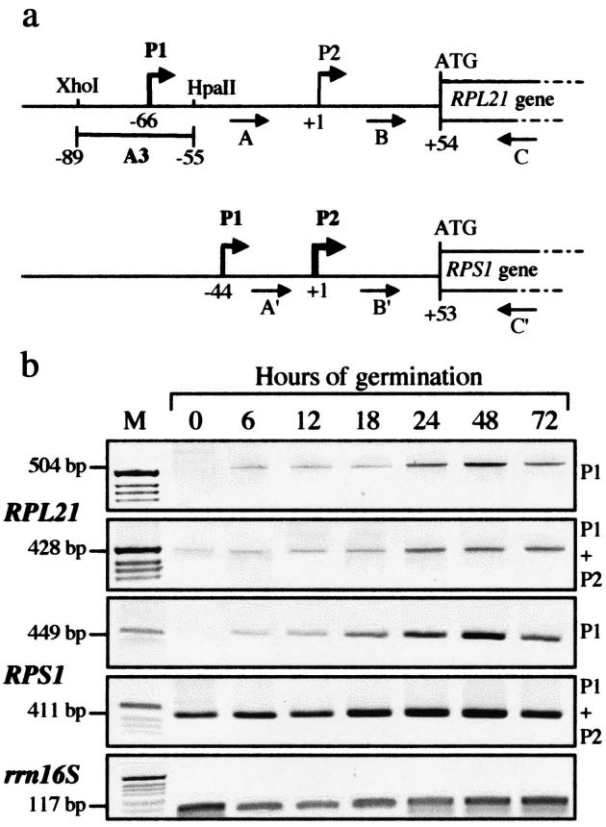

Fig. 2. Appearance of P1-initiated transcripts during the first hours of seed imbibition. a: Schematic representation of the P1 and P2 promoters of the spinach *RPL21* and *RPS1* genes. A3, DNA fragment used in gel-shift assays. b: Semi-quantitative measurements of different types of transcripts by RT-PCR using A or A′ and C or C′ primers (P1-initiated transcripts), B or B′ and C or C′ primers (P2+P1-initiated transcripts). The precursor of the plastid 16S rRNA (*rrn16S*) was used as control of RNA used in the experiments. M, DNA size markers. The number of bases indicates the expected size of P1- or P2-initiated transcripts.

2.6. Cross-linking analysis

Labelled A3 fragment was prepared by PCR using the pCHΔ6 clone previously described [10], α[^{32}P]dCTP and the following oligonucleotides: 5′-CCAGTGTGCTGGAATTCGGC-3′; 5′-GGTTTATCACTTTATCCTATCC-3′. After DNA–protein binding reaction performed as described for gel-shift assays, the binding mixture was irradiated with UV light at 254 nm at a distance of 4.5 cm for 15 min. The cross-linked protein–DNA complexes were incubated in the presence of 278 U of DNase I (Gibco-BRL) for 10 or 20 min. Then, the mixture was separated on a 15% SDS–polyacrylamide gel and the polypeptides bound to the DNase-resistant labelled nucleotides were revealed by autoradiography.

3. Results

3.1. Optimisation of germination conditions

At first, we have optimised the conditions for spinach seed germination. Tests were performed as described in Section 2. The optimal temperature for germination of spinach seeds ranges between 20 and 27°C (Fig. 1, insert). No germination occurs at temperatures over 37°C or under 5°C. Optimal water content was determined as 4 ml/dish (or 40 μl/cm^3; Fig. 1). Under optimised conditions, we obtained 80% of germinated seeds within 9 days. Shortest time to observe germination is about 4 days. If not otherwise indicated these conditions have been used in all further experiments.

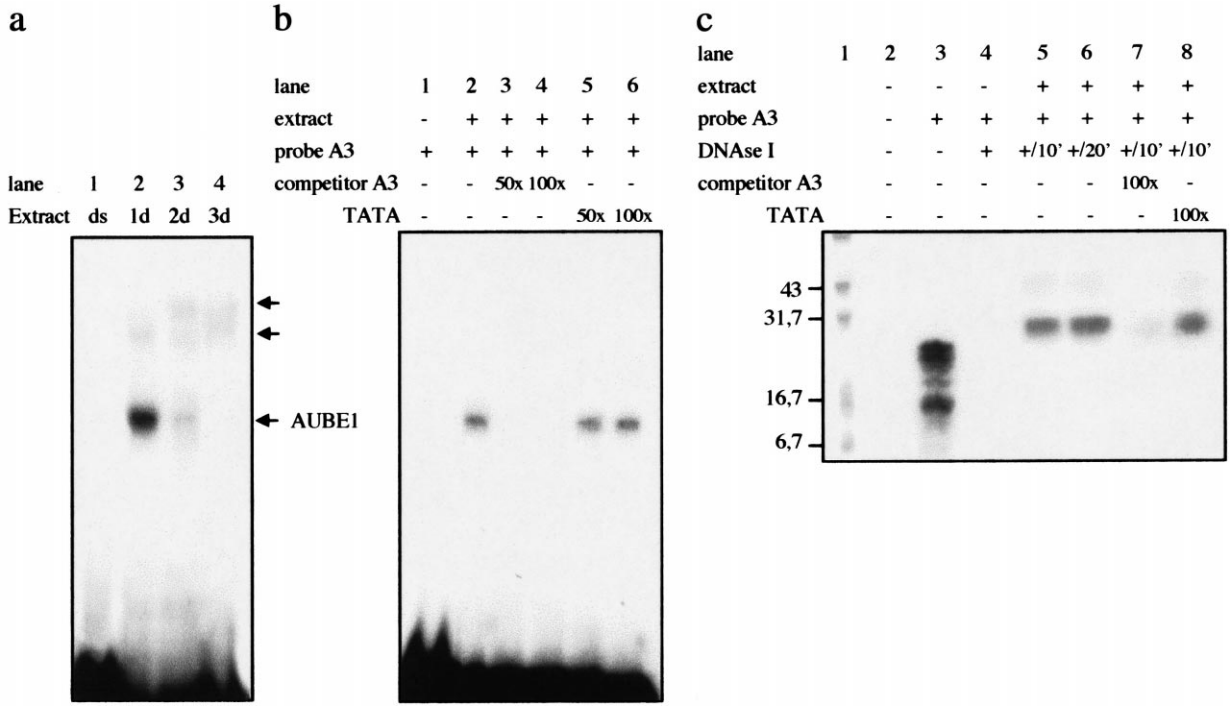

Fig. 3. Transient accumulation of a *trans*-acting element binding to A3 during germination. Position of the A3–DNA fragment in the *RPL21* promoter is represented in Fig. 2a. a: Gel-shift experiments were made as described in Section 2 using ^{32}P-labelled A3 fragment and 20 μg of total proteins extracted from dry seeds (ds, lane 1) or from germinating seeds, 1–3 days (1–3d) after the onset of imbibition. The main factor, AUBE1, and faintly identified factors (arrows) are indicated. b: Competition assays. The radiolabelled A3 fragment (lanes 1–6) was incubated with (+) or without (–) protein extracts from seeds after one day of germination. Incubation was made in the absence (–) or the presence of a 50- or 100-fold molar excess of either unlabelled A3 (lanes 3 and 4) or unlabelled and unspecific TATA-containing fragment corresponding to part of the *RBCS-1* promoter (lanes 5 and 6). c: Determination of the molecular size of AUBE1. The PCR-labelled A3 fragment (lanes 3–8) was incubated with (+) or without (–) protein extract from seeds after 1 day of imbibition, in the presence or not (–) of cold A3 (lane 7) or unspecific (lane 8) competitor. The mixture was irradiated by UV light, followed by DNaseI treatment (+, 10′ or 20′). The radiolabelled complexes and the UV-treated probe were separated on 15% SDS-gels and revealed by autoradiography. Exposure time was overnight except for the control (lane 2) and the irradiated probe (lanes 3–4), exposed for 3 h. Size of markers (lane 1) are indicated in kDa.

3.2. Transient usage of the P1 promoter of the RPL21 and RPS1 genes during germination

We previously identified early transcriptional expression of nuclear-encoded plastid ribosomal protein genes during germination [8]. Genes of this family are present in one copy per haploid genome. A sub-family, including the *RPL21* and the *RPS1* genes, encoding the plastid L21 and S1 ribosomal proteins, respectively, possesses two promoters P1 and P2 [9,10] which are differentially used. A schematic representation of the *RPL21* and *RPS1* promoters is shown in Fig. 2a. Transcripts of both genes using P2 are constitutively expressed at a low level whereas high expression is made by using P1 in green organs [9]. To precisely know the time of P1 activation during germination, a time-dependent semi-quantitative RT-PCR experiment was performed covering the minimum period of germination, i.e. day 1–3 after the beginning of imbibition (see Fig. 1). We observed that P1 promoter starts to function at about 6 h following seed imbibition. P1 transcripts accumulate up to 48 h. The amount of P1 transcript is stable or diminishes slightly thereafter (Fig. 2b). The production of P1 transcript increases again later in relation with the development of greening tissues, like cotyledons and leaves [9]. The functioning of the P2 promoter could be deduced from the measure of P1 transcripts and of total transcripts (Fig. 2b). For both genes, P2 transcripts scarcely accumulate during the first 2 days of germination. We expected that the constitutive promoter would be used during embryogenesis. This assumption was shown to be true as transcripts originated at P2 are present in dry seeds (Fig. 2b).

3.3. A transcription factor of RPL21 is early and transiently synthesised during germination

In order to analyse the mechanism of P1 activation in more detail, we searched for *cis*- and *trans*-acting elements that could be related to the *RPL21*-P1 promoter usage. Preliminary experiments had shown that a 35 bp DNA fragment of the *RPL21* promoter, called A3 (Fig. 2a), was able to bind nuclear proteins (not shown). This short promoter DNA fragment encompasses the P1 start site and was further characterised. Gel-shift assays were performed using A3 and total proteins extracted from dry seeds and from imbibed seeds at several steps during germination. As shown in Fig. 3a, a transcription factor, named AUBE1, binds to the A3 fragment after 1 day of imbibition. Competition experiments show that this binding is specific for A3 (Fig. 3b). AUBE1 is scarcely detected in dry seeds (it is not visible in Fig. 3a but is detected after a longer exposure time). Its amount decreases drastically after 2 days of imbibition and it has disappeared after 3 days. Additional factors or dimers of AUBE1 (arrows in Fig. 3a), faintly detected, might be present. To further characterise AUBE1, its size was determined by SDS–PAGE, after UV cross-link of the AUBE1/A3–DNA complex, followed by DNase digestion. The apparent molecular size of AUBE1 is of 28–30 kDa (Fig. 3c). When cold competitor

prevents the formation of the labelled complex (Fig. 3b, lanes 3 and 4) the proteins detected after the UV and DNase treatment are scarcely visible (Fig. 3c, lane 7). Thus, the 30 kDa corresponds to AUBE1.

3.4. Effects of water and osmotic-pre-treatment (priming) on germination

We reason that the AUBE1 factor could be used as a marker for initiation of germination. A pre-treatment of seeds called priming is currently made in order to obtain a higher rate of germination [11]. This pre-treatment consists in starting imbibition for a short period of time under controlled conditions followed by desiccation. Thus, seeds that are initiated for germination can be stored. We reasoned that, if AUBE1 is really activated in the early germination period, it should already be synthesised during priming pre-treatment. Moreover, it might be present in primed stored seeds, i.e. after desiccation. AUBE1 would thus define a new marker of germination initiation. To test these hypotheses we first determined the optimal conditions to perform priming of spinach seeds. As shown in Fig. 4, seeds primed either with distilled water or with −0.5 MPa PEG solution [13] germinate earlier than control seeds. The optimal priming duration is 18 h for water-priming (Fig. 4, top) and 48 h for osmotic-priming (Fig. 4, bottom). Both treatments allow to obtain 80% of germinated seeds 3–4 days before control seeds, and increase the final percentage of germinated seeds by about 5–10% (Fig. 4). Thus, priming of spinach seeds represents a valuable tool to homogenise germination.

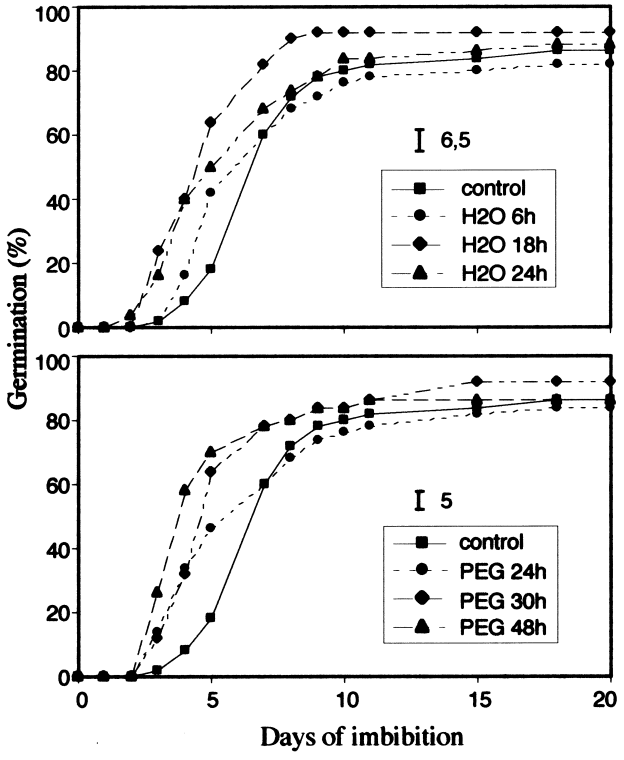

Fig. 4. Optimalisation of priming conditions. Top: Germination after a pre-treatment with distilled water. Bottom: Germination after a pre-treatment with a −0.5 MPa PEG solution. Seeds were germinated in the dark at 10°C. The bar reported with 1 value represents the maximum standard deviation observed during experiments.

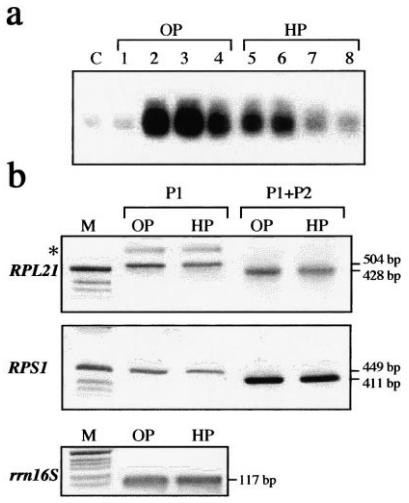

Fig. 5. AUBE1 and P1-transcripts accumulate during seed priming. a: Gel-shift assays. Labelled A3 fragments were incubated with 20 μg of total protein extracts from control seeds (C) and from seeds primed either with −0.5 MPa PEG solution (OP) or with distilled water (HP) for 1, 2, 3 or 4 days, respectively. Exposure time was 36 h. b: Measurements of different transcripts (P1 and P1+P2) by RT-PCR using the same primers as in Fig. 2 and RNA isolated from 48 h osmotic-primed seeds (OP) or from 18 h hydro-primed seeds (HP). Seeds were desiccated after the pre-treatment (see Section 2). The *rrn16S* was used as a control of the amount of RNA used in the experiments. M, DNA size marker (bp). Asterisk, bands corresponding to contaminating genomic DNA that includes the first intron [9]. These bands are slightly visible in the P1+P2 experiment, after longer exposure.

3.5. The AUBE1 factor is accumulated during priming

In the presence of −0.5 MPa PEG solution, AUBE1 appears on day 2, accumulates up to day 3 but decreases on day 4, probably due to degradation (Fig. 5a, OP lanes). To note, the accumulation of AUBE1 is observed in dry seeds that have been pre-treated (primed seeds). Seeds primed in water show a similar AUBE1 accumulation pattern but the factor is observed already after 24 h of treatment and decreases on day 3 (Fig. 5a, HP lanes). Presumably, the lower level of AUBE1 in water-primed seeds results from higher degradation rate of this protein at higher water potential than used for the osmotic-priming. In both priming treatments, the accumulation of AUBE1 in seeds depends on the duration of the pre-treatment. The accumulation reaches an optimum at 2–3 days after the onset of osmotic-priming and 1–2 days after the onset of water-priming. If the seeds are pre-treated for a longer time than this optimum, the quantity of the factor binding to A3 decreases. Interestingly, the accumulation of AUBE1 during priming correlates with the optimal priming period, which is shorter for water-priming than for osmotic-priming (compare Figs. 4 and 5a). The variation in the AUBE1 factor is without significant influence on the total content of protein in the experiments (not shown). We have to ask the question whether this induction is also correlated with transcription activation at the *RPL21* P1 promoter. Analyses of *RPL21* P1 transcripts in primed dry seeds were performed by RT-PCR. Fig. 5c shows that water- and osmotic-primed seeds, in contrast to non-primed seeds (Fig. 2b, lane 0), contained transcripts initiated at P1 for the *RPL21* gene. The same holds true for the *RPS1* gene (Fig. 2b)

4. Discussion

Germination is a morphologically well-defined process that encompasses swelling of seeds in the presence of water up to the protrusion of root tips. During imbibition, cells recover their activity. Many studies have been made showing that germination is under hormone control (for review see Section 1). But, the entire process of germination is not well understood at a molecular level. For instance, it is not known whether the newly synthesised products are essential for this intermediary phase of development or are accumulated as a storage for subsequent tissue development. In the present paper we analyse the early expression of one gene encoding a plastid-localised ribosomal protein in more detail. We show that AUBE1 binds to the P1 promoter and is related to the appearance of the P1 transcripts suggesting a function in transcription initiation.

Surprisingly, AUBE1 is synthesised only during a short period of time during germination and is transiently accumulated. The expression of the *RPL21* gene is also transiently activated but the transcripts are stable. The similar results obtained for the early expression for the *RPS1* gene suggest the existence of a common control, at least for the two genes. Transcription activation by the usage of P1 will be re-activated later during seedling development, as previously observed [8], to provide components for the plastid translational apparatus necessary for chloroplast development. What is then the significance of the early and transient synthesis of the AUBE1 factor? In the mature embryo, proplastids are present. After germination, proplastids develop into etioplasts or chloroplasts, depending on the presence or the absence of light. Early synthesis of AUBE1 may represent the first step of a series of events that take place during germination to set up the plastid translational apparatus, long before the building of the photosynthetic apparatus. In agreement, the mature form (devoid of transit peptide) of the plastid translation elongation factor EF-Tu accumulates in early phases of *Arabidopsis* seed germination [6]. Plastid ribosomes would be required before cell division, which occurs after germination, to participate to plastid housekeeping functions (e.g. plastid fatty acid synthesis). The P1 usage in germination could correspond to specific tissue since in seedlings and adult plants P1 is restricted to green tissues. Our observations on *RPL21* and *RPS1* gene expression at the very beginning of imbibition, stresses the importance of ribosome biosynthesis for plastid development.

AUBE1 interacts with the *RPL21* gene promoter. The same factor might be effective for the expression of other nuclear ribosomal protein genes that share in common the P1 initiation site, and thereby the same mechanism of gene activation. This hypothesis is supported by the early activation of P1 promoter in the *RPS1* gene. If confirmed, AUBE1 would represent a general factor that would trigger the synthesis of plastid ribosomes, a step necessary for plastid reactivation during germination.

The correlation between the presence of AUBE1 and P1 transcripts is verified with primed spinach seeds. Also, AUBE1 is not degraded in primed seeds and might contribute, at least in part, to the improvement of germination efficiency. Thus, our results have a practical consequence for the priming of spinach seeds. AUBE1 could be used as a marker for germination initiation. In particular, an excess in the imbibition treatment could be detected by the disappearance of the factor. Thus, AUBE1 is a marker useful to precisely determine the time necessary for priming.

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