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RESEARCH PAPER

Elicitor-dependent expression of the ribosome-inactivating protein beetin is developmentally regulated*

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Abstract

BE27 and BE29 are two forms of beetin, a virus-inducible type 1 ribosome-inactivating protein isolated from leaves of *Beta vulgaris* L. Western blot analysis revealed the presence of beetin forms in adult plants but not in germ or young plants, indicating that the expression of these proteins is developmentally regulated. While beetins are expressed only in adult plants, their transcripts are present through all stages of development. In addition, the treatment of *B. vulgaris* leaves with mediators of plant-acquired resistance such as salicylic acid and hydrogen peroxide promoted the expression of beetin by induction of its transcript, but only in adult plants. The plant expresses three mRNAs which differ only in their 3′ untranslated region. All these observations suggest a dual regulation of beetin expression, i.e. at the post-transcriptional and transcriptional levels. Additionally, total RNA isolated from leaves treated with hydrogen peroxide, which express high levels of active beetin, is not de-adenylated by endogenous beetin, nor *in vitro* by the addition of BE27, thus suggesting that sugar beet ribosomes are resistant to beetin.

Key words: Beetin, *Beta vulgaris*, hydrogen peroxide, ribosome-inactivating protein (RIP), salicylic acid, 3′ UTR.

Introduction

Ribosome-inactivating proteins (RIPs) are a group of proteins with *N*-glycosidase activity present in a number of plants and some bacteria and fungi that remove adenines, in particular, the specific adenine from the sarcin/ricin loop of the large rRNA, in both prokaryotic and eukaryotic ribosomes (Endo *et al*., 1987; reviews in Barbieri *et al*., 1993; Ferreras *et al*., 2000; Girbes *et al*., 2004; Park *et al*., 2004; Stirpe, 2004; Stirpe and Battelli, 2006). RIPs are conventionally classified into two types: type 1, composed of a single polypeptide chain with *N*-glycosidase activity; and type 2, which are heterodimers consisting of an A chain, functionally identical to a type 1 RIP, linked to a sugar-binding B chain (reviews in Barbieri *et al*., 1993; Girbes *et al*., 2004; Hartley and Lord, 2004). The B chain can mediate the entry of the A chain into the cell through binding of exposed cell-surface galactosyl residues. Some of the type 2 RIPs, such as ricin, which is the best known RIP, are extremely toxic, whereas others have low toxicity, due at least in part to the reduced ability of the B chain to bind to the polysaccharide chains present at the surface of plasma membrane proteins and to a different intracellular pathway (Pascal *et al*., 2001; review in Girbes *et al*., 2004). The toxicity of type 1 RIPs is also low since they lack the lectin moiety and hence are unable to bind to cells as well as type 2 RIPs.

RIPs have been observed in several plant tissues such as seeds, leaves, bark, fruits, and latex (reviews in Barbieri *et al*., 1993; Girbes *et al*., 2004). The RIP levels in plant tissues are highly variable and RIPs may be present with many isoforms in the different tissues (review in Ferreras *et al*., 2000; Bolognesi *et al*., 2002). The expression of RIP genes is also influenced by different phenomena such as age, season, wounding, chemical, and environmental conditions (Stirpe *et al*., 1996; Rippmann *et al*., 1997). It has been suggested that at least two pathway types may be operating to promote type 1 RIP induction. In this sense, it has been shown that jasmonate promotes the expression of

* The authors dedicate this article to the beloved memory of Enrique Méndez who passed away on 23 July 2007.

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several RIPs, such as JIP60 in barley (Chaudhry *et al*., 1994) and PIP2 in *Phytolacca insularis*, but the latter protein is not induced by salicylic acid (SA), which suggests that the systemic expression of PIP2 is SA-independent (Song *et al*., 2000). On the other hand, SA induces the transcription and accumulation of a RIP from bitter melon (Xu *et al*., 2007). Recent studies with ME1 from the roots of *Mirabilis expansa* indicate that jasmonic acid induces ME1 transcript expression, suggesting a post-transcriptional regulation (Vepachedu *et al*., 2003).

Although the biological role of RIPs in plants is not yet clear, it has been proposed that RIPs would play important roles in plant defence as antiviral agents by triggering ribosome inactivation in infected cells and hence cell death (Taylor *et al*., 1994; Hong *et al*., 1996; Krishnan *et al*., 2002; review in Park *et al*., 2004). It has also been reported that many RIPs can attack their own ribosomes (Prestle *et al*., 1992; Bonness *et al*., 1994). To prevent damage to ribosomes, these RIPs are compartmentally isolated in the cell wall, intercellular space, or vacuoles (Tully and Beevers, 1976; Ready *et al*., 1986). However, in response to attack by pathogens, RIPs can enter the cytosol and kill infected cells, thereby inhibiting the spread of infection to the whole plant body. Nevertheless, new evidence suggests that the defence mechanism of RIPs may proceed by directly targeting invading pathogens rather than host ribosomes (review in Park *et al*., 2004). Furthermore, RIPs can confer resistance to plants against different viruses when applied externally or when expressed in transgenic plants (Taylor *et al*., 1994; Iglesias *et al*., 2005; reviews in Girbes *et al*., 2004; Stirpe, 2004).

BE27 and BE29 are two forms of beetin, a type 1 RIP from sugar beet (*Beta vulgaris* L.) inducible upon infection by plant RNA viruses or following treatment of virus-free beet leaves with SA or hydrogen peroxide (H_2O_2), which are mediators of the systemic acquired resistance of plants (Girbes *et al*., 1996; Iglesias *et al*., 2005). Both forms of beetin represent different levels of glycosylation of the same polypeptide chain (Iglesias *et al*., 2005). In the present study, the relationship between the presence of beetin transcript and protein expression during development and after induction with SA or H_2O_2 is investigated. In addition, the presence of three mRNAs coding for beetins with differences in their 3′ untranslated region (3′ UTR) are reported. 3′ UTRs have been shown to play a significant regulatory role at both transcriptional and translational level in the expression of proteins related to plant development (review in Rafalski *et al*., 1984; Rothnie, 1996).

Materials and methods

Primers

Primer J1 [5′-CGTCTAGAGTCGACTAGTCG $(T)_{20}$] was used in the reverse-transcription of 3′ RACE and primer J2 (5′-CGTCTAGAGTCGACTAGTCG-3′) was used as the flanking primer. Primer BVF (5′-GCAGATGTAACTTTTGAC-3′) was designed from the N-terminal amino acid sequence of the protein and was used to amplify cDNA and genomic DNA. Primer BCR (5′-GTACTTGAATCTTGCTGCCTC-3′) was derived from the conserved sequence of the active site of RIPs and was used to amplify the beetin dsDNA probe and in semi-quantitative RT-PCR. Primer UL (5′-GCATATGGCCTGATAGCCAATG-3′) was synthesized according to the sequence of the 3′ UTR of the long mRNA and used for the amplification of genomic DNA and long mRNAs. Primer US (5′-ATAATTGGGTTGTAGAAATCCCA-3′) was designed from a sequence of the 3′ UTR of the short mRNA.

Induction of beetins by treatment of sugar beet leaves with elicitors

Treatment of laboratory-grown sugar beets with either H_2O_2 or SA was carried out by spraying dilute solutions (5 mM) every 24 h over 3 d. Crude protein extracts from beet leaves or germ were prepared by grinding in a mortar with liquid nitrogen 100 mg of control or either H_2O_2- or SA-treated leaves or germ, and extracted overnight at 4 °C with 10 vol of a solution containing 140 mM NaCl and 5 mM sodium phosphate (pH 6.6). Then, the extracts were centrifuged at 12 900 *g* and the clarified supernatant was stored at –20 °C until needed. Protein concentrations were determined as described by Kalb and Bernlohr (1977).

PCR labelling of dsDNA probe with digoxigenin

Probe labelling was accomplished by PCR amplification, using dUTP-DIG from Roche Diagnostics S.L. (Barcelona, Spain). The PCR reaction was carried out in a final volume of 20 µl containing 100 pg of DNA template (plasmid PCR 2.1-cDNA beetin), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM dNTPs (dATP, dCTP, and dGTP) 0.065 mM dTTP, 0.035 mM DIG-dUTP, 1 µM of the primers BVF and BCR, and 2 units of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA, USA). The PCR reaction was performed using the following thermal program: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 90 s at 55 °C, and 2 min at 72 °C. The reaction was stopped by chilling the tubes to 4 °C. The concentration of DIG-dUTP probe was quantified with a dot-blot test using the digoxigenin-labelled PBR328 control, as indicated by the manufacturer.

DNA and RNA preparation

DNA was purified from 4-week-old leaves of *B. vulgaris* using a Genomic Prep-Cell and tissue DNA isolation Kit (GE-Healthcare, Madrid, Spain). Leaves (100 mg) were ground in a mortar with liquid nitrogen and the resulting powder was treated as indicated by the manufacturer. Total RNA from germ and leaves of 2- and 4-week-old plants after different treatments was isolated using the RNeasy plant mini-kit (Qiagen, Stanford, CA, USA).

RNA gel blot analyses

Five micrograms of total RNA from the germ and leaves of 2- and 4-week-old plants under different treatments were separated on a 1% formaldehyde–agarose gel and transferred onto a nylon membrane (Hybond N^+, GE Healthcare). The membranes were hybridized overnight with a DIG-dUTP probe in a solution of 0.25 M sodium phosphate (pH 7.2), 1 mM EDTA, and 10% SDS at 62 °C. Membranes were washed with 20 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 1% SDS three times for 20 min at 60 °C under high-stringency conditions (Engler-Blum *et al*., 1993). Immunological detection of the DIG-labelled probe was done using a chemiluminescent substrate CDP-star (Roche).

Relative quantitative RT-PCR

Two micrograms of total RNA from sugar beet leaves were reverse-transcribed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) in a reaction mixture containing 10 mM TRIS–HCl (pH 8.3), 50 mM KCl, 1 mM of dNTPs, 5 mM $MgCl_2$, 1 unit of RNase inhibitor, 2.5 μM of Random Hexamers, and 2.5 units of MuLvReverse transcriptase (murine leukaemia virus reverse transcriptase). The incubation was performed as indicated by the manufacturer. Quantitative PCR amplification was accomplished using aliquots of the resulting cDNA that contained the same amount of the 18S rRNA quantified by PCR using specific 18S RNA primers and competimers at a 9:1 ratio (Quantum RNA™ 18S internal standards; Ambion, Austin, TX, USA) in a final reaction volume of 20 μl containing 10 mM TRIS–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM of dNTPs, 1 μM of primers BVF and BCR, and 2 units of Amplitaq DNA polymerase (Applied Biosystems). Amplification was performed in the linear range, using the following thermal profile: 5 min at 95 °C, followed by 30 s at 95 °C, 1 min at 60 °C, and 2 min at 72 °C, for 25 cycles. The reaction was stopped by chilling the tubes to 4 °C and the PCR products were analysed in a 0.8% agarose gel. The membrane was scanned and the total volume per band was calculated using the Scion image program (derived from Macintosh NIH Image program designed by Wayne Rasband from the National Institutes of Health, USA).

Molecular cloning of 3′ ends of cDNA and genomic DNA for BE27

The molecular cloning of cDNA coding for beetin was done by PCR amplification using the 3′ RACE technique. One microgram of total RNA was reverse-transcribed using the oligonucleotide$(T)_{20}$ contained in primer J1. Two micrograms of the resulting cDNA were amplified by PCR using a specific primer deduced from the N-terminal sequence of the protein (BVF), and the synthetic oligonucleotide J2, which overlaps part of the sequence of the J1 primer. The amplified DNA products were separated on 1% agarose gel and two different bands were purified, cloned into the pCR 2.1 vector (original TA cloning kit; Invitrogen, Barcelona, Spain) and sequenced in both directions (Servicio de Secuenciación de DNA., Centro de Investigaciones Biológicas, Madrid).

RT-PCR of the mRNAs from germ and 4-week-old plants treated with H_2O_2 was carried out by reverse transcription with a primer, J1-oligod$(T)_{20}$, as indicated above, followed by PCR amplifications with the corresponding pairs of primers: BVF–UL or BVF–US. Both UL and US were designed based on the 3′ UTR deduced from the previously cloned cDNAs.

The genomic sequence was obtained by PCR using two primers corresponding to the 3′ -and 5′-terminal sequences of the previously cloned cDNA. The reverse primer (UL) was designed from the sequence next to the poly(A) tail of the longer 3′ UTR. PCR amplification was carried out in a final volume of 20 μl containing 20 ng of DNA template, 10 mM TRIS–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM of dNTPs, 1 μM of DNA primers BVF and UL, and 2 units of Amplitaq DNA polymerase (Applied Biosystems). The PCR product was cloned into the PCR 2.1 vector and sequenced.

Assay of N*-glycosidase activity*

To investigate whether ribosomes of *B. vulgaris* were depurinated *in planta* by an active beetin, 0.5 g of leaves treated with H_2O_2 were prepared by grinding in a mortar with liquid nitrogen. Then, 100 mg of the resulting powder was processed for RNA isolation as indicated above and 30 mg of the powder was extracted with 10 vol of PBS (140 mM NaCl, 5 mM sodium phosphate, pH 6.6) to obtain a crude protein extract. Three micrograms of the total RNA were treated with aniline and electrophoresed as indicated elsewhere (Iglesias *et al.*, 2005). The *N*-glycosidase activity of the crude protein extract was assayed by the addition of 10 μl to a reaction mixture containing 80 μl of rabbit reticulocyte lysate. The mixture was incubated for 30 min at 37 °C, followed by aniline treatment and electrophoresis.

The *N*-glycosidase activity of BE27 in RNA substrates was assayed in samples of total RNA from *B. vulgaris* leaves and rabbit reticulocyte lysate. Each reaction mixture (25 μl) containing 15 μg of RNA in TE buffer (pH 7.5) was incubated for 30 s at 90 °C to denature RNA substrates. Reactions were quenched by placing the tubes on ice, followed by the addition of 4 μg of BE27. Then, the reaction mixtures were incubated at 37 °C for 30 min. The aniline treatment was carried out as described above and 3 μg of RNA samples was electrophoresed on 5% acrylamide gel (Girbes *et al.*, 1993).

Results

Effects of H_2O_2 and SA on beetin expression in three growth stages

To study the expression pattern of beetin forms during plant growth, protein levels and RNA transcript levels were analysed at three different stages of development of *B. vulgaris* plantlets: germ and 2- and 4-week-old plants. Western blot analyses using rabbit anti-beetin antibodies revealed the presence of beetins only in 4-week-old plants (Fig. 1A). To investigate beetin transcript levels, total RNA was purified from germ and 2- and 4-week-old plants. Northern blot analyses (Fig. 1B) revealed RNA transcripts of beetin to be constitutively expressed at high levels in the three arbitrary stages of growth but they were not translated in germ and 2-week-old plants. These results suggested a possible mechanism by which beetin protein levels would be regulated post-transcriptionally during the growth of *B. vulgaris*. Treatments of germ, and 2-and 4-week-old plants, with SA and H_2O_2, both stress-related signal molecules, indicated that H_2O_2 induced the expression of beetins only in 4-week-old plants, while SA,

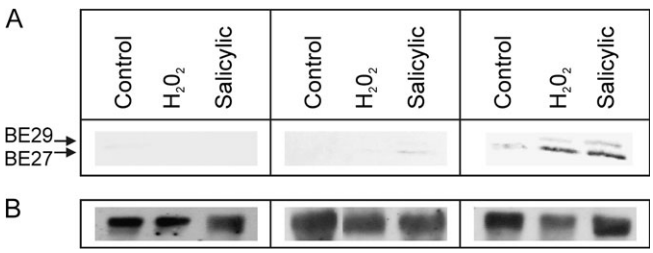

Fig. 1. (A) Thirty micrograms of crude protein extracts from germ and leaves of 2- and 4-week-old plants of sugar beet, either sprayed with water (control) or treated with SA or H_2O_2, were analysed by western blotting using rabbit anti-beetin antibodies. (B) Northern blot analysis: 5 μg of total RNA from germ and leaves of 2- and 4-week-old plants of sugar beet, either sprayed with water (control) or treated with SA or H_2O_2, were electrophoresed, blotted, and hybridized with a DIG-probe as described in the Materials and methods. Each experiment was done using 5 μg of TMV genomic RNA as a negative control.

which seemed to be a stronger elicitor, induced the expression of beetins in 4-week-old plants and a very weak expression in 2-week-old plants (Fig. 1A).

Semi-quantitative RT-PCR of beetin mRNA

Although northern blot experiments revealed similar amounts of mRNA in all cases, a quantitative RT-PCR assay was performed to determine the exact mRNA levels in 2- and 4-week-old plants treated with 5 mM H_2O_2 and in untreated controls (Fig. 2). The RT-PCR reaction was under linear conditions, using the same amount of total RNA as indicated by the amplification of standard 18S rRNA. The results in Fig. 2A point to amplification in all samples, but the amount of beetin transcripts was higher in 4-week-old plants treated with H_2O_2. Figure 2B depicts the amount of each amplification band, analysed by a densitometry programme. The amount of transcripts in plant samples without treatment was almost the same in 2- and 4-week-old plants. Treatment with H_2O_2 did not have any effect on 2-week-old plants, while in 4-week-old plants the elicitor promoted a 3-fold increase in beetin transcript levels, which corresponded to the highest expression level of protein (Fig. 1A). The results obtained clearly suggest a mechanism for the induction of expression that acts at transcription level, promoted by this elicitor. Thus, in *B. vulgaris* two different mechanisms of regulation of beetin production seem to coexist—control of translation and control of transcription—that seem to be developmentally regulated.

Analysis of the 3′ UTR of BE transcripts

In order to clarify the mechanism of regulation of beetin expression, the presence of different mRNAs in adult plants was investigated. For this purpose, total mRNA from 4-week-old leaves was reverse-transcribed to cDNA. Three cDNA fragments coding for beetin were cloned and they indicated the presence of three mRNA transcripts differing only in the 3′ UTR following a common open reading frame (Iglesias *et al*., 2005). The 3′-UTR variants of the beetin cDNAs were designated I, II, and III (Fig. 3). A more detailed analysis of the nucleotide sequence of the 3′ UTR revealed 94% sequence homology between clones I and II. They contained two putative polyadenylation signals [near-upstream elements (NUE)], AATAAT and AATAAA, and a poly(A)-rich tail. The 3′ UTRs of mRNA I and III were identical except for the lack of a 55 bp fragment preceding the site of poly(A) in clone III, hence lacking the second consensus polyadenylation signal. It is therefore possible that the short mRNA would be derived from the same gene as the long mRNA after undergoing differential polyadenylation. The presence of two genes encoding beetins was confirmed by PCR using genomic DNA extracted from adult plants. The gene fragments were amplified using a primer from the amino-terminal sequence of the protein and another one from the sequence preceding the site of poly(A) in the long mRNA. The fragments were cloned and sequenced, and they indicated the presence of two genes (GenBank accession numbers AM900410 and AM900411), one of them encoding a previously described mRNA sequence (Hornung *et al*., 1996) corresponding to the 3′-UTR variant II (Fig. 3), whereas the other encoded an mRNA sequence carrying the 3′-UTR variant I shown in Fig. 3. In agreement with this, a Southern blot of genomic DNA digested with several restriction enzymes also revealed the presence of two genes encoding beetins (data not shown).

It is widely accepted that the sequence and structural motifs of the 3′ UTR can regulate mRNA stability and that they play important roles in regulating the expression of proteins, determining intracellular localization, controlling polyadenylation, and assisting in translation (Ingelbrecht *et al*., 1989; review in Rafalski *et al*., 1984; de Sauvage *et al*., 1992; Rothnie *et al*., 1994; Rothnie, 1996). Compilation of the conservative elements of the 3′ UTR in *Arabidopsis thaliana* and *Oryza sativa* indicated that plant poly(A) signals can be roughly grouped in four major groups: (i) far-upstream elements (FUE); (ii) near-upstream elements (NUE; an AATAAA-like element); (iii) T-rich elements (TRE); and (iv) cleavage sites (CS) (Loke *et al*., 2005; Dong *et al*., 2007). As shown in Fig. 3A, all these conservative elements were found throughout the 3′ UTRs of beetin transcripts located at the expected positions. Differences among the three 3′ UTRs concerning the number and sequence of NUE could affect the regulation of polyadenylation.

mRNA stability can be regulated by the sequence and the potential secondary structures that may be formed within the 3′ UTR (Rott *et al*., 1998; Komine *et al*.,

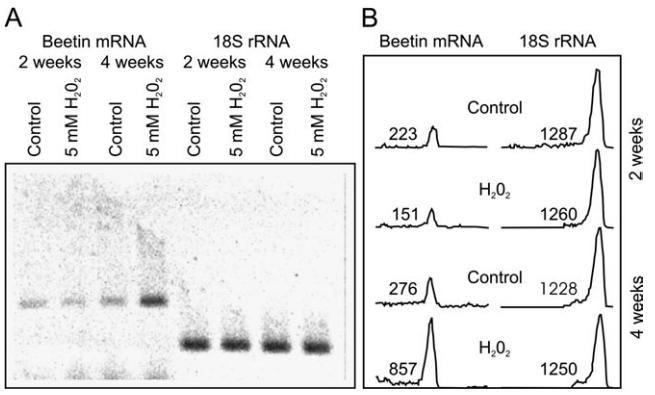

Fig. 2. (A) Quantitative RT-PCR analysis of samples from 2- and 4-week-old plants sprayed with water (control) or treated with 5 mM H_2O_2. Lanes 1–4 correspond to PCR amplifications of beetin mRNA with the following primers: BVF from the N-terminal sequence of the protein (ADVTFD), and BCR from the highly conserved internal amino acid sequence EAARFDY (active site of RIPs). Lanes 5–8 correspond to PCR amplification of 18S rRNA of the same samples with internal standard primers and competimers at a ratio of 9:1 which gives a product of 488 bp. (B) Densitometry analysis.

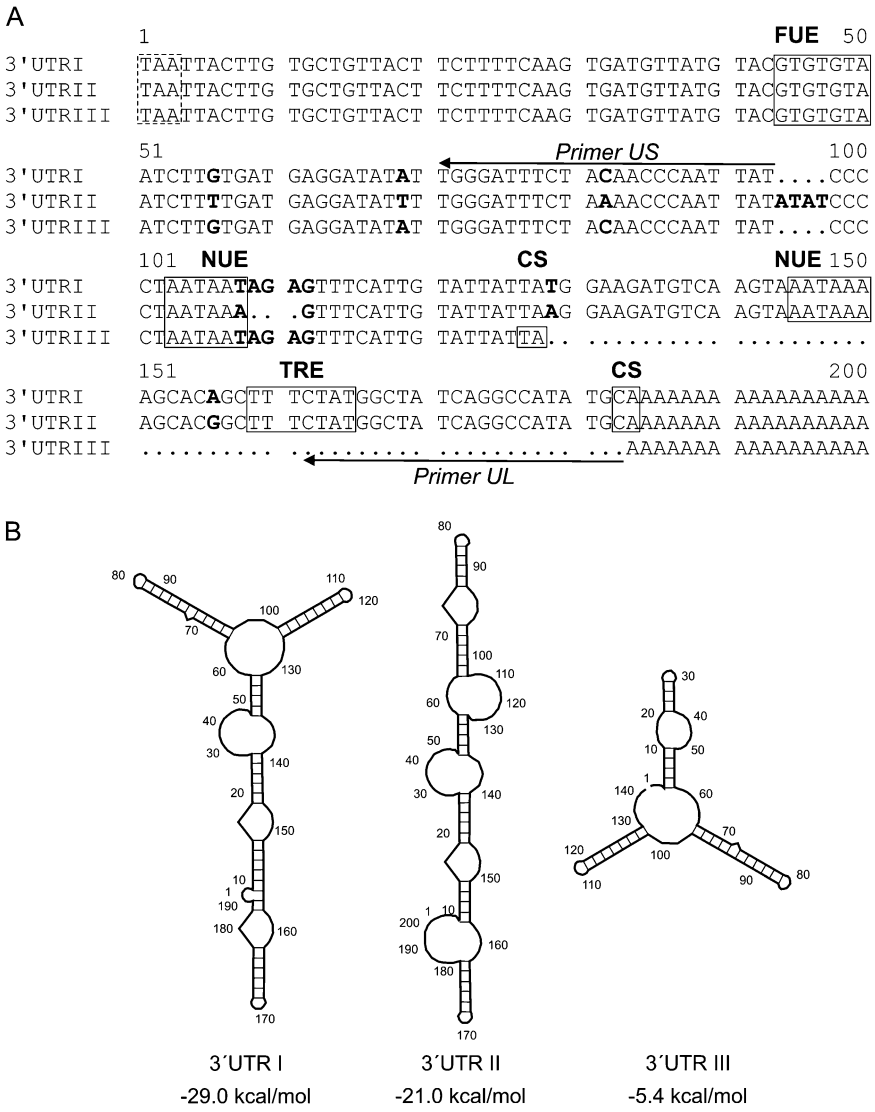

Fig. 3. (A) Alignment of cDNA sequences of the 3′-UTR regions of beetins. (B) Proposed secondary structures of the 3′ UTRs. Prediction of RNA secondary structure and thermostability was calculated with the program given online at http://www.genebee.msu.su/services/rna2_reduced.html.

2002). Figure 3B shows the possible secondary structure of each 3′ UTR and the free energy required to maintain these structures. The three potential secondary structures displayed significant differences that could affect recognition by protein factors, as has been reported previously (Loke *et al*., 2005). The thermostability values were –29 kcal mol^{-1} for I, –21 kcal mol^{-1} for II, and –5.4 kcal mol^{-1} for III. Thus, it is possible that the different 3′ UTRs could be degraded at different rates. The low value calculated for the 3′ UTR of mRNA III indicated that it is probably not stabilized by secondary structures and hence could be easily degraded.

In an attempt to discriminate between the 3′-UTR variants of the beetin transcripts present in germ and those present in induced adult plants, RT-PCR experiments were performed. For this purpose, two pairs of primers that were a combination of a forward primer (BVF) and two reverse primers were used. One of the reverse primers (US) annealed to a sequence inside the 3′ UTR of the short mRNA (variant III) hence recognizing variants I, II, and III, while the other (UL) annealed to the end of the 3′ UTR of the long mRNAs (variants I and II) (Fig. 3). The presence of only long and short mRNAs could be detected, since, owing to the high sequence homology, it was not possible to discriminate between the two long mRNAs (I and II). Two fragments of the predicted size (840 bp and 927 bp) were amplified (Fig. 4). Figure 4 also shows a more intense signal for the short fragment than for the larger one, as expected if the short mRNA was present. The result indicated that both short and long mRNAs were present in germ. A similar result was obtained with adult plants induced with H_2O_2 (Fig. 4).

The apparent presence of the same mRNAs in both germ and induced adult plants, which were untranslatable in germ, suggests the involvement of proteins present in induced plants that could bind to sequences located in the 3′ UTR of some of these mRNAs, thus activating their translation.

In vivo *depurination of* Beta vulgaris *total RNA*

Previous work had indicated that both beetin forms are extracellular proteins that can be recovered from the apoplastic fluid (Iglesias *et al*., 2005). Assuming the possibility that the sugar beet ribosomes would be sensitive to beetin, it was thought that beetin confinement to the apoplastic space might prevent the depurination of its own ribosomes and perhaps other host RNAs. To check such a possibility, the potential *in vivo* depurination of native sugar beet ribosomes was analysed depending on the presence of induced beetin. As shown in Fig. 5A, neither germ sugar beet RNA, which lacks beetin, nor H_2O_2-treated plantlets, which do contain beetin, displayed a significant degree of depurination. To determine whether the H_2O_2-treated plantlets contained beetin in an active form, the *in vitro* depurination activity of such extracts was assayed on a rabbit reticulocyte lysate system. As shown in Fig. 5B, both the beetin-containing extracts from the H_2O_2-treated plantlets and purified BE27, as a control, triggered depurination splitting the Endo's fragment, characteristic of the action of RIPs.

The *in vitro* effect of added beetin on extracted total RNA from 4-week-old leaves of *B. vulgaris* was also studied, and it was found that beetin did not apparently promote depurination (Fig. 6A). Thus, the sugar beet RNA was not affected by the *in vivo* accumulation of beetin due to induction by H_2O_2, nor by the *in vitro* treatment of that total RNA with a large excess of highly purified beetin.

Discussion

Previous studies have shown that viral infection and the molecular mediators of viral infection such as H_2O_2 and SA trigger the expression of sugar beet RIPs (Girbes *et al*., 1996; Iglesias *et al*., 2005), but the controls operative in such expression remain unclear. To determine whether the response of beetin expression to elicitors could be developmentally regulated, plants were used from three growth stages: germ, 2-week-old plantlets, and 4-week-old plantlets. It was observed that the beetin transcript levels were similar in all cases except in the H_2O_2-treated 4-week-old plantlets. By contrast, the protein was not expressed in germ and 2-week-old plantlets; only a very slight trace of beetin was observed upon treatment with SA. In control 4-week-old plantlets, a very low amount of beetin was expressed and its expression

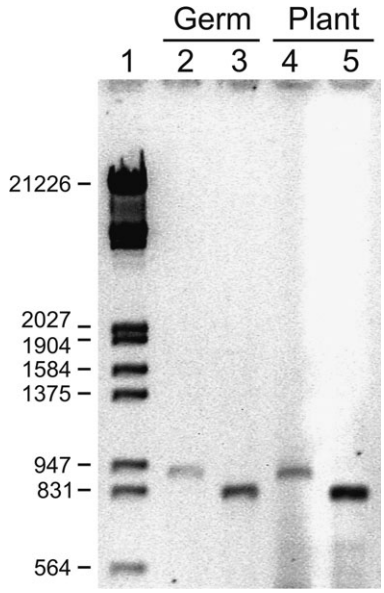

Fig. 4. RT-PCR analysis of specific mRNAs from germ (lanes 2 and 3) and 4-week-old plants induced with H_2O_2 (lanes 4 and 5). Reactions were carried out using the corresponding pairs of oligonucleotide primers. Lanes 2 and 4, primers BVF–UL; lanes 3 and 5, primers BVF–US. Numbers on the left indicate the size of the standards in nucleotides (lane 1).

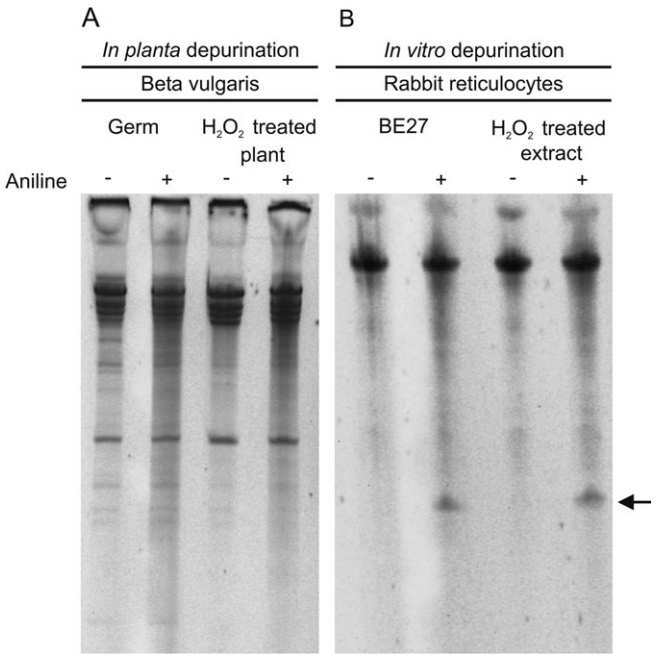

Fig. 5. Depurination triggered by beetin. (A) *In vivo* depurination of *B. vulgaris* rRNA. Three micrograms of total RNA from untreated germ and 4-week-old plants treated with 5 mM H_2O_2 were extracted, treated with acid aniline, and separated on a 5% (w/v) urea–polyacrylamide gel. (B) *In vitro* depurination of rabbit reticulocyte lysates: a crude protein extract was obtained from the same 4-week-old plants treated with 5 mM H_2O_2 as indicated in (A). The *N*-glycosidase activity of the crude protein extract was assayed by incubation of 80 µl of rabbit reticulocyte lysate with 10 µl of the protein extract or 2 µg of BE27 as a control of activity. The arrow shows the fragment split by acid aniline treatment of rabbit reticulocyte lysates.

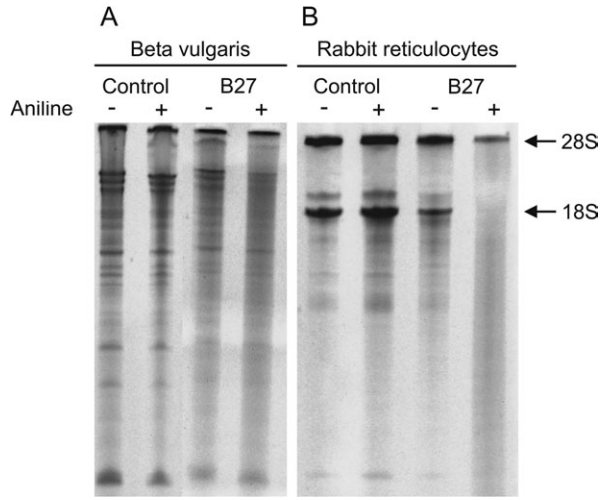

Fig. 6. Sensitivity to BE27 of extracted total RNA. (A) Depurination of RNA from *B. vulgaris*. Total RNA extracted from *B. vulgaris* leaves was denatured, incubated with BE27, treated with aniline, and fractionated on a 5% (w/v) urea–polyacrylamide gel. (B) Depurination of RNA from rabbit reticulocyte lysate. Total RNA extracted from rabbit reticulocyte lysate was treated with BE27 as above. By contrast to the sugar beet resistance to beetin, the rabbit reticulocyte total RNA is highly sensitive to beetin.

was greatly enhanced upon treatment with H_2O_2 and SA. On comparing the level of transcripts with that of the expressed protein, it seems clear that some kind of transcriptional and post-transcriptional regulatory mechanisms would be operating in the induction and expression processes.

The lack of a correlation between protein and mRNA levels suggests that constitutive mRNAs are defective or untranslatable. Such a possibility has been argued for ME1 transcripts (Vepachedu *et al*., 2003). The expression of ME1 transcripts, but not of the corresponding ME1 protein, occurs in response to exposure to jasmonic acid in cell cultures, which indicates a possible post-transcriptional regulation prior to translation (Vepachedu *et al*., 2003). Other reported inducible RIPs such as the *Mesembryanthemum crystallinum* RIP (Rippmann *et al*., 1997) or PIP2 (Song *et al*., 2000) seem to be regulated only at transcription level.

It was also found that induction of beetin with H_2O_2 seemed to take place only in 4-week-old plantlets, indicating that the elicitor-dependent expression of beetin is developmentally regulated. By contrast, the PIP2 gene was induced by wounding in leaves, regardless of their developmental stage (Song *et al*., 2000). Other studies have shown that many genes induced during the senescence associated with age are also induced by stress or stress-related hormones (Stirpe *et al*., 1996; Weaver *et al*., 1998).

It appears that the *B. vulgaris* genome contains two genes distinguished by sequence variation in the 3′ UTR. Three beetin transcripts were cloned and all three showed the same open reading frame, together with differences in their 3′ UTR. Two of the 3′ UTRs differ in their length, the short variant displaying only one polyadenylation site. The other variant showed differences in the 3′ UTR sequence. Several sequence motifs in the 3′ UTR have been implicated in the control of stability of mRNA and in post-transcriptional regulation (de Sauvage *et al*., 1992). It has previously been shown that cloned transcripts from *M. crystallinum* RIP1 (a stress-inducible RIP) have identical 3′ UTRs, although differing in length. The use of different polyadenylation sites correlated with transcripts from stressed or unstressed tissues has been reported for other stress-related proteins from *M. crystallinum* (Rippmann *et al*., 1997). The 3′-UTR variants for beetin had different predicted secondary structure thermostability energies, but it was found that the short transcripts, which had the lowest energy and could therefore have less stability, were present not only in germ but also in mature leaves. Nonetheless, the thermostability of the 3′ UTR does not preclude their presence, and other factors seem to be involved (Baek and Skinner, 2006).

It has been reported that beetin essentially accumulates in the intercellular fluid (Iglesias *et al*., 2005). The full-length beetin sequence shows that this RIP has a signal peptide, which is absent from the mature protein (Hornung *et al*., 1996; Iglesias *et al*., 2005). The present results indicate that ribosomes from *B. vulgaris* leaves are not depurinated *in planta* by endogenous beetins, nor *in vitro* by the addition of a large amount of highly purified BE27 (Figs 5, 6). This clearly indicates that sugar beet ribosomes are resistant to beetin and probably also to other type 1 RIPs. Additional support for this comes from complementary experiments that revealed that *B. vulgaris* S-30 extracts were insensitive to the highly active PAP-S (see Fig. S1 in Supplementary data at *JXB* online). Under the same conditions, *V. sativa* ribosomes treated with PAP-S released the Endo's fragment upon treatment with acid aniline (see Fig. S1 in Supplementary data at *JXB* online), as do *Escherichia coli* ribosomes in recombinant bacteria expressing BE-27 (Iglesias *et al*., 2005).

The above is in agreement with previous data reported for a large number of RIPs indicating that, in general, plant ribosomes are much less sensitive to RIP inactivation than mammalian ribosomes (Krawetz and Boston, 2000; Barbieri *et al*., 2004). On the other hand, some plants lacking measurable RIP activity contain cytosolic ribosomes that are highly sensitive to plant RIPs, such as those of *V. sativa* (Arias *et al*., 1992; Iglesias *et al*., 1993). Beetin is able to depurinate plant ribosomes from *V. sativa* (Iglesias *et al*., 2005). These differences between plants ribosomes have been reported previously (review in Barbieri *et al*., 1993). The targeting of beetin to the apoplasm could be to fulfil an as yet unknown biological role. One possibility would be to prevent virus propagation. Beetin could enter the cell from the apoplasm

together with viral particles and, once inside, attack their RNA, in keeping with the line hypothesized by Park *et al.* (2004). In fact, the direct multidepurination of purified tobacco mosaic virus (TMV)-RNA by beetin had been reported previously (Iglesias *et al*., 2005). The prevention by beetin of sugar beet infection by direct application of artichoke mottled crinkle virus particles supports our view (Iglesias *et al*., 2005). Further studies will address the potential involvement of the 3′ UTRs in the expression, stability, and translation of the different mRNAs.

Supplementary data

Supplementary data can be found at *JXB* online.

Figure S1. Effects of beetin and PAP-S on *Beta vulgaris* and *Vicia sativa* germ S-30 extracts.

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