The jasmonate-induced 60 kDa protein of barley exhibits *N*-glycosidase activity in vivo

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Abstract Upon jasmonate treatment barley leaf segments express a putative ribosome-inactivating protein (JIP60). The influence of this protein on translation in planta has been analysed by using barley plants and tobacco plants transformed with a barley cDNA encoding JIP60. In both plant systems JIP60 exhibited *N*-glycosidase activity in vivo. The depurination of the 25S rRNA of tobacco and barley ribosomes led to accumulation of translationally inactive polysomes.

Key words: Ribosome-inactivating protein; RNA *N*-glycosidase; *Hordeum vulgare*; *Nicotiana tabacum*

1. Introduction

In barley leaf segments treatment with jasmonic acid (JA) or its methyl ester (MJ) leads to de novo synthesis of proteins, so-called jasmonate-induced proteins (JIPs [1]). Based on sequence homology of the N-terminal region and in vitro studies, the JIP with a molecular mass of 66 kDa (JIP60) was identified to be a ribosome-inactivating protein (RIP [2,3]). RIPs are *N*-glycosidases that hydrolyse an adenine residue from a conserved loop of the large rRNA subunit (α-sarcin loop; A_{4324} in the case of rat liver ribosomes [4]). The α-sarcin loop is involved in binding of elongation factors (EFs) to ribosomes [5]. Therefore, modification of this loop leads to inactivation of the 60S ribosomal subunit and cessation of protein synthesis at the elongation step [6].

RIPs must enter the cytosol in order to reach their substrates. However, it has been demonstrated that most RIPs are targeted into cell walls, vacuoles or protein bodies or they are synthesised as inactive precursors [7]. Therefore, conspecific ribosomes cannot be affected by their own RIPs. So far RIP activity has been analysed using in vitro assays. In barley leaves the JIP60 is located in the cytosol [8]. Specific proteolysis of JIP60 in vivo was also demonstrated [2]. The artificial N-terminal derivative lacking 21 internal amino acids exhibited high RIP activity as analysed in vitro. Based on these facts, in vivo RIP activity of JIP60 might be assumed in barley. However, its analysis is complicated by the fact that besides JIP60 other JIPs accumulate simultaneously in the leaf upon jasmonate treatment. Among them there is the most abundant JIP of 23 kDa (JIP23), which was recently shown to be involved in post-transcriptional control as well [9]. To analyse RIP activity of JIP60 independently of other barley JIPs, we used transgenic tobacco plants constitutively expressing JIP60. Here we present evidence that JIP60 depurinated 25S rRNA of transgenic tobacco and barley in vivo. In both systems translationally inactive polysomes accumulated in the presence of JIP60.

2. Materials and methods

2.1. Plant material, incubation with jasmonate and tobacco transformation

Primary leaves of 7-day-old seedlings of barley (*Hordeum vulgare* L. cv. Salome) were used. Growth of seedlings and incubation conditions were performed as described [10]. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown axenically or in soil in a growth chamber at 25°C and a 16/8-h light/dark cycle with an irradiance of 95 μmol photons m^{-2} s^{-1}. Construction of the *Agrobacterium* vectors and transformation were performed as described recently [11].

2.2. Isolation of polysomes

For isolation of polysomes, 5–10 g of frozen tobacco leaves or barley leaf segments were ground in liquid nitrogen and subsequently dissolved in five volumes of buffer A (200 mM Tris-HCl, pH 8.9, 200 mM KCl, 35 mM $MgCl_2$, 0.6 M sorbitol, 12.5 mM EGTA, 15 mM DTT). After centrifugation at $10\,000\times g$ and $30\,000\times g$ (S-30 fraction), respectively, the homogenate was filtered through Miracloth. After adding Triton X-100 to a final concentration of 2% (w/v), the polysomes were pelleted by centrifugation at $160\,000\times g$ for 3.5 h through 1.5 M sucrose in buffer B (40 mM Tris-HCl, pH 8.9, 20 mM KCl, 10 mM $MgCl_2$, 1.5 M sucrose, 5 mM EGTA, 5 mM DTT). The pellet was gently resuspended in buffer C (10 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$) and centrifuged at $8000\times g$ in a microcentrifuge. The polysomes were frozen in liquid nitrogen and stored at −70°C until use. The purification state of polysomes was indicated by an OD ratio at 260/280 nm of about 1.8–1.9. In order to determine the polysomal profile, the polysomal preparation was loaded onto a linear sucrose gradient prepared in buffer C (15–55%, w/v) and was subsequently centrifuged at $260\,000\times g$ for 70 min. The gradient was monitored at 254 nm, and the percentages of polysomes and monosomes were estimated via the corresponding peak areas.

Ribosomes from yeast (*Saccharomyces cerevisiae*) were obtained by centrifugation of 12S supernatant at 55 000 rpm for 3.5 h through a sucrose cushion. The sedimented ribosomes were dissolved in a buffer containing 10 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$.

2.3. RNA isolation, ribosomal RNA depurination

In order to isolate RNA, polysomal fractions were treated with a mixture of phenol-chloroform and isoamyl alcohol, followed by precipitation with ethanol and resuspension in water. One portion of the polysomal RNA was treated in < 3-μl aliquots with 20 μl 1 M aniline, adjusted to pH 4.5 with acetic acid, at 50°C for 2 min to cleave the phosphodiester backbone at the site of depurination, reprecipitated in ethanol, and suspended in water. The aniline-treated and untreated RNAs (3–6 μg each) were separated by electrophoresis in 4.5% polyacrylamide gels in TBE buffer. Subsequently, the gels were washed

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Abbreviations: JA, jasmonic acid; JIP, jasmonate-induced protein; MJ, methyl jasmonate; RIP, ribosome-inactivating protein; eEF-2, eukaryotic elongation factor 2

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with fresh TBE buffer for 15 min, followed by staining with ethidium bromide for 30 min.

2.4. Cell-free translation

The standard assay was performed in 25 μl of reaction mixture containing 20 mM HEPES-KOH, pH 7.6, 4.5 mM Mg-acetate, 120 mM K-acetate, 2 mM DTT, 1 mM Na_2-ATP, 20 mM GTP, 8 mM creatine phosphate, 1 μg creatine kinase, 100 mM spermidine, 0.1–0.3 A_{260} units of the isolated polysomes (see above) and 250 μCi [^{35}S]methionine (3.7 MBq/mM). Protein synthesis was performed at 26°C with the S-30 fraction of wheat germ. Samples of 5 μl were withdrawn from the reaction mixture at the times indicated and the radioactivity was determined by scintillation counting according to Mans and Novelli [12]. Specific translational activities of polysomes were calculated via the percentage of polysomes within each preparation. Although the total amount of radioactivity incorporated varied between different incubations, the profile was similar in each individual incubation.

2.5. Separation and analysis of proteins

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose (Sartorius), and immunoblot analysis was performed with a 1:2000 dilution of rabbit polyclonal antibody raised against JIP60 using the Western light chemiluminescence detection kit (Tropix Inc.) as described previously [11].

3. Results and discussion

3.1. Generation of a proteolytic fragment (an active RIP) from JIP60 expressed in MJ-treated barley leaves and transgenic tobacco plants

RIPs are divided into two main groups depending on the absence (RIP type I) or presence (RIP type II) of a lectin chain. The majority of RIPs type I are synthesised as precursors, with N-terminal extensions that direct the RIP into the endoplasmic reticulum [13]. Many precursors also possess C-terminal extensions similar to those known to be required for the vacuolar targeting of several plant proteins [14]. RIPs type II are most probably synthesised as a preproprotein containing both the A- and the B-chain. They have to be cleaved post-translationally into the two domains to yield the active form. Based on amino acid sequence, molecular weight of JIP60 and its activity in vitro [2], its processing can be expected as well. To investigate the expression and the processing of JIP60 in MJ-treated barley leaf segments and transgenic tobacco plants Western blot analysis was performed. Several fragments were detected, which differed in number and molecular mass between both plants. In barley immunoreactive polypeptides of 66 and 11 kDa were detected (Fig. 1A), whereas in transgenic tobacco polypeptides with molecular masses of 37, 30, 27 and 20 kDa were found (Fig. 1B). It is not yet clear whether all these sub-fragments are formed by specific proteolysis. Probably, some low abundant fragments might be formed by non-specific cleavage. However, the molecular masses of some of them, such as the 30-, 17- and 11-kDa polypeptides, correspond precisely to fragments which have to be expected from the primary sequence [2].

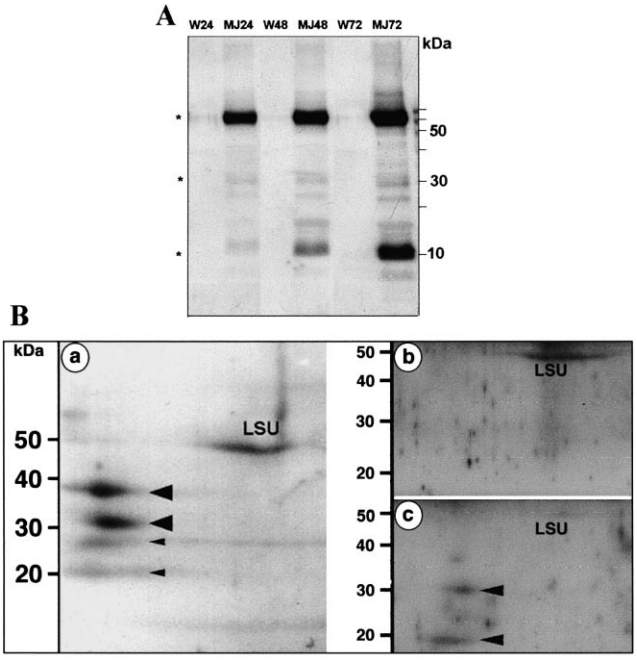

Fig. 1. Western blot analysis showing the expression of JIP60 in barley leaf segments after different times of MJ treatment (A) and wild-type (B,a) or transgenic tobacco plants (B,b). JIP60 was detected using polyclonal antiserum raised against JIP60. W24, MJ24, W48, MJ48, W72 and MJ72 are respective plant S-30 fraction proteins from leaf segments floated on water or MJ for 24, 48 and 72 h. B,c: Immunoblot of protein extract from transgenic plants using antibodies raised against the N-terminus of JIP60. Immunoreactive polypeptides are indicated by asterisks and arrows.

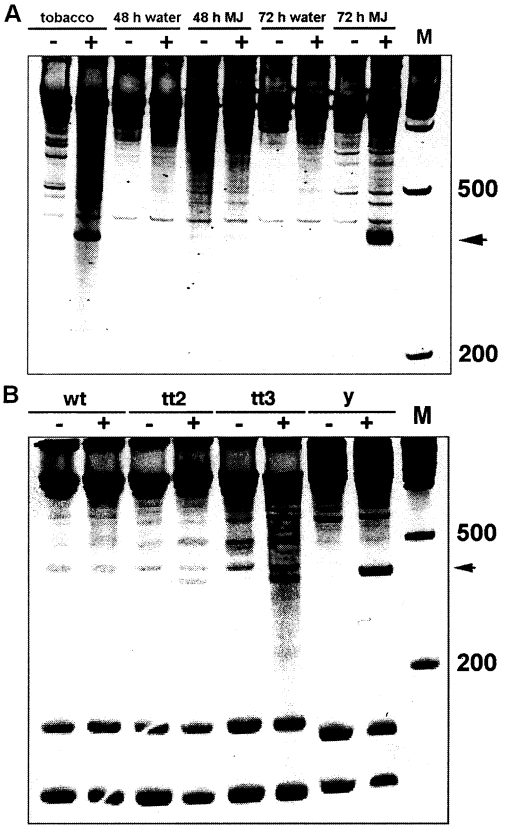

Fig. 2. Detection of depurination of barley (A) and tobacco ribosomes (B) in vivo. Ribosomes were isolated from 48- and 72-h water- and MJ-treated barley leaf segments and from tobacco plants (wild-type (wt) and classes 2 (tt2) and 3 (tt3)) and treated with aniline. 'tobacco' represents rRNA isolated from tobacco plant ribosomes treated with 10 μg/ml of ricin, 'y' designates rRNA from yeast ribosomes treated with 10 ng/μl of ricin. + and − denote the presence and absence of aniline treatment, respectively, and the arrow indicates the position of the diagnostic rRNA fragment of 390 nt.

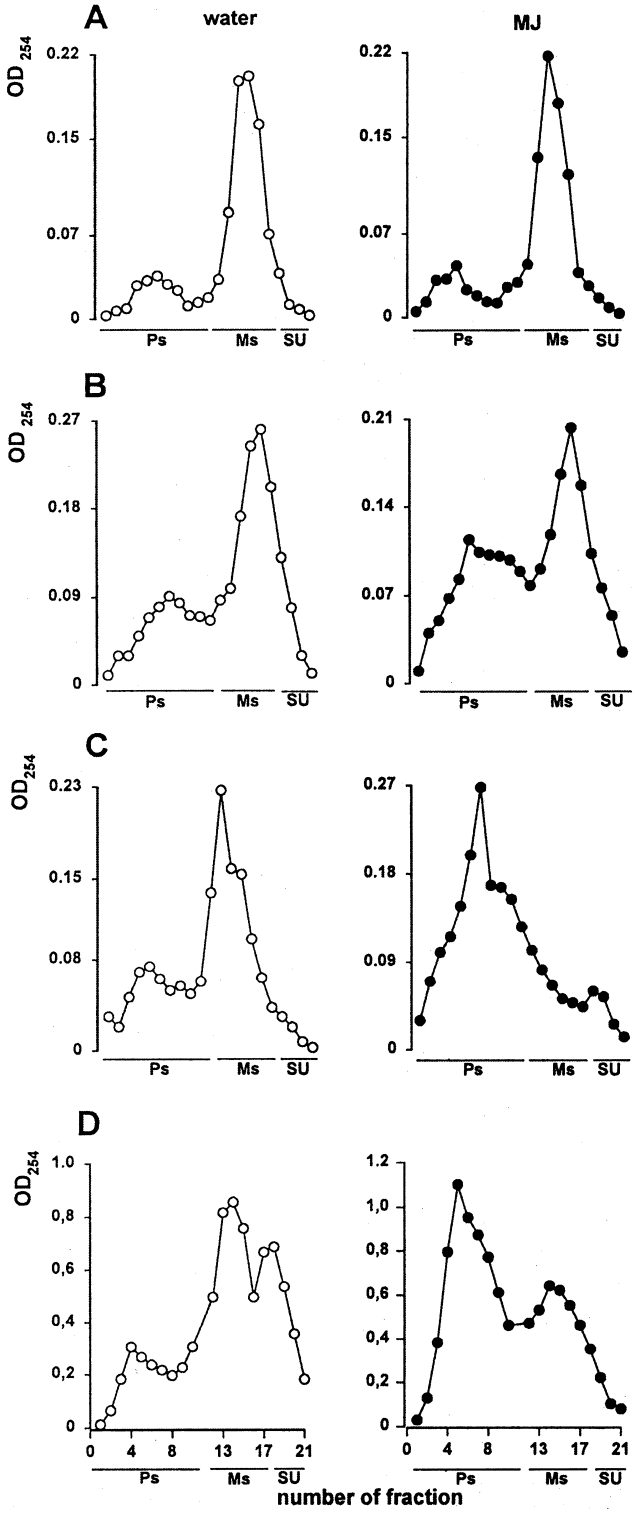

Fig. 3. Sucrose density gradient profiles of total ribosomes from water- and MJ-treated barley leaf segments. Ribosomes were isolated from 7-day-old leaf segments after 10 h (A), 24 h (B), 48 h (C), or 72 h (D) of water or MJ treatment. Ps: polysomes; Ms: monosomes; SU: subunits.

3.2. N-Glycosidase activity of JIP60 in vivo

RIPs are known to be *N*-glycosidases that specifically depurinate 23S/25S/26S/28S rRNA of the large subunit of bacterial, plant, yeast and mammalian ribosomes, respectively, by cleavage of *N*-glycosidic bonds and cause phosphodiester bonds to become sensitive to chemical hydrolysis [4,7]. Aniline treatment results in the formation of the diagnostic rRNA fragment specific for RIP activity since aniline cleaves the sugar phosphate bonds at modified nucleotides in the RNA [15]. Therefore, isolated polysomes of barley or tobacco were treated with aniline to inspect whether one or more polypeptide fragments shown in Fig. 1 exhibit *N*-glycosidase activity (Fig. 2). In barley the specific rRNA fragment was detected only upon 72-h MJ treatment (Fig. 2A), whereas ribosomes of water-treated leaf segments failed to generate this fragment. The appearance of the fragment at 72 h corresponds to an increased number and amount of JIP60 cleavage products. Interestingly, in tobacco, a higher level of the diagnostic fragment was observed in such transgenes, which exhibit a more dramatically altered phenotype (Fig. 2B) [11]. Thus, the depurination of ribosomes in both plant systems is direct evidence that JIP60 acts as a RIP in planta.

3.3. The effect of depurination on the ribosome function

Ribosomes treated with RIPs are unable to bind the eEF-2/GTP complex. This leads to a blockage of the protein synthesis at elongation step [6]. However, there are some reports that RIPs also affect initiation [16]. An alteration of the rates

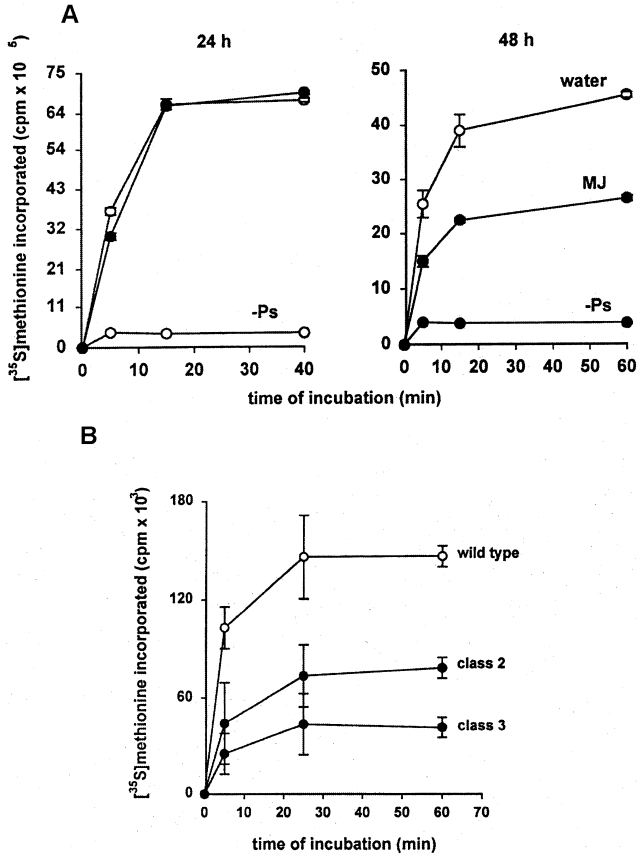

Fig. 4. Time course of incorporation of [^{35}S]methionine into protein with polysomes isolated from 24-h and 48-h water- (open circles) and MJ- (solid circles) treated barley leaf segments (A) and from wild-type and transgenic tobacco plants class 2 and class 3 (B). The bars represent the standard deviation from the average of three independent measurements of incorporation. −Ps represents protein synthesis in the wheat germ S-30 fraction without addition of barley or tobacco polysomes.

of translation steps results in a change in the polysome/monosome ratio [17,18]. The examination of polysome profiles, in conjunction with protein synthesis studies in vitro, makes it possible to identify which step of translation is effected. Fig. 3 represents sucrose density gradient profiles of ribosomes, isolated from barley leaf segments treated with water or MJ for 10–72 h. During the first 10 h MJ hardly affected the polysome/monosome ratio. However, after 24 h of MJ treatment the polysome proportion slightly increased. The effect was more pronounced after 48 h of MJ treatment. This shift towards polysomes can result either from an activated initiation or from a retarded elongation. Our previous data from transgenic tobacco plants expressing different amounts of JIP60 suppose an impairment of elongation by altered polysomal profiles and changes in the in vivo translational activity [11].

To investigate the capability of ribosomes to maintain the elongation step in vitro, polysomes prepared from both plants were used to monitor completion of a nascent polypeptide in a wheat germ in vitro translation system (run-off translation system). With barley ribosomes incorporation of labelled amino acids was not altered after 24 h of MJ treatment, but it was diminished after 48 h (Fig. 4A). Also in transgenic tobacco carrying JIP60, the translational activity was lower than in the wild-type (Fig. 4B).

The translation in vitro was not impaired by m^7 GTP (data not shown), suggesting that most if not all of the incorporated amino acids complete a nascent polypeptide chain associated with isolated polysomes. Hence, the reduction of the in vitro translational activity of polysomes in both plant systems was due to impairment of translation at the step of elongation or termination of both of them.

A conjunction of polysome profile analysis data with one of the translation activity in vitro leads to the conclusion that the accumulation of polysomes in vivo occurred due to impairment of the elongation step.

Thus the comparison of JIP60 activity in MJ-treated barley leaf segments and transgenic tobacco plants expressing JIP60 showed that JIP60 acted in both plant systems in the same manner: ribosomes are the target for JIP60 action. The detection of *N*-glycosidase activity in MJ-treated barley leaf segments and transgenic tobacco plants confirmed that JIP60 was active as a RIP in planta. The depurination of ribosomes caused the accumulation of polysomes and the reduction of their in vitro translation activity in both systems.

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