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Overexpression of the jasmonate-inducible 23 kDa protein (JIP 23) from barley in transgenic tobacco leads to the repression of leaf proteins

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Abstract We investigated transgenic tobacco lines which express different amounts of the barley JIP 23. In these plants the amount of several proteins decreased proportionally to increasing amounts of JIP 23 whereas the transcript levels were constant as determined for the small and the large subunit of RuBPCase. However, the translation initiation of the *rbcS* transcript was found to be less efficient than in the wild type. In contrast, the *jip 23* transcript was efficiently initiated, indicating that no unspecific impairment of initiation occurred. The data suggest that the barley JIP 23 leads to discrimination among certain tobacco transcripts during translation initiation.

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Key words: Jasmonate-inducible 23 kDa protein; Ribulose-1,5-bisphosphatase carboxylase/oxygenase; Translational control; Ribosome, initiation; *Nicotiana tabacum*

1. Introduction

Jasmonates are widely distributed plant hormones [1] which affect a variety of different physiological and developmental processes [2,3]. Barley leaf segments respond to treatment with methyl jasmonate (MJ) with the accumulation of newly synthesised proteins (JIPs) [4–6]. This de novo synthesis is accompanied by a down-regulation of several proteins, such as the subunits of ribulose-1,5-bisphosphatase carboxylase/oxygenase (RuBPCase) [4,5,7–9]. Recent studies showed that the lower amount of these proteins upon MJ treatment is not correlated with a lower amount of the corresponding mRNAs and seems to occur by a repressed translation initiation of these transcripts [10]. In contrast, for the MJ-induced mRNAs high rates of translation were found, contributing to accumulation of JIPs. Therefore, a post-transcriptional control was suggested, which discriminates between pre-existing and MJ-induced transcripts [10,11]. Since the most abundant JIP (JIP 23) starts to accumulate about 3 h before the onset of the repression of protein synthesis [5], we wondered whether this protein is able to repress translation. To exclude multiple effects such as simultaneous up-regulation of several proteins which occur in barley leaf segments upon MJ treatment, we generated tobacco plants expressing different amounts of JIP 23. A functional consequence appearing on JIP 23 accumulation in the transformants was a preferential down-regulation of a number of proteins, such as the small and the large subunit of RuBPCase, accompanied by a decreased photosynthetic activity.

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Abbreviations: JIP, jasmonate-induced protein; RuBPCase, ribulose-1,5-bisphosphatase carboxylase/oxygenase; SSU, small subunit of RuBPCase; LSU, large subunit of RuBPCase; LHCP, light harvesting chlorophyll protein complex

2. Materials and methods

2.1. Construction of the Agrobacterium vectors

All manipulations were done according to standard techniques [12]. In brief, the JIP 23 cDNA containing the entire coding region [13] was fused as an *Eco*RI fragment to a pBi121 derivative between the CaMV 35S promoter and the NOS polyadenylation signal [14] (S. Melzer, personal communication). The resulting plasmid pBi*JIP* 23s was transformed into *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw technique described by [15].

2.2. Transformation and molecular analysis of plants

Transformation of tobacco (*Nicotiana tabacum* L. cv. Samsun NN) was performed according to [16]. Transformed plants were grown axenically or in soil in the growth chamber at 25°C and a 16/8 h light/dark period with 95 μmol/m^2/s. DNA and RNA were extracted simultaneously as described previously [17]. The number of cDNA integrations was determined by genomic Southern blot analysis. High molecular weight DNA was digested with appropriate restriction enzymes (Gibco BRL Life Technologies) according to the manufacturer's protocol. The fragments were separated on a 0.8% agarose gel and blotted according to [12] using charged nylon membranes (Qiagen).

The expression of the genes of interest was investigated by Northern blots [12]. 20 μg total RNA were applied to a denaturing 1.2% agarose gel, separated and transferred onto uncharged nylon membranes (Qiagen) via capillary blot. To control the uniform loading, the gel was stained with ethidium bromide prior to blotting.

DIG-labelled probes specific for *rbcS*, *rbcL*, and *jip 23* mRNA were synthesised using the DIG labelling and detection kit (Boehringer Mannheim). Hybridisation and detection with CSPD (Tropix) followed the protocol of [18].

2.3. Isolation of polysomal bound and free mRNA

All operations were carried out at 0–4°C according to [19]. Leaf segments (2–5 g) of barley or young tobacco leaves were ground in liquid nitrogen and homogenised 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 two low-speed centrifugations (10 000×*g*, 10 min and 30 000×*g*, 20 min), the supernatant was filtered through Miracloth (Calbiochem-Novabiochem), and Triton X-100 was added to a final concentration of 2% (w/v). Polysomes were collected by centrifugation at 160 000×*g* for 3.5 h through a sucrose cushion (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), and subsequently resuspended in buffer C (10 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$). The RNA was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation (2.5 vol.). To obtain free RNA the supernatant from the high speed centrifugation was treated as above, except for an additional ethanol precipitation step (0.1 vol.) to remove excess polysaccharides prior to the precipitation of the RNA. The RNAs were dissolved in TE buffer and stored at −70°C. The amount of the isolated RNA was determined spectrophotometrically, while its quality was assayed by in vitro translation.

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2.4. In vitro translation assay

Rabbit reticulocyte lysate (Novagen) was supplemented with 10 μg of either polysomal bound or free RNA and 10 μCi [^{35}S]methionine, and was subsequently used according to the manufacturer's instructions. The translation products were separated in 7.5–25% gradient SDS polyacrylamide gel electrophoresis (PAGE), fixed, dried, and exposed to X-ray film [4].

2.5. Analysis of proteins

Total proteins were extracted from young, not fully expanded tobacco leaves that were of similar age. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Schleicher and Schuell) as described previously [20]. Rabbit polyclonal antibodies raised against JIP 23 overexpressed in *Escherichia coli* according to [21] were used in a dilution of 1:2400. JIP 23 was detected with the Western Light Chemiluminescent Detection Kit (Tropix) following the manufacturer's instructions. Two-dimensional SDS-PAGE of total proteins was carried out according to [22] with the exception that SDS solubilisation buffer was used [23].

2.6. Measurement of the assimilation rate

The gas exchange was measured in an open system (Waltz) comprising a binos 100 infrared gas analyser (Rosemount) and a climatised cuvette located inside a phytotron. Measurements were performed at 270, 500, and 1000 μmol photons/m^2/s. After adjustment of the mounted leaf to the ambient conditions, the steady-state assimilation and transpiration were measured for 15 min. Leaves of similar age (nearly full expanded) and still attached to the plant were analysed. Transgenic and wild type plants were analysed at the same time of day to prevent interference by the circadian rhythm.

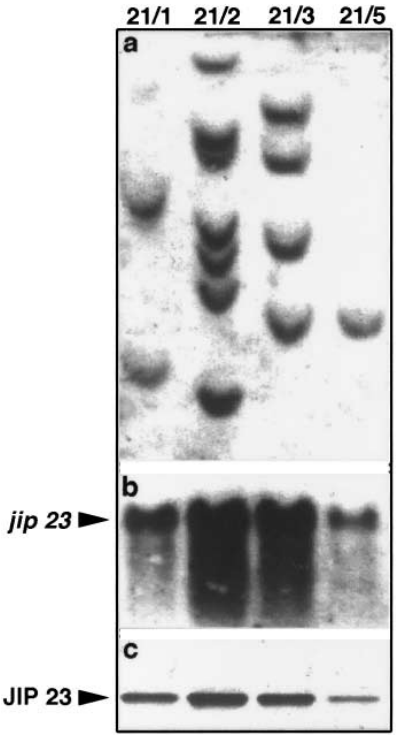

Fig. 1. Analysis of the integration and expression of JIP 23 cDNA. a: Southern analysis of *Hin*dIII digested genomic DNA from transformed tobacco lines. 20 μg of genomic DNA was electrophoresed, blotted and subsequently hybridised with a DIG-labelled JIP 23 probe. The copy numbers of the JIP 23 cDNA in the independent lines were estimated according the detectable fragments: line 21/1: two copies; line 21/2: seven copies; line 21/3: four copies; line 21/5; one copy. b: Northern analysis of the JIP 23 mRNA present in the transgenic lines. 20 μg of total RNA was electrophoresed, blotted and hybridised with a DIG-labelled JIP 23 probe. c: Western blot analysis of JIP 23 present in the transgenic lines. Total proteins of leaves (5 μg per lane) were separated and blotted. JIP 23 was detected using polyclonal antiserum raised against JIP 23 of barley. Arrows indicate the JIP 23 mRNA and the JIP 23 protein.

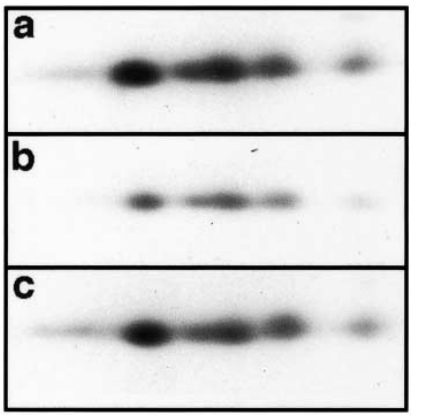

Fig. 2. Identification of JIP 23 isoforms in the transgenic tobacco plants. 5 μg of total protein was separated by two-dimensional SDS-PAGE, blotted and detected using polyclonal antiserum raised against JIP 23 of barley. Isoforms present in (a) transgenic tobacco line 21/2, (b) MJ-treated barley leaf segments, and (c) a combination of both extracts.

3. Results and discussion

3.1. Regeneration and analysis of transgenic tobacco plants expressing JIP 23 cDNA

Among the barley JIPs the most prominent is a protein with a molecular mass of 23 kDa (JIP 23). The function of this protein is still unknown, particularly as the cDNA clone which has been isolated shows no similarities to sequences available in the databases [13]. To obtain some insight into the possible role of JIP 23 we transformed tobacco plants with the full length cDNA of JIP 23 under the control of the cauliflower mosaic virus promoter CaMV 35S. Transformants were propagated in vitro and proved to be independent transformants by Southern blot analysis. Four primary transformant lines were selected carrying one (line 21/5), two (line 21/1), four (line 21/3) or seven (line 21/2) integrations of the JIP 23 cDNA (Fig. 1a). Inspection of these lines by Northern and Western blot analysis revealed that the amounts of mRNA and of JIP 23 correlated with the number of integrations (Fig. 1b, c) indicating that all *jip 23* transgenes were functional.

In barley leaf segments six isoforms of JIP 23 are detectable upon MJ treatment [24]. To estimate which of them were heterologously expressed in the transgenic lines, total protein extracted from MJ-treated barley leaf segments and from young leaves of the transgenic line 21/2 were separated by 2-D SDS-PAGE and analysed by Western blots. For transgenic tobacco leaves six JIP 23 isoforms could be detected, which were identical to those of MJ-treated barley leaf segments as shown by analysis of combined protein extracts (Fig. 2). These results suggest that (i) tobacco modifies JIP 23 post-translationally like, if not the same as barley, and (ii) one *jip 23* gene out of at least two present in barley (E. Görschen, unpublished results) is sufficient to generate all six isoforms occurring in barley leaf segments upon MJ treatment. So far, the nature of the modifications leading to the isoforms of JIP 23 in barley leaf segments and in transgenic tobacco is unknown.

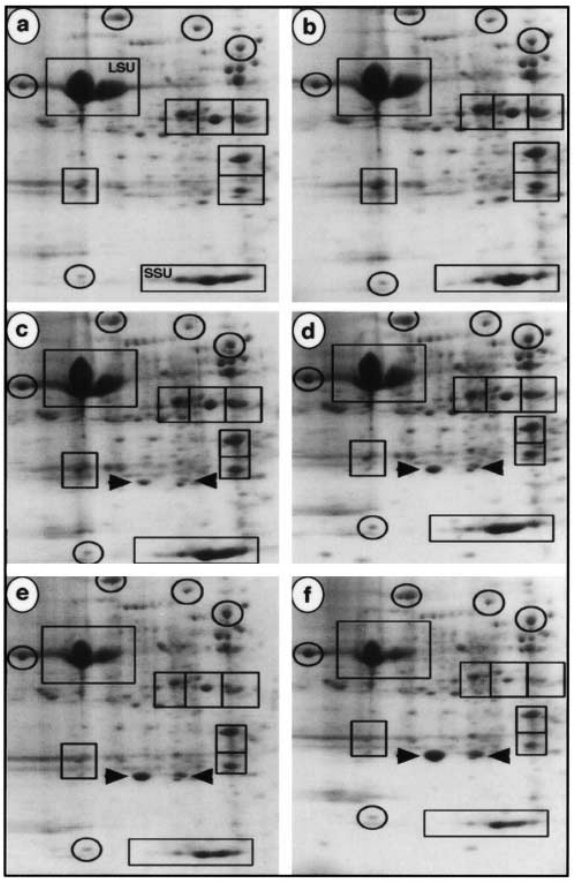

Fig. 3. Protein pattern from leaves of wild type and transgenic tobacco plants. 25 μg of total proteins were separated by a 2-dimensional SDS-PAGE and stained with Coomassie blue. (a) wild type tobacco plants, (b) transformed control tobacco plant containing the JIP 23 in antisense orientation, (c-f) transgenic tobacco lines containing one (line 21/5), two (line 21/1), four (line 21/3), and seven (line 21/2) copies of the JIP 23 cDNA. Boxes indicate proteins which are reduced in the transgenic lines, circles indicate proteins which are constitutively expressed, arrows indicate JIP 23.

3.2. *The protein composition in transgenic tobacco is altered in the presence of JIP 23*

In barley leaf segments mRNA encoding JIP 23 accumulates 3–5 h after onset of MJ treatment [5]. Later on the amount of various nuclear- and chloroplast-encoded proteins decreases [4,5,8,10,11,25] which is not reflected by the amounts of the corresponding mRNAs for at least 36 h (e.g. SSU, LSU, LHCP, 65 and 68 kDa proteins of photosystem I) [10,25,26]. This fact together with other data led to the suggestion that the initiation of translation of these mRNAs might be impaired [10,11,25] due to the presence of JIPs (JIP 23, JIP 60). To test whether the expression of JIP 23 in transgenic tobacco alters the polypeptide composition, we compared two-dimensional protein pattern of the wild type with that of the transgenic lines 21/1, 21/2, 21/3, and 21/5 carrying two, seven, four, and one copy of the JIP 23 cDNA, respectively (Fig. 3). The inspection revealed a proportional increase of JIP 23 with the number of transgene integrations (Fig. 3c–f, arrowheads; Fig. 1c). However, the amount of several proteins, such as the small and the large subunit of RuBPCase (SSU, LSU), decreased (Fig. 3, boxes). The amounts of other proteins appeared to be constant indicating the equal loading of gels (Fig. 3, circles). The protein pattern of control plants transformed with JIP 23 cDNA in antisense orientation did not differ from that of the wild type (Fig. 3b). This fact, and the strict correlation between the amount of JIP 23 and that of other proteins in the transgenic plants, argues against artificial effects caused by the transformation/regeneration process [27].

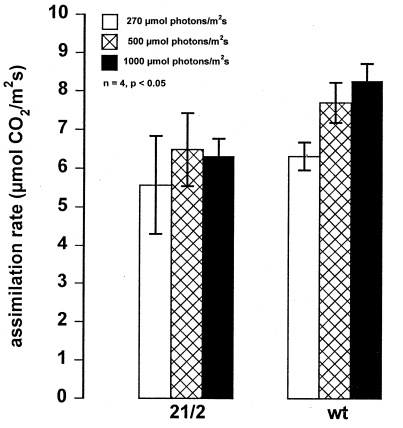

Fig. 4. Comparison of the calculated assimilation rate of transgenic tobacco line 21/2 and wild type plants (wt). Leaves were mounted in a cuvette and gas exchange parameters were measured under different light intensities. Each column represents the means of four leaves of different plants, measured three times each. The indicated standard deviation is within the 95% confidence interval.

For the further experiments described below we chose the transgenic tobacco line 21/2 which exhibited the most obvious alterations at the protein level.

3.3. *The photosynthetic activity is decreased in tobacco leaves expressing barley JIP 23*

The lower amounts of RuBPCase suggest an altered photosynthetic activity. Therefore, we analysed under different light intensities the gas exchange of single leaves from wild type plants and from the transgenic line 21/2 carrying the lowest amount of RuBPCase (Fig. 3f). The rate of assimilation, calculated from the gas exchange data, displayed no significant differences between the transgenic plants and the wild type plants at light intensities of 270 and 500 μmol photons/m^2/s (Fig. 4, open and cross-hatched columns). However, at a light intensity of 1000 μmol photons/m^2/s the assimilation rate was significantly reduced in the transgenic line compared to that of the wild type plants (Fig. 4, solid columns). The decreased assimilation rate corresponds to the low amount of RuBP-

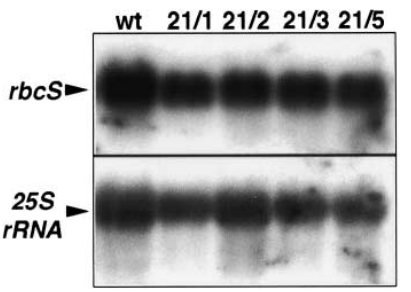

Fig. 5. Northern analysis of *rbcS* mRNA present in the transgenic tobacco lines and in wild type plants. 20 μg of total RNA was electrophoresed, blotted and hybridised with a DIG-labelled *rbcS* probe. Uniformly loading of the gel was proofed with a 25S rRNA probe.

Case present in this transgenic line (Fig. 3f). All transgenic tobacco lines expressing JIP 23 exhibited no altered phenotypes, most likely due to the relatively low light intensities (95 μmol photons/m^2/s) in the growth cabinet.

3.4. *The rbcS transcript is bound to polysomes less stringently in JIP 23 expressing tobacco plants*

In order to determine whether the decreased amount of certain proteins in the transgenic tobacco lines is transcriptionally regulated, we compared the amount of *rbcS* mRNA from wild type plants with that of the transgenic tobacco line 21/2 by Northern blot analysis. As shown in Fig. 5 no significant difference was detectable suggesting any type of translational control, either during the initiation or elongation. Therefore, we analysed the amount of *rbcS* and *jip 23* mRNA in polysomal and in free RNA fractions from young leaves of wild type and transgenic plants. Any arrest of elongation would contribute to accumulation of mRNAs in the polysomal fraction, whereas upon impaired initiation mRNAs would occur mainly in the free RNA fraction. As shown in Fig. 6a, in the fraction of free RNA prepared from the wild type plant only a minute amount of *rbcS* transcript was detectable. In contrast, in leaves expressing JIP 23 a substantial amount of *rbcS* transcript was present in the fraction of free RNA, making evident that the *rbcS* transcript is bound less stringently to the ribosomes. However, the transcript was also detectable in the polysomal RNA fraction of the transgenic plants, indicating that the rejection from the ribosomes is not complete. An unspecific effect by JIP 23 on ribosomes is very unlikely since the *jip 23* mRNA was found to be almost completely bound to polysomes in the transgenic plants (Fig. 6b). The data suggest that within the transgenic plants a discrimination of transcripts such as *rbcS* occurred during translation most likely due to the presence of JIP 23.

So far it is an open question whether the decreased amount of LSU whose mRNA amount is also unchanged in the transgenic lines (Fig. 7) is due to a similar mechanism. A direct influence of the JIP 23 on the synthesis of the LSU is very unlikely due to its extraplastidic location [28]. However, the

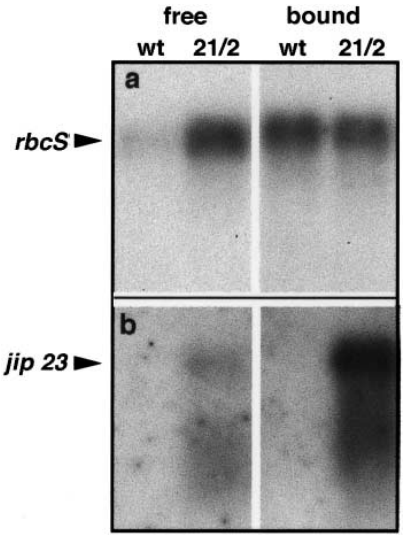

Fig. 6. Free and polysomal bound *rbcS* (a) and *jip 23* mRNA (b) in the transgenic tobacco line 21/2 and wild type plants. 20 μg of either RNA fraction was electrophoresed, blotted and hybridised with a DIG-labelled *rbcS* and a DIG-labelled *jip 23* probe, respectively.

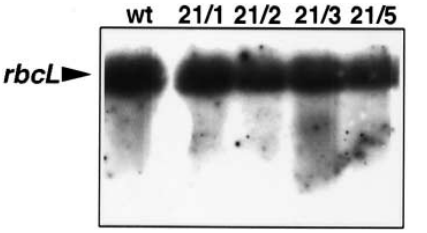

Fig. 7. Northern analysis of the *rbcL* mRNA present in the transgenic tobacco lines and in wild type plants. 20 μg of total RNA was electrophoresed, blotted and hybridised with a DIG-labelled *rbcL* probe.

assembly of chimeric nuclear- and plastid-encoded proteins such as the RuBPCase, were found to be highly co-ordinated (reviewed in [29]), e.g. the synthesis of LSU ceases if the synthesis of the SSU is blocked [30–32]. Furthermore, the synthesis of the RuBPCase subunits seems to be regulated at initiation of translation [33]. These facts suggest that a decreased amount of the SSU caused by JIP 23 as described above would contribute to a decrease in translation initiation of *rbcL* transcripts.

Taken together, the data presented here lead to the suggestion that JIP 23 has the potential to reduce the binding of certain mRNAs to ribosomes in transgenic tobacco plants. We hypothesise that JIP 23 might act in a similar manner in MJ-treated barley leaf segments. At least the time course of JIP 23 synthesis and translation repression of certain mRNAs in MJ-treated barley leaf segments as well as the fact that similar proteins were reduced in both barley and transgenic tobacco would support this idea. Furthermore, the identification of the MJ-induced protein JIP 60 as an active RIP [17,34,35] implies that the regulation of translation at the initiation and the elongation might be an important mechanism of controlling gene expression in MJ-treated barley leaves.

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