Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells

Bae Hyunjong*, Dae-Seok Lee* and Inhwan Hwang†

Center for Plant Intracellular Trafficking, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, South Korea

Received 28 June 2005; Accepted 20 October 2005

Abstract

One of the limiting factors in the production of recombinant proteins in transgenic plants is the low level of protein accumulation. A strategy was investigated for a high level of protein accumulation in plant cells. A fungal xylanase encoded by XYLII of Trichoderma reesei was chosen as the model protein because xylanases have a high potential for applications in environment-related technologies. Xylanase was expressed in the cytosol or targeted either to chloroplasts or peroxisomes alone, or to both organelles simultaneously. When xylanase was targeted to both chloroplasts and peroxisomes simultaneously the amount of xylanase accumulated was 160% of that in chloroplasts alone and 240% of that in peroxisomes alone although the transcript levels were similar among these constructs. The growth stage of the transgenic plants also affected the total amount of xylanase; the highest level of accumulation occurred at the time of flowering. This study provides genetic and biochemical data demonstrating that a high level of protein accumulation in transgenic plants can be obtained by targeting a protein to both chloroplasts and peroxisomes at the same time.

Key words: Chloroplast, dual targeting, peroxisome, transgenic plants, xylanase.

Introduction

Expressing exogenous proteins in transgenic plants is potentially one of the most economical systems for the large-scale production of recombinant protein (Daniell et al., 2001; Leelavathi et al., 2003). To date, many commercially important recombinant proteins have been successfully expressed in plants. However, to be economically successful, a high level of protein accumulation is a critical factor for the production of recombinant proteins. Various approaches have been used to manipulate the expression level of these proteins in plants. Despite recent advances, a low protein level is a major hurdle in the use of plants as bioreactors in order to produce pharmaceutically and industrially important proteins on a large scale (Gidding et al., 2000). A possible approach for increasing recombinant protein production in transgenic plants is the development of artificial targeting systems to localize proteins at different organelles. In fact, it has been shown that a soluble protein can be accumulated at high levels when it is targeted to chloroplasts. The possibility is exploited here of targeting a protein to two different organelles simultaneously using two different targeting signals. Most of the experiments reported before involved targeting of a protein to a single organelle. Therefore, targeting a protein to two different organelles at the same time could, in theory, provide more space for protein accumulation, thus resulting in a higher level of protein accumulation in the cell. However, in the majority of cases, proteins have a signal sequence that is specific for an organelle. An exception to this is a few proteins that are dually targeted to both chloroplasts and mitochondria (Akashi et al., 1998; Silva-Filho, 2003; Wall et al., 2004). To prove this hypothesis, a protein was designed that can be targeted to both the chloroplast and the peroxisome. It is well known that the N-terminal transit peptide is necessary and sufficient for targeting a protein to the chloroplast (Van den Broeck...
et al., 1985; Smeekens et al., 1986; Bassham et al., 1991; Bruce, 2000; Lee et al., 2002). In contrast, the C-terminal three amino acid residues, SKL, are necessary and sufficient for targeting a protein to the peroxisome (Nito et al., 2002; Sparkes and Baker, 2002). The gene, XYLII encoding xylanase was cloned from Trichoderma reesei (Carter et al., 1992) and fused with the N-terminal transit peptide of a stromal protein Rubisco activase for targeting to the chloroplast and the C-terminal SKL sequence for targeting to the peroxisome. Evidence is provided here that transgenic plants expressing xylanase targeted to both the chloroplasts and the peroxisome accumulated significantly higher levels of protein than those expressing xylanase targeted to chloroplasts or peroxisomes alone.

**Materials and methods**

**Plant transformation and growth**

Arabidopsis thaliana (ecotype Columbia) was transformed by the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were selected on Murashige and Skoog (1962) (MS) agar plates supplemented with kanamycin (50 mg l\(^{-1}\)). Plant lines containing a single T-DNA insertion were selected based on the ratio of kanamycin-resistant/sensitive plants of the T2 generation.

**Construction of plant expression vectors**

To construct RA:GFP, a DNA fragment encoding the N-terminal transit peptide (80 amino acid residues) of Rubisco activase (RA, GenBank accession number A2g39730) (Wernke and Ogren, 1989) was amplified by polymerase chain reaction (PCR) using specific primers 5'-ATGGCCGCCCAGTTCCTCCACCTCCCTC-3' and 5'-CCATCCATCTGGCTCCATGTTGTTT-3' and fused to the N-terminus of the GFP coding region. To generate GFP:SKL, the C-terminal SKL sequence was added to GFP by PCR using specific primers 5'-ATGAGTAAAGAGAAGAACATT-3' and 5'-TTATAGTTTCGATTTGTATAGTGAT-3'. To construct pCamRbc-XYLII for the expression of xylanase in the cytosol, XYLII that encodes endo-β-1, 4-xylanases (EC 3.2.1.8) of Trichoderma reesei (Carter et al., 1992) was placed under the control of the promoter of an alfalfa RbcS gene, RbcSK-IA (Khoudi et al., 1997). To construct pCamRbc-RA:XYLII for targeting xylanase to chloroplasts, the N-terminal transit peptide of RA was fused to the N-terminus of xylanase. To construct pCamRbc-XYLII:SKL for targeting xylanase to peroxisomes, three amino acid residues SKL (Ser-Lys-Leu) were added to the C-terminus of xylanase by PCR using primers 5'-ATGGGTTGCTTTTCCAGC-3' and 5'-CTATACTAGTTGACACTCTGTG-3'. To generate pCamRbc-RA:XYLII:SKL for dual targeting of xylanase to the chloroplast and the peroxisome, the N-terminal RA transit peptide and the C-terminal SKL motif were fused to the N- and C-termini, respectively, of xylanase by PCR using two specific primers 5'-GCTCTAGAAGTGCCGGCGCAGTTTCCC-3' and 5'-CTATACTAGTTGACACTCTGTG-3'.

In vivo targeting of fusion protein

For transient expression of proteins in protoplasts, plasmid DNAs were introduced into Arabidopsis protoplasts prepared from wild seedlings by the polyethylene-glycol-mediated transformation method (Jin et al., 2001). Expression of the fusion protein was monitored at various time points after transformation, and images were captured with a cooled charge-coupled device camera equipped to a Zeiss Axioplan fluorescence microscopy (Zeiss, Jena, Germany).

**Generation of transgenic plants**

Transgenic Arabidopsis plants expressing these xylanase fusion proteins were generated according to the floral dip method (Clough and Bent, 1998). Kanamycin-resistant plants were selected from the T1 generation and used for RT-PCR and protein analyses. Genomic DNA was isolated from Arabidopsis plants (T2 generation) and used as template for PCR amplification. T1 plants were analysed for the presence of the transgenes by PCR using primers 5'-CGAGCTCATGGTCTCCTCCACCTCCCTC-3' and 5'-GCTCTAGACTAGTTGCTGACACTCTGTG-3' that recognizes the XYLII gene.

For RT/PCR, total RNA was extracted from 100 mg of frozen mature leaves using the lithium chloride method described by Aufzay and Rougeon (1980) and treated with DNase I (RNase free). RNA was extracted, ethanol precipitated, and resuspended in 50 µl of sterile distilled water. cDNAs were generated from 5 µg of RNA with the Superscript reverse transcriptase (Gibco/BRL) using a gene-specific primer 5'-GCTCTAGACTAGTTGCTGACACTCTGTG-3'. Subsequently, the cDNA was used for PCR amplification using the same primers as those used to examine the presence of the XYLII gene from the genomic DNA.

**Western blot analysis**

Protein extracts were prepared from mature leaf tissues of T2 plants as described previously (Loulakis et al., 2002). The western blot was proved with anti-xylanase antibody and subsequently developed with the enhanced chemi-luminescence kit (Amersham). Rabbit anti-XYLII polyclonal antibody was used at 1:2000 dilution and incubated for 1 h at room temperature. HRP-conjugated goat polyclonal anti-rabbit IgG antibody (Sigma) was used at 1:1000 dilution and incubated for 1 h. The ECL detection system was used for detection (Amersham Pharmacia Biotech). The amount of protein was determined by comparing the intensity of recombinant xylanase bands on western blots with those of purified xylanase bands obtained from E. coli extracts. The protein standard curve was obtained using serial dilution of purified xylanase. The protein concentration of purified xylanase was determined by the Bradford method (1976).

**Enzyme assays**

Xylanase activity was determined by measuring the amount of reducing sugars released from a 1% (w/v) xylan solution in 0.1 M phosphate buffer (pH 6.8) for 30 min at 50 °C. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per 30 min. The amount of reducing sugars released was determined using the dinitrosalicylic acid (DNS) method (Miller et al., 1959).

To determine the enzymatic activity of organelle-targeted recombinant xylanase, chloroplast and peroxisomal fractions were obtained from whole cell homogenates as described below. Homogenates of transgenic leaves were prepared and applied to a Percoll step gradient, and the chloroplast fraction was collected as previously described (Robinson and Barnett, 1988). The purified chloroplasts were lysed and subjected to centrifugation at 10 000 g at 4 °C for 10 min to obtain the supernatant. The supernatant was used for enzyme assay. To obtain a peroxisomal fraction, the whole cell homogenates were subjected to equilibrium density centrifugation as previously described (Smith et al., 2000). The peroxisomes were found in the crude organelle pellet together with mitochondria and lysosomes. The peroxisomes in the pellet fraction were lysed and used for enzyme assay.
Results

**Dual targeting of a protein to both chloroplasts and peroxisomes in protoplasts**

Various approaches have been used to achieve a high level of protein production in plants (Daniell *et al*., 2001; Leelavathi *et al*., 2003). As an approach, it was hypothesized that targeting of a protein to two different organelles at the same time could increase the amount of protein accumulation in the cells significantly. This possibility was examined first in protoplasts. The protoplast system using the transient expression of introduced genes is convenient to examine this possibility quickly and easily (Jin *et al*., 2001). To this end, a number of different constructions were generated (Fig. 1A) and the fusion constructs expressed transiently in protoplasts by PEG-mediated transformation. The green fluorescent protein (GFP) coding region was fused to the N-terminal transit peptide (tp) of Rubisco activase (RA) for targeting to the chloroplast and the C-terminal SKL sequence for targeting to the peroxisome in order to investigate the possibility of targeting GFP to two different organelles simultaneously. The fusion construct, RA:GFP:SKL, was introduced into *Arabidopsis* protoplasts using the PEG-mediated transformation method (Jin *et al*., 2001), and the localization of GFP was examined by fluorescence microscopy. GFP signals gave two different staining patterns,

![Diagram showing DNA constructs and their localization](https://example.com/diagram.png)

**Fig. 1.** Targeting of protein to the chloroplast and the peroxisome. (A) Schematic presentation of DNA constructs. (a) RA:GFP:SKL; (b) RA:GFP; (c) GFP:SKL; and (d) RFP:SKL. (B) *In vivo* targeting. Protoplast were transformed with RA:GFP:SKL plus RFP:SKL and localization of green and red fluorescent signals was examined 12 h and 24 h after transformation. Green, red, and blue fluorescent signals indicate GFP, RFP, and chlorophyll, respectively. Note that the colour of the autofluorescent signal of chlorophyll is depicted in blue. (C) Western blot analysis of RA:GFP and RA:GFP:SKL. Protein extracts were prepared from transformed protoplasts and used for western blot analysis using anti-GFP antibody.
chloroplast and punctate staining patterns at various time points after transformation (Fig. 1B, panels a and e), suggesting that RA:GFP:SKL may be targeted to both the chloroplast and the peroxisome. To examine the identity of the organelle that gives the punctate staining pattern, RA:GFP:SKL was co-transformed into protoplasts together with RFP:SKL, a marker for the peroxisome, and localization of these proteins was examined (Lee et al., 2002). The GFP signals in the chloroplast closely overlapped the red autofluorescent signals (depicted in blue) of chlorophyll (Fig. 1B, panels a, c, and d), indicating that RA:GFP:SKL is targeted to the chloroplast. In addition, the green fluorescent signals at the punctate stains closely overlapped the RFP signals at peroxisomes (Fig. 1B, panels a, b, and d), indicating that RA:GFP:SKL is also targeted to the peroxisome. Together these results indicate that the artificially designed dual-targeting protein, RA:GFP:SKL, is targeted to both the chloroplast and the peroxisome.

To obtain independent evidence for the dual targeting of RA:GFP:SKL, protein extracts obtained from the transformed protoplast were analysed by western blot analysis. Protein extracts were prepared from protoplasts transformed with RA:GFP or RA:GFP:SKL and used for western blot analysis using the anti-GFP antibody. In control protoplasts transformed with RA:GFP, the GFP band was detected at the size of 32 kDa, an expected size of the processed form of RA:GFP (Fig. 1C), indicating that RA:GFP is targeted to the chloroplasts. By contrast, protoplasts transformed with RA:GFP:SKL gave two different protein bands, at positions 36 kDa and 32 kDa, which correspond to the precursor and processed forms, respectively, of RA:GFP:SKL (Fig. 1C). The accumulation of the upper band was specific for the construct with the peroxisomal targeting, suggesting that the protein species in the upper band is targeted to the peroxisome. These results are consistent with the data obtained from image analysis of transformed protoplasts expressing RA:GFP:SKL.

Expression of recombinant proteins in transgenic plants

In the next step, it was examined whether the dual targeting system can direct a protein to two different organelles in transgenic plants. Four different constructs were designed using the XYLII gene encoding xylanase (Fig. 2A). In RA:XYLII:SKL, xylanase was fused with the N-terminal transit peptide of RA and the C-terminal SKL motif at the N- and C-termini, respectively. The chimeric gene was under the control of the strong RbcS (RbcSK-1) promoter from alfalfa (Khoudi et al., 1997). In RA:XYLII and XYLII:SKL, xylanase was fused with the N-terminal transit peptide at the N-terminus and the C-terminal SKL motif at the C-terminus, respectively. In UTR:XYLII, only the 5'-untranslated region (UTR) of the Arabidopsis small subunit of Rubisco complex (RbcS, accession number At1g67090) was added to the N-terminus of the xylanase coding region. Transgenic plants harbouring RA:XYLII:SKL, RA:XYLII, XYLII:SKL, or UTR-XYLII were generated by Agrobacterium-mediated transformation (Clough and Bent, 1998) and the expression of these constructs was examined. For each construct, 20 independent transgenic lines of the T1 generation were obtained. The expression of transgenes in these transgenic plants was examined first. To compare the relative mRNA levels of XYLII in the T2 transgenic plants, total RNA was isolated from transgenic plants and the transcription levels were examined using semi-quantitative RT-PCR analysis. The amount of PCR products produced from the PCR was normalized using the PCR product obtained from 18S rRNA.
rRNA that served as an internal standard (Fig. 2B). The expression level of various forms of xylanase constructs was quantified as the ratio of the PCR product of xylanase mRNA to that of 18S RNA (Fig. 2C). The level of XYLIII transcripts was nearly the same in all transgenic plants examined. No PCR products were obtained from the control non-transformed plant. This study showed that the transit peptide or the peroxisome targeting signal does not affect the transcription level of these transgenes in transgenic plants. Approximately ten plants per independent transgenic line were selected for further analysis. To examine the level of xylanase encoded by XYLII, total soluble protein extracts were obtained from leaf tissues of transgenic plant harbouring various forms of XYLII constructs and were analysed by western blotting using the anti-xylanase antibody. As a control, total soluble protein extracts were obtained from wild-type plants and included in the western blot analysis. All the transgenic plants expressed xylanase despite the difference in the localization of expressed protein (Fig. 3A). However, the protein level

![Western blot analysis of xylanase encoded by various XYLII constructs in transgenic Arabidopsis.](https://academic.oup.com/jxb/article-abstract/57/1/161/442084)

**Fig. 3.** Western blot analysis of xylanase encoded by various XYLII constructs in transgenic Arabidopsis. (A) Western blot analysis of xylanase expression in transgenic plants. Protein extracts were prepared from leaf tissues of transgenic plants and used for western blot analysis using the anti-xylanase antibody. Soluble protein was prepared by centrifugation of the homogenate at 10,000 g for 30 min. An equal amount of total soluble protein (10 µg) was analysed by western blot analysis. As a load control, the level of the ER localized chaperonin, binding protein (BiP), was examined by western blot analysis using the anti-BiP antibody. (a) UTR:XYLII (b) XYLII: SKL (c) RA: XYLII (d) RA:XYLII:SKL. (B) The amount of xylanase produced in transgenic plants. The amount of xylanase was expressed as the percentage of total soluble protein (TSP). Numbers indicate independent transgenic lines for each construct shown in (A). (C) Quantification of xylanase expressed in transgenic plants. The intensity of the protein bands was quantified by image analysis software. To get a linear correlation between the intensity of the band and the amount of protein, western blots were exposed at several different exposure times. The intensity of protein band was normalized based on the level of the large subunit of the Rubisco complex.
varied slightly depending on the individual transgenic lines. The amount of protein accumulated in the cell was correlated with the expression level of mRNA (Fig. 3B). The amount of protein detected by the anti-xylanase antibody was significantly higher in the transgenic plants harbouring RA:XYLII:SKL than the transgenic plants harbouring the other constructs such as RA:XYLII, XYLII:SKL, and UTR:XYLII (Fig. 3B). The level of xylanase accumulated in the plants ranged from 1.2% to 4.8% of total soluble proteins (Table 1). The higher protein level in the transgenic plants harbouring the dual targeted construct is probably due to the accumulation of xylanase in two different organelles. Overall the transgenic plants harbouring the dual targeting construct accumulated xylanase 2–4-fold higher than the single-targeted constructs in the plant.

**Xylanase encoded by RA:XYLII:SKL is targeted to both chloroplasts and peroxisomes in transgenic plants**

Next, the subcellular localization of xylanase in transgenic plants was examined by immunohistochemistry. Protoplasts obtained from transgenic plants harbouring different constructs were immunostained by the anti-xylanase antibody. As shown in Fig. 4A, xylanase encoded by RA:XYLII was targeted to the chloroplasts as indicated by the overlap of green fluorescent signal with the red autofluorescent signals of chlorophyll. By contrast, the control non-transgenic plants did not show any green fluorescent signals (data not shown), indicating that xylanase is correctly targeted to chloroplasts. In the case of XYLII:SKL, xylanase gave a punctate staining pattern and closely overlapped the red fluorescent signals of RFP:SKL (Fig. 4B), indicating that xylanase is correctly targeted to peroxisomes. When localization of xylanase encoded by RA:XYLII:SKL was examined, the green fluorescent signals gave two different staining patterns as observed by transiently expressed dual-targeted xylanase in protoplasts (Fig. 4D). The disc pattern of green fluorescent signals detected by the anti-xylanase antibody closely overlapped the red autofluorescent signals of the chlorophyll. In addition, the green fluorescent signals of xylanase at the punctate stains closely overlapped the red fluorescent signal of RFP:SKL, indicating that xylanase encoded by RA:XYLII:SKL is targeted to both chloroplasts and peroxisomes. By contrast, transgenic Arabidopsis plants harbouring UTR-XYLII gave a diffuse green fluorescent signal in the cytoplasm, indicating that xylanase encoded by UTR-XYLII is present in the cytoplasm (Fig. 4C).

**Growth stage-dependent protein accumulation in the transgenic plants**

To investigate the possible difference in protein accumulation during the growth stages, the amount of xylanase encoded by various XYLII constructs was compared throughout the life cycle by western blot analysis using the anti-xylanase antibody. The protein level in transgenic plants harbouring RA:XYLII:SKL was highest after 5 weeks of germination (Fig. 5). Transgenic plants expressing xylanase in cytoplasm showed a decline in the protein level after 3 weeks of germination. However, the amount of xylanase in transgenic plants expressing organelle-targeted xylanase was continuously increased until maturity.

**Organelle-targeted xylanase is active in transgenic plants**

The enzymatic activity of xylanase encoded by various XYLII constructs in transgenic plants was examined next. The enzymatic activity was assayed using xylan as the substrate and the activity was determined by measuring the amount of reducing sugars released from xylan. Regardless of the constructs used, all the transgenic plants showed xylanase activity, indicating that xylanase encoded by the XYLII constructs in the transgenic plants is active despite the difference in subcellular localization. The level of enzymatic activity in transgenic plants was then compared. As shown in Table 1, the level of xylanase activity was variable depending on individual transgenic plants. Nevertheless, the overall level of xylanase activity was highest in the transgenic plants harbouring RA:XYLII:SKL.

To demonstrate that the recombinant xylanase localized to chloroplasts and peroxisomes is active, peroxisomal and chloroplast fractions were obtained from a Percoll gradient and used to measure the activity of the recombinant xylanase. Both fractions showed high levels of xylanase activity when birch wood xylan was used as substrate (Table 2). These results suggested that xylanase targeted to various organelles is properly folded and exists as an active form.

**Table 1. The amount and enzymatic activity of xylanase expressed in transgenic plants harbouring various XYLII constructs**

The protein level was quantitated by western blot analysis. Enzyme activity was measured in a triplicate experiment to obtain means. The specific activity of purified xylanase was 490 U mg⁻¹. The total soluble proteins were approximately 30–50 mg. TSP: total amount of soluble protein; CH, chloroplast; PE, peroxisome.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Localization</th>
<th>Protein level (% TSP)</th>
<th>Enzyme activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA:XYLII:SKL</td>
<td>CH and PE</td>
<td>4.8</td>
<td>19.4</td>
</tr>
<tr>
<td>RA:XYLII</td>
<td>CH</td>
<td>3.0</td>
<td>14.2</td>
</tr>
<tr>
<td>XYLII:SKL</td>
<td>PE</td>
<td>1.7</td>
<td>13.8</td>
</tr>
<tr>
<td>UTR-XYLII</td>
<td>Cytosol</td>
<td>1.2</td>
<td>11.3</td>
</tr>
</tbody>
</table>

**Discussion**

In this study, the possibility of targeting a protein to two different organelles in the cell, such as chloroplasts and peroxisomes, was exploited. In most cases, proteins that are encoded by single genes in the nucleus and translated in the cytoplasm are targeted to single organelles, although a few proteins encoded by single genes are shown to be
targeted to two different organelles such as chloroplasts and mitochondria in the cell (Akashi et al., 1998; Silva-Filho, 2003; Wall et al., 2004). The proteins that are targeted to two different organelles are referred to as dual targeted proteins (Elgersma et al., 1995; Peeters and Small, 2001; Satoh et al., 2003).

Previous studies have demonstrated that the chloroplast is an efficient protein production system (Svab et al., 1990; Staub et al., 2000). The proteins that are targeted to two different organelles are referred to as dual targeted proteins (Elgersma et al., 1995; Peeters and Small, 2001; Satoh et al., 2003).

Previous studies have demonstrated that the chloroplast is an efficient protein production system (Svab et al., 1990; Staub et al., 2000). The benefit of a dual targeting strategy involving two different organelles may further increase the amount of protein produced in the plant. Transgenic plants expressing exogenous xylanases did not show any noticeable morphological alteration. Transgenic plants harbouring RA: XYLII (A), XYLII: SKL (B), UTR: XYLII (C), and RA: XYLII: SKL (D) and immunostained with the anti-xylanase antibody as the primary antibody and FITC-labelled anti-rabbit IgG antibody as the secondary antibody. To confirm the localization of a protein in the peroxisome, protoplasts obtained from the transgenic plants were transformed with RFP: SKL and immunostained using anti-xylanase antibody. Red fluorescent signals of RFP:SKL were directly observed from the immunostained protoplasts. Bar=20 μm.

Fig. 4. Localization of xylanase in transgenic plants harbouring various XYLII constructs. Protoplasts were prepared from leaf tissues of Arabidopsis transgenic plants harbouring RA:XYLII (A), XYLII:SKL (B), UTR-XYLII (C), and RA:XYLII:SKL (D) and immunostained with the anti-xylanase antibody as the primary antibody and FITC-labelled anti-rabbit IgG antibody as the secondary antibody. To confirm the localization of a protein in the peroxisome, protoplasts obtained from the transgenic plants were transformed with RFP:SKL and immunostained using anti-xylanase antibody. Red fluorescent signals of RFP:SKL were directly observed from the immunostained protoplasts. Bar=20 μm.

Also, xylanase encoded by the dual targeted construct was correctly targeted to both organelles (Fig. 4). One possible explanation for the higher protein level is that there is more subcellular space available for the accumulation of proteins in the cell. In general, translational efficiency is affected by the factors including the base composition, length and secondary structure of the 5’ UTR, the context of translation initiation, and G/C content of the mRNA. Thus, the possibility cannot be completely ruled out that the different constructs used in this experiment might have affected the translation rate of XYLII mRNAs. Another factor affecting the accumulation of xylanase was the growth stage of the plants. Interestingly, the amount of organelle-targeted proteins appears to increase with age up to 5 weeks of germination, whereas the amount of protein accumulated in the cytoplasm declined after 3 weeks of germination. Previously it has been suggested that proteins in the cytoplasm are much more susceptible to protease digestion (Horvath et al., 2000). These results suggest that
the harvest time of plants for the extraction of recombinant proteins may be important and need to be determined depending on the subcellular localization of protein. Another important issue in the expression of recombinant protein in plants is whether the proteins targeted to organelles are active in the cell. Xylanase targeted to organelles of the chloroplast and the peroxisome was biologically active (Table 2).

In conclusion, these findings demonstrate that dual and simultaneous targeting of xylanases to chloroplasts and peroxisomes could increase the amount of protein expressed in the cell. This method can be used to achieve a high level of protein accumulation in transgenic plants for the purpose of molecular farming a protein of interest.

### Table 2. Xylanase activity in chloroplast and peroxisomal fractions CH, chloroplast; PE, peroxisome

<table>
<thead>
<tr>
<th>Construct</th>
<th>Localization</th>
<th>Total activity (Units)</th>
<th>Specific activity (U mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA:XYLII</td>
<td>CH</td>
<td>1.1</td>
<td>24</td>
</tr>
<tr>
<td>XYLII:SKL</td>
<td>PE</td>
<td>0.78</td>
<td>8</td>
</tr>
</tbody>
</table>

**References**


