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Production and Analysis of Transgenic C3-C4 Intermediate *Moricandia arvensis* Expressing a Maize C4 Phosphoenolpyruvate Carboxylase Gene

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Abstract

Moricandia arvensis (L.) DC. has been identified as a C3-C4 intermediate species based on its low CO_2 compensation point, low photorespiratory activity and partial degree of Kranz anatomy. However, *M. arvensis* does not have key enzymes for C4 photosynthesis such as phosphoenolpyruvate carboxylase (PEPC). We introduced maize C4 PEPC cDNA under the control of the cauliflower mosaic virus 35S promoter into *M. arvensis* by *Agrobacterium*-mediated gene transfer. Most of the transgenic plants accumulated large amounts of the transcripts of the introduced PEPC gene, although the amount of the PEPC protein was rather small. The amounts of the maize PEPC protein in the transgenic plants were positively correlated with the activity of PEPC. Elevated PEPC activity, twice as high as that of the wild-type control, was observed in some transgenic plants.

Introduction

Moricandia arvensis (L.) DC. is classified as belonging to the family Brassicaceae. It is distributed in semi-arid areas such as the Sahara desert and the Mediterranean region. *M. arvensis* has been identified as being C3-C4 intermediate because of its low CO_2 compensation point (16 ± 3 μl at 21% O_2 and 25 ℃), low photorespiratory activity and partial degree of Kranz anatomy characterized by the differentiation of mesophyll cells and prominent bundle sheath cells (Holaday *et al.*, 1981, Holaday and Chollet, 1984, Devi *et al.*, 1995). In mesophyll cells, photorespiration is depressed because mesophyll cells lack the P-protein of glycine decarboxylase, which is a key mitochondrial enzyme in photorespiratory activity (Morgan *et al.*, 1993). In bundle sheath cells, mitochondria and peroxisome are distributed centripetally against the cell wall adjacent to the vascular bundle and are overlaid by chloroplasts (Rawsthorne, 1992). CO_2 released from the mitochondria is therefore likely to be efficiently recaptured by the chloroplasts in the bundle sheath cells. This unique photorespiratory pathway and Kranz-like leaf anatomy are considered to result in a rather low CO_2 compensation point (Rawsthorne, 1992). However, the activities of key C4 photosynthetic enzymes such as PEPC, NAD-malic enzyme and pyruvate Pi dikinase (PPDK) are reported to be considerably low or undetectable (Holaday *et al.*, 1981). There is no evidence for the existence of a C4 photosynthetic pathway in *M. arvensis*.

It is of interest, therefore, to introduce genes for key C4 enzymes into *M. arvensis* for the study of the transition from C3 photosynthesis to C4 photosynthesis. In addition, transformation of *M. arvensis* is feasible by *Agrobacterium*-mediated transformation (Rashid *et al.*, 1996). We therefore consider that *M. arvensis* is potentially an ideal plant for the study of transformation with genes for key C4 enzymes. There have been no reports on the introduction of C4 photosynthetic genes into C3-C4 intermediate plants, including *M. arvensis*.

In this study, we transformed *M. arvensis* by using a maize cDNA for C4 PEPC, one of the most important enzymes because it fixes CO_2 in the first step of the C4 photosynthetic pathway. We investigated transcripts and protein of the introduced PEPC genes and PEPC activity. Some transgenic plants showed a level of PEPC activity twice as high as that of wild-type *M. arvensis*.

Materials and Methods

Transformation of M. arvensis

The chimeric vector, pEM126, containing the cauliflower mosaic virus (CaMV) 35S promoter, PEPC cDNA of maize, and the nopaline synthase terminator was the same as that used in a previous study (Kogami *et al.*, 1994). The transformation of *M. arvensis* plants was carried out by the method of leaf disc-transformation mediated by *Agrobacterium* (Rashid *et al.*, 1996). Transgenic plants were grown in a greenhouse. Day and night temperatures were maintained at 20 ℃ and 15 ℃, respectively.

Southern blot analysis

Total genomic DNA (2 μg) digested with *Bam*HI was separated by electrophoresis in 1% (w/v) agarose gel and blotted onto a nylon membrane. The membrane was then hybridized with digoxigenin-labeled probe DNA, a *Bam*HI fragment of PEPC cDNA consisting of 2480 bp of the coding region (Kogami *et al.*, 1994). Labeling and detection were carried out according to the instruction manual of Digoxigenin Labeling and Luminescent Detection Kit (Boehringer Mannheim, Germany). Hybridization was carried out in 5 x SSC, 0.5% blocking reagent (Boehringer Mannheim), 0.1% sodium N-lauroyl sarcosinate and 0.02% SDS at 65 ℃. The membrane was washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each and twice in 0.1 x SSC, 0.1% SDS at 65 ℃ for 20 min each. The hybridization signals were detected using CSPD (Tropix, USA) as the substrate.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from expanded leaf tissues (ca. 100 mg) of maize and *M. arvensis* by using Isogen Kit (Nippon Gene, Japan). For Northern blot analysis, total RNA (10 μg) was fractionated by electrophoresis in a 1% (w/v) agarose gel after denaturation with glyoxal, and then blotted onto a nylon membrane. This membrane was then hybridized with the same probe as used for Southern blot analysis. Hybridization, washing and detection were carried out under the same conditions as described for Southern blot analysis.

Western blot analysis

Frozen leaves were ground in liquid nitrogen into a fine powder, and soluble proteins were extracted with a buffer containing 50 mM HEPES-NaOH (pH 7.5), 10 mM dithiothreitol, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM PMSF, 5% glycerol, 15% ethylene glycol and 1% insoluble polyvinylpyrrolidone (Tokyo-Kasei) and then centrifuged at 12,000 x g for 10 min. Soluble protein from maize leaves (1 μg) or from *M. arvensis* leaves (50 μg) were separated by thin-layer polyacrylamide gel isoelectric focusing (IEF; p*I* 3.5-9.5, Pharmacia) or SDS-PAGE (7% polyacrylamide gel). After electrophoresis, proteins were electroblotted onto an Immobilon-P membrane (Millipore, USA) and probed with a polyclonal antibody raised against the maize PEPC and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. The relative amounts of C4 PEPC protein of transgenic plants were semi-quantitated using a densitometer (ATTO Densitograph software library, ATTO). A range of dilution of maize extracts was used as a standard, and relative PEPC amounts of *M. arvensis* were indicated as percentages of that of maize.

Enzyme extraction and assay

Approximately 100 mg of leaf tissue was frozen in liquid N_2 in the daytime and stored at -80 ℃ before use. The frozen tissues were ground into a powder in liquid N_2 using a pestle. After extraction in the same buffer as used for Western blot analysis, the crude extracts were centrifuged at 12, 000 x g for 5 min at 4 ℃ and the supernatant was used for enzyme assay. Enzyme activity was determined spectrophotometrically at 30 ℃. The standard assay mixture contained 100 mM HEPES-NaOH (pH 8.0), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM DTT, 1 mM EDTA, 0.2 mM NADH, 2 mM phosphoenolpyruvate and 3 IU malate dehydrogenase in a total volume of 1 m*l* (Chu *et al.*, 1990). One unit of PEPC activity corresponds to the oxidation of 1 μ mole of NADH min^{-1} by the coupling enzyme. Protein concentration was determined using a Bradford Protein Assay Kit (BIORAD, USA) with bovine serum albumin as a standard.

Results

Production of transgenic M. arvensis

Leaf discs of *M. arvensis* were inoculated with *Agrobacterium tumefaciens* carrying cDNA of the maize C4 PEPC gene under the control of the CaMV 35S promoter. A total of 28 independent transformants were selected on a medium containing 100 mg/*l* kanamycin, and resistant shoots were regenerated into plants. Transgenic plants were transferred to a greenhouse for further growth.

Southern blot analysis

Stable integration of the introduced transgene was

confirmed by Southern blot analysis. The total genomic DNA was digested with *Bam*HI, which cut a unique site within the vector construct, and hybridized with a part of PEPC cDNA as a probe. The number of bands are expected to reflect the number of copies of the integrated transgene. **Fig. 1** shows some representative transgenic plants used for further analysis. A single band was detected in 27 out of 28 independent transformants. The size of the band was different in each transgenic plant. No bands were detected in the wild-type control. We therefore conclude that a single copy of the PEPC transgene was integrated into the genome of the transgenic plants.

Northern blot analysis

Northern blot analysis was carried out on 12 representative transgenic plants to study the steady-state level of the transcripts of the introduced PEPC gene. As shown in **Fig. 2**, PEPC mRNA was detected in the leaf tissue of transgenic *M. arvensis* plants in the same position as that of maize. No detectable bands were found in wild-type *M. arvensis* plants. Transgenic P5 and P2 showed almost the same band intensity as that of the maize, indicating that the introduced genes were highly

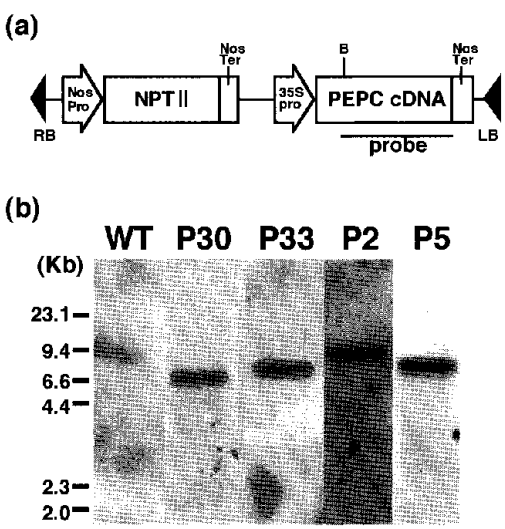

Fig. 1. Southern blot analysis of transgenic *M. arvensis* plants with maize C4 PEPC gene.
(a) Construction of chimeric PEPC gene used for transformation, PEPC; phosphoenolpyruvate carboxylase cDNA of maize, 35S Pro; CaMV 35S promoter, Nos Pro; nopaline synthase promoter, Nos Ter; 3' signal of nopaline synthase, RB; Right border, LB; Left border, B; *Bam*HI. (b) Southern blot analysis of transgenic M. arvensis plants (P30, P33, P2 and P5) and wild type (WT). Total genomic DNA was digested with *Bam*HI and the blots were probed with a part of the maize PEPC cDNA.

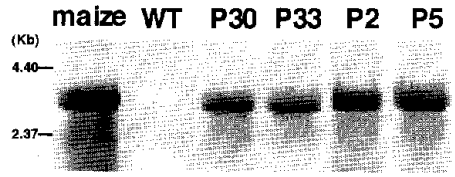

Fig. 2. Northern blot analysis of transgenic *M. arvensis* with the maize C4 PEPC gene.
Total RNA was isolated from leaves of transgenic *M. arvensis* (P30, P33, P2 and P5), wild-type *M. arvensis* (WT) and maize. The blot was probed with a part of the maize PEPC cDNA. Each lane contained 10 μg of total RNA.

expressed at the transcriptional level in the transgenic plants.

Western blot analysis

To determine if the transgenic *M. arvensis* plants produced elevated levels of the maize PEPC protein, Western blot analysis of leaf proteins was carried out using an anti-maize PEPC antibody. The anti-maize PEPC antibody detected a 107 kd band in a wild-type *M. arvensis* in SDS-gel blot analysis, indicating that the antibody cross-reacted with the endogenous PEPC protein of *M. arvensis*. The size of this band was quite similar to that of maize, which showed 110 kd in the SDS-gel blot. All the 12 transgenic *M. arvensis* plants showed a 110 kd band corresponding to maize PEPC protein in addition to the 107 kd band of the endogenous PEPC protein (data not shown).

The IEF-gel blot clearly discriminated the maize PEPC protein from the endogenous PEPC protein of *M. arvensis*, as shown in **Fig. 3**. Wild-type *M. arvensis* showed a rather faint, broad band at pI 5.6, while wild-type maize showed an intense band at pI 5.3. Most of the transgenic *M. arvensis* showed both bands at pI 5.6 and 5.3. The intensity of the band at pI 5.3, which corresponds to the maize PEPC protein, differed among transgenic plants. The amounts of maize PEPC protein were semi-quantitated using a range of dilution of maize leaf extract as a standard. The amount of the maize protein in transgenic *M. arvensis* was positively correlated with the amount of the transcripts of the introduced maize PEPC ($r=0.65$, significant at 5% level, data not shown).

Activities of PEPC

Activities of PEPC were measured for the 12 transgenic *M. arvensis* plants in which the maize PEPC transcripts and protein were detected. The activity of PEPC was higher than that of the wild-type *M. arvensis*, although it varied among the 12 plants. The highest activity was obtained in the P5

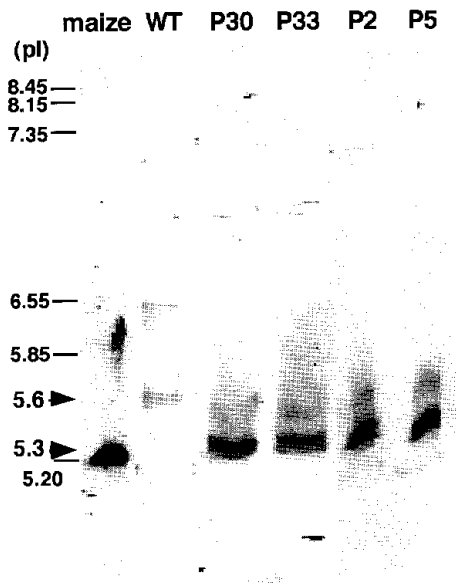

Fig. 3. Western blot analysis of transgenic *M. arvensis* with the maize C4 PEPC gene.
Soluble proteins were extracted from leaves of transgenic *M. arvensis* (P30, P33, P2 and P5), wild-type *M. arvensis* (WT) and maize, and separated by isoelectric focusing. PEPC protein was detected by anti-maize PEPC antibody. The amounts of protein loaded were one μg for maize extract and 50 μg each for extracts from transgenic lines and the wild type of *M. arvensis*.

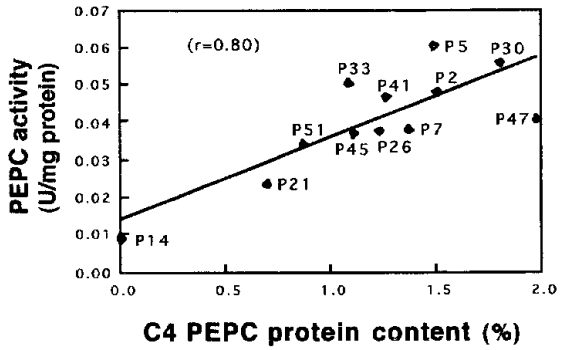

Fig. 4. The correlation between C4 PEPC protein content and PEPC activity in transgenic *M. arvensis* plants with the maize C4 PEPC gene. The relative amounts of the maize C4 PEPC protein were estimated by Western blot analysis and are indicated as a percentage of PEPC content in maize. PEPC activity is indicated as U/mg protein.

plant, which was 2.1-fold higher than that of the wild-type plants. This P5 plant also showed high levels of maize PEPC mRNA and protein. The activities of PEPC and the levels of the maize PEPC protein in transgenic plants were positively correlated (r=0.80, significant at 1% level, **Fig. 4**). However, the specific activity of the PEPC in the crude extract of transgenic *M. arvensis* was less than 3.6% of that of maize at most.

Expression in the selfed progeny

Bud pollination was carried out to obtain selfed progeny, because *M. arvensis* has a property of self-incompatibility. Some of the selfed progeny were not so healthy, probably because of the inbred depression as is often observed in outbreeding species. The expression of the maize PEPC gene was investigated by Western blot analysis in the progeny of transgenic lines, P2 and P5. The intensity of the band corresponding to the maize PEPC protein varied among the selfed progeny. An intense band was detected in three plants out of the 12 transgenic plants (data not shown).

Discussion

C4 plants possess higher CO_2 fixation efficiency than C3 plants and are considered to be superior to them under arid, hot, and light-intensive environmental conditions because they have an efficient CO_2 concentration system. Several attempts have therefore been made to overexpress C4 photosynthetic genes in C3 plants in order to increase photosynthetic efficiency and to understand the evolutionary transition from C3 photosynthesis to C4 photosynthesis.

Among enzymes in the C4 photosynthetic pathway, PEPC is one of the most important because it performs primary fixation of atmospheric CO_2. There have been some studies in which a C4 PEPC gene was introduced into C3 plants. For example, the maize C4 PEPC gene was introduced into a C3 tobacco plant under the control of either the *cab* gene promoter (Hudspeth *et al.*, 1992) or the CaMV 35S promoter (Kogami *et al.*, 1994). PEPC genes from various origins were introduced into a C3 potato (Gehlen *et al.*, 1996). However, the activities of the PEPC in transgenic plants in their reports were low and consequently no significant impact on the physiology of photosynthesis was observed. Since C3 plants suffer from no cell differentiation of Kranz anatomy, it seems to be difficult to introduce C4 traits into C3 plants.

In this respect, C3-C4 intermediate species having a Kranz anatomy are potentially superior materials for the study of introduction of genes for C4 key enzymes and for understanding the evolutionary transition from C3 photosynthesis to C4 photosynthesis. C3-C4 intermediate species have been identified in *Eleocharis, Neurachne, Panicum, Mollogo, Alternanthera, Flaveria, Parthenium* and *Moricandia* (Rawsthorne, 1992). There has been no report on the introduction of C4 photosynthetic genes into C3-C4 intermediate plants. We have

previously reported that the leaf-disc transformation of *M. arvensis* is feasible by *Agrobacterium*-mediated transformation (Rashid *et al.* 1996).

In this study, transgenic *M. arvensis* plants expressing the maize C4 PEPC gene under the control of the CaMV 35S promoter were successfully produced. This is the first report on the introduction of a key C4 enzyme into a C3-C4 intermediate species. In transgenic *M. arvensis*, as many transcripts of the introduced PEPC were detected as those in maize. However, the amounts of PEPC protein were less than 2% of that of maize. One of the reasons for the low accumulation of the PEPC proteins can be attributed to features of the introduced genes. The cDNA used in the present study did not contain any 5' untranslated leader sequence. Employment of 5' untranslated leader sequences has been reported to enhance the PEPC activity and PPDK activity in transgenic potatoes (Gehlen *et al.*, 1996, Ishimaru *et al.*, 1998). Recently, genomic DNA was shown to be superior to cDNA for high expression of the introduced PEPC gene (Matuoka *et al.*, 1998, Ku *et al.*, 1999). They reported that introduction of the maize genomic PEPC gene, which contained all exons and introns and its own promoter and terminator sequences, into rice caused 30-100-fold higher PEPC activity than non-transgenic rice. The source of the introduced gene may also affect the translational efficiency. The PEPC cDNA was taken from maize. This is a monocot species and is distantly related to *M. arvensis* in dicot. It is possible that the introduced cDNA was not translated efficiently in *M. arvensis* partly due to a biased codon usage in the C4 PEPC genes of monocots (Kawamura *et al.*, 1992). Therefore, we suggest that the high level accumulation of PEPC protein and consequently high PEPC activity can be achieved by improvement of the source and design of the introduced genes. Introduction of the genomic PEPC gene from a C4 dicot should greatly enhance PEPC activity in transgenic *M. arvensis*.

It is noteworthy that the activities of PEPC were positively correlated with the amounts of PEPC proteins (**Fig. 4**). It would seem to be of interest to investigate *M. arvensis* as to whether the maize PEPC is also subject to regulatory phosphorylation as in maize (Vidal and Chollet, 1997). The highest PEPC activity obtained in this study is 2.1-fold higher than that of wild-type *M. arvensis* (**Fig. 4**). Almost the same elevated levels of PEPC protein were reported to be obtained in transgenic tobacco when the same construct was introduced (Kogami *et al.*, 1994).

In the present study, we demonstrated that the PEPC activity was enhanced in *M. arvensis* by genetic engineering. We obtained transgenic plants with PEPC activity significantly higher than that of the wild type. The introduced PEPC gene was shown to be inherited and expressed in selfed progeny. Further studies are now in progress to introduce some other genes for C4 photosynthetic enzymes such as those of PPDK and NAD-malic enzyme. *M. arvensis* has a partial degree of Kranz anatomy with reduced photorespiration. *M. arvensis* can potentially serve as a model plant for the study of the transition from C3-C4 intermediate to C4 plants.

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