Note

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Photosynthetic Carbon Assimilation of Transgenic Tobacco Plant Expressing Cytoplasmic Carbonic Anhydrase cDNA from Mouse

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Plant biotechnology in photosynthesis can be applied to the study of rate limiting process of the carbon assimilation pathway in cells. The photosynthetic carbon assimilation (PCA) of C3 plants appear to be unsaturated under ambient CO_2 concentration in contrast to that of C4 plants. Thus, a small increase in CO_2 concentration leads to an increase of C3-PCA. If the CO_2 transfer resistance in mesophyll cells were minimized by any means of cellular engineering, PCA would be improved by the resulting elevation of available CO_2 at the CO_2 fixing enzyme site. A possible candidate in this context is the genetic introduction of carbonic anhydrase [EC 4. 2.1.1., CA] activity into mesophyll cytoplasm, since CA facilitates inorganic carbon diffusion through bilayer lipid membranes [1] and CA activity scarcely exists in contrast to its chloroplastic abundance in C3 plants [2]. Previously, Okabe observed that the introduction of liposome encapsulated bovine CA (Type II) into tomato cotyledonous protoplasts by fusion technology (*ca.* 8% increase of intrinsic control protoplastic CA activity) enhanced photosynthetic O_2 evolution under a lower HCO_3^- condition [3]. Price *et al.* reported that anti-sense RNA transgenic tobacco plants in which intrinsic CA activity was reduced to the level of 1 - 2% of the wild type showed a 4.4% reduction of PCA with a concomitant decrease in inorganic carbon diffusion conductance of *ca.* 27% [4]. However, the question remains to be clarified whether the extremely low CA activity in the C3 plant cytosol is sufficient for maximizing PCA. In this communication, we describe the generation of transgenic tobacco progenies expressing animal CA peptide in the cytosol and the effect of genetically introduced foreign cytoplasmic CA activity on those PCA.

We chose a mouse CA cDNA rather than one of plant origin for this study (1) because anti-bovine-CA antiserum does not cross with plant CA but mouse CA peptide does, which enabled the detection of the mouse CA peptide expression in the leaf extracts of the transgenic plants. (2) Mouse CA expresses its activity in monomer form whereas plant CA requires assembling in hexamer or octamer. (3) Also mouse CA is more tolerant of oxidative conditions than plant CA. Therefore, mouse CA appeared suitable for expressing its activity in oxidative cytoplasm.

The *Agrobacterium* binary vector (pBmmCA010) harboring partially modified mouse CA Type II cDNA (mmCA010, ADVANCE, Japan) was constructed by replacing the β- glucuronidase gene from the binary plasmid pBI121 (Toyobo, Japan) at Hind III-Eco R I site with the mmCA cDNA cassette (single CaMV35S promoter - mmCA010, 1.1 kbp fragment size - NOS-terminator). The mmCA010 cDNA cassette was first confirmed by the expression of the 29 KD mmCA peptide in *Escherichia coli* (*E. coli* JM109) on SDS-PAGE and Western analysis using highly potent purified bovine CA type II antiserum (ADVANCE / COSMO Bio., Japan). The sequence consisted of a Xba I and Bam H I linker (Takara, Japan) at the 5′ flanking end (5′-TCTAG-GAGGATCCCCCATGGCTCGATG-3′) followed by the same sequence as the reported database of mouse CA II cDNA [5] after the 27th code from its 5′ terminal. Thus, there was a sequence of corresponding code for a total of 252 amino acids with a deletion of its 3' poly-A signal sequence. The Ti plasmid pBmmCA010 was introduced into *Agrobacterium tumefaciens* (LBA4404, Toyobo, Japan) and then infected to tobacco leaf discs (*Nicotiana tabacum* cv. Xanthi) according to the method of Horsch *et al.* [6].

Out of 49 plantlets regenerated from the leaf discs on the selection medium, 23 plantlets proved to express significant levels of mmCA peptide in leaf extracts. Among them, the seedlings of strain C-4 were selected for further analysis because it had the highest expression level of mmCA peptide. From the kanamycin tolerance test of the T1 seeds, the strain C-4 was estimated to carry 2 copies of mmCA cDNA cassette in the genome. Actually , T1 seedlings of C-4 were sorted into 3 phenotypes by the mmCA expression peptide levels in the leaf extracts (High CA, Low CA and Null/Wild phenotypes).

Assessment of the locality of expressed mmCA protein in the transgenic leaves was carried out according to Tsuzuki *et al.* [2] by isolating mesophyll

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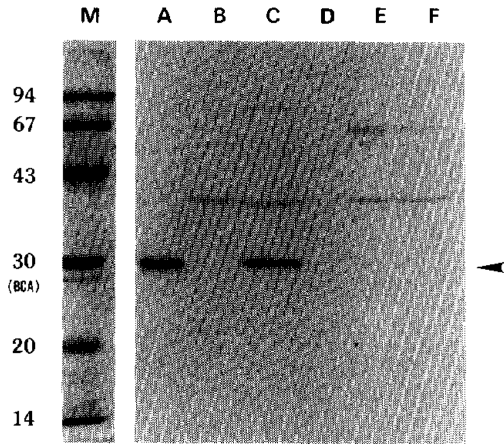

Fig. 1 Detection of mmCA in extra-chloroplastic fraction of transgenic tobacco mesophyll cells.

Western blotting of SDS-PAGE (12%) of the leaf extracts (lane A and D), chloroplastic fraction (lanes B and E) and extra-chloroplastic fraction (lanes C and F) with the detection of anti-bovine CA II rabbit serum. The lanes A, B, and C correspond to the High mmCA phenotype and the lanes D, E and F correspond to the Null/Wild phenotype. Lane M shows the molecular markers (Pharmacia, Japan) with CBB staining. The arrow indicates the immuno detection band of about 29 KD.

protoplasts followed by the centrifugal fractionation of the chloroplasts. The chloroplastic and extra-chloroplastic fractions were analyzed by SDS-PAGE and Western analysis. The animal CA signal band was detected only in the extra-chloroplastic fraction (**Fig. 1**). The fact that mmCA cDNA had no "transit" peptide for organelle transport coincided well with the cytoplasmic location of expressed mmCA peptides.

To discriminate the expressed mmCA activity from leaf intrinsic CA, we used differential sensitivities of each CA origin against oxidative condition and a specific inhibitor, acetazolamide. Leaf samples were ground oxidatively at 50°C for 6 min with 4% PVPP, sea-sand and extraction buffer without -SH reagent in a warmed mortar and pestle. The supernatant was collected and further oxidized with 3% H_2O_2 for 10 min at room temperature. We confirmed that this process of leaf extract preparation decreased the endogenous CA activity by more than 95% without affecting the animal type CA activity. After 1 min centrifugation at 4 °C and 15,000×g, a supernatant aliquot of 50μL was used to measure the remaining CA activity with and without 10^{-7} M acetazolamide which was confirmed to inhibit 99% of animal CA activity but had no effect on plant CA activity in the preliminary tests. The mmCA activities with this method were calculated to be 5.2±0.5, 3.0±0.4 and -0.1±0.4 mg • Chl^{-1}, for the High CA, Low CA, and Null/Wild phenotypes, respectively (**Table 1**). The specific activities of mmCA in High and Low CA phenotypes were almost the same. On the other hand, total CA activities in the leaf extracts which were prepared under the reductive conditions showed no significant difference among these phenotypes. Considering these facts, the highest expression of mmCA activity was estimated to be at least 1.1% of the endogenous CA activity.

The plants of self-pollinated T2 seeds of the C-4, grown on sand-cult.
ure in the controlled green house at 25/20 °C (day/night), were further analyzed on the leaf PCA properties and the expression levels of mmCA, RubisCO, soluble protein and Chlorophyll in the leaves as shown in the **Table 2**. The expression levels of High and Low CA phenotypes were calculated to be 250-300 and 130-200 ng mouse CA • cm^{-2}, respectively. Assuming that the endogenous CA as low as 0.6% of the soluble protein in the tobacco leaf [4], the content of mmCA in High CA plants was estimated to be *ca.* 0.9% of the total leaf CA protein. No other significant difference was observed among these phenotypes with

Table 1. Total and mmCA activities in the leaf extracts of mmCA transgenic plants determined by the differential measurements. (The values were means±S.E..)

Phenotype		Total CA	After oxidation*1	Acetazolamide*2	mmCA Activity*3	Specific Activity
		unit • $mgChl^{-1}$				unit/mg mmCA
High CA	(n = 5)	533± 50	30.0±3.7	24.9±3.6	5.2±0.5	841± 58
Low CA	(n = 3)	578±143	23.2±6.5	20.2±6.6	3.0±0.4	851±163
Null/Wild	(n = 5)	505± 84	19.3±3.2	19.1±4.4	−0.1±0.2	—

*1 CA activities in the leaf extracts prepared by grinding under oxidative condition and incubated with H_2O_2. (See Materials and Methods)

*2 Additive treatment of 10^{-7} M acetazolamide after oxidation with 3% H_2O_2.

*3 Calculated from the assumption from the preliminary experiment that the acetazolamide inhibited the mouse and plant CA by 99 and null %, respectively.

Table 2. Levels of mmCA, RubisCO, Soluble protein and Chl in the youngest fully expanded leaves of mmCA transgenic plants. (The values were means ± S.E..)

Phenotype		mmCA	RubisCO	Soluble protein	Chl
		ng·cm^{-2}	μg·cm^{-2}	μg·cm^{-2}	μg·cm^{-2}
High CA	(n = 9)	277.0± 8.1	203.0± 4.8	488.6±11.4	40.4±0.6
Low CA	(n = 6)	157.8±14.6	214.5±10.4	491.3±24.5	41.0±0.7
Null/Wild	(n = 8)	—	207.8± 4.1	492.7± 4.7	41.7±0.8

Table 3. PCA characteristics of the youngest fully expanded leaves of mmCA transgenic plants measured at various CO_2 concentration under strong light intensity. (The values were means ± S.E..)

Phenotype		PCA rate at CO_2 concentrations of		
		Ci 600 ppm	Ci 250 ppm	Ci 150 ppm
			μmol CO_2·m^{-2} s^{-1}	
High CA	(n = 9)	25.7±0.6	18.6±0.2	9.84±0.15*
Low CA	(n = 6)	26.4±0.6	18.5±0.3	9.70±0.19
Null/Wild	(n = 8)	26.1±0.6	18.4±0.3	9.36±0.22

(* : $P < 0.025$)

regard to the RubisCO, soluble protein and Chlorophyll.

The PCA rates of these phenotypes under high light intensity at 21% O_2 conditions were determined at varying CO_2 concentrations (**Table 3**). Under ambient CO_2 condition (Ci=250 ppm), these plants showed little difference in their PCA rates. However, at the lower intercellular CO_2 concentration (Ci = 150 ppm), the High CA phenotype showed a significantly higher PCA rate (5. 1%) than those of the Null/Wild phenotypes.

Our consistent observation of the significant increase of PCA in our transgenic plants under CO_2 limiting conditions suggests the importance of cytoplasmic CA activity in the C3 plant PCA capacity. In this regard, Graham *et al.* [7] and Evans *et al.* [8] reported that the CO_2 conductivity from the cell membrane to the inner surface of chloroplasts is not an important factor for the determination of PCA. They based this on the observation that chloroplasts in the mesophyll cells of C3 plants were arranged along the cell membrane facing the stomatal cavity and deduced that most of the CO_2 molecules which penetrated into the cytoplasm might diffuse into chloroplasts directly through this short-cut distance, without being converted into HCO_3^- or CO_2. If this is the case, introduction of extra CA into the cytoplasm of mesophyll cells of C3 plants would not have produce any effects on the PCA capacity. We have presently two assumptions to explain the mechanism as follows : (1) There exists a significant volume of inorganic carbon movement through the cytoplasm to the chloroplasts by a so-called "indirect passage". (2) Cytoplasmically localized CA could increase the concentration of HCO_3^-, the preferred substrate of PEP carboxylase (PEPCase), and enhance PCA in transgenic leaves. If these assumptions are true in the cytoplasm of transgenic plants, the expressed mmCA might have contributed to the increased rate of PCA.

In conclusion, the generation of transgenic progenies expressing animal CA peptide in cytoplasm in this study suggested one possible way to improve PCA of C3 plants though further work is needed to optimize CA activity using stronger promoter systems or an increased number of CA genes.

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