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Transgenic Improvement of Photosynthetic Property in Tobacco Using El2 Ω Promoter System to Express Higher Level of Mouse Carbonic Anhydrase Activity in Cytoplasm

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Abstract

Genetic challenge to express higher carbonic anhydrase (CA) activity in the cytoplasm of tobacco mesophyll cells was carried out by an improved promoter (El2 Ω) with a cDNA of partially modified mouse CA. In leaves of T1 progenies of transgenic tobacco (ffCA plant), the expressed mouse CA peptide was confirmed with 1.5 - 3.2 times higher than the transgenic tobacco having foreign mouse CA controlled by simple CaMV 35S promoter (fCA plant). The ffCA plants manifested higher photosynthetic carbon assimilation (PCA) rate under ambient CO_2 condition than wild type plants. The chlorophyll fluorescence measurement analysis revealed that the ffCA plant's improved PCA rate is related to the reduction of mesophyll CO_2 transfer resistance (Rr) with little change in the stomatal resistance (Rs) or mesophyll CO_2 fixation resistance (Rx). Moreover, ffCA plants showed a higher growth rate under high light condition.

Abbreviations

CA: carbonic anhydrase, CaMV: cauliflower mosaic virus, ffCA plants: the transgenic tobacco having foreign mouse CA controlled by El2 Ω promoter, fCA plants: the transgenic tobacco having foreign mouse CA controlled by simple CaMV 35S promoter, PCA: photosynthetic carbon assimilation, Rr: mesophyll CO_2 transfer resistance, Rs: stomatal resistance, Rx: mesophyll CO_2 fixation resistance, WA unit: Wilbur-Anderson CA activity unit

1. Introduction

Plant biotechnology in photosynthetic improvement is based on finding the limiting steps in the metabolic pathway in the plant's cellular physiology. Carbonic anhydrase [EC4.2.1.1, CA], which catalyzes the conversion reaction between CO_2 and HCO_3^- and also promotes the inorganic carbon diffusion through lipid membranes (Broun *et al.*, 1970), is highly related to the flow of carbon in the leaf photosynthesis. Although there have been some reports suggesting the significant existence of extra-chloroplastic CA isozymes in several C3 species (Utsunomiya and Muto, 1993; Rumeau, 1996), most CA activity characteristically localizes in the chloroplastic stroma and not in its envelope (Tsuzuki *et al.*, 1985). In the previous report (Jinushi *et al.*, 1998), based on a very low level of cytoplasmic intrinsic CA activity we generated transgenic tobacco (fCA plants) which expresses foreign mouse CA in the mesophyll cytoplasm controlled by a conventional cauliflower mosaic virus 35S (CaMV 35S) promoter. The fCA plants which expressed foreign CA at high levels showed a significant and consistent enhancing tendency in the photosynthetic carbon assimilation (PCA) rate under lower CO_2 conditions. However, the strain that expressed foreign CA at a lower level did not show such an enhancement. These facts lead to hypothesis that the introduction of higher foreign CA activity into the cytoplasm would enhance its PCA activity to a greater extent.

Among the unique techniques available two reports were suggestive; (1) Kay *et al.* (1987) reported that the duplication of CaMV35S promoter sequences enhanced foreign gene expressions. (2)

Mitsuhara *et al.* (1996) constructed a new CaMV35S promoter (El2 Ω) containing two extra-enhancer sequences; one is El2 sequence which is in the upstream region of CaMV35S promoter, and the other is Ω sequence which is the 5'-untranslated region of tobacco mosaic virus genomic RNA. They were observed to have more than 10 times higher expression of the marker gene using El2 Ω promoter compared with a single CaMV35S promoter in the transgenic tobacco.

In this paper we describe a study using El2 Ω promoter in the production of unique transgenic tobacco plants (ffCA), into which have been introduced a partially modified mouse CA cDNA and which expressed mouse CA peptide in the mesophyll cytoplasm. We measured those mesophyll resistances for CO_2 transfer (Rr) in photosynthesis with chlorophyll fluorescence methods in order to elucidate the detailed mechanisms of introduced foreign CA on PCA.

2. Materials and Methods

2.1 Construction of gene cassette with improved promoter and transformation

The vector plasmid pBffCA010 was constructed by replacing the sequence of β-glucuronidase gene and nos-terminator of the pBE2113-GUS (Mitsuhara *et al.*, 1996) with the sequence of the partially modified mouse CA cDNA and nos-terminator in the pBfCA010 (Jinushi *et al.*, 1998) of 861 bp at *Bam*HI-*Eco*RI site (**Fig.1**). It was introduced into leaf discs of tobacco (*Nicotiana tabacum* line SR-1) according to the same method as previously reported (Jinushi *et al.*, 1998; Horsch *et al.*, 1985). Transgenic tobacco plants were screened by antibiotic Km resistance and the expression level of foreign CA peptide using anti-serum against bovine CA type II which could discriminate it from plant intrinsic CA peptides.

2.2 Plant Materials

T1 seeds of the transgenic tobacco plants, the strain ffCA-3, which showed an extremely high level of foreign CA expression, were sown on sand. The seedlings were grown in the growth cabinet where the temperature was maintained at 25℃/20℃ (day/night) with a 12 hour day length. The light intensity was kept constant at 1000 μmol · m^{-2} · s^{-1} during day time. Two sets of the plants were prepared; one for the measurement of leaf intrinsic CA and foreign CA activity, and the other for the measurement of leaf PCA properties.

2.3 Determination of CA Activity

In the leaves of line SR-1, which originated from the plastid genome mutation (Svab and Maliga, 1991), there were extremely low levels of endogenous CA activity compared to those of c.v. Xanthi, which we had previously tested to generate fCA transgenic plants (Jinushi *et al.*, 1998). Fifteen cm^2 of the leaves was ground with the mortar and pestle for 2 min on ice with 4 % of polyvinyl polypyrrolidon, sea-sand and 1.4 m*l* of the extraction buffer (pH 8.3) containing 50 mM sodium barbital, 1 mM Na_2-EDTA, 2 % glycine, 2 % polyvinyl pyrrolidon 1 % BSA, 10 % glycerol and 0.2 mM phenylmethanesulfonylfluoride. After centrifugation at 4 ℃ and 15,000 x g for 5 min, an aliquot of the supernatant was used for the measurement of CA activity (Tsuzuki *et al.*, 1985). The foreign CA activity in the leaf extract was estimated by differential measurement in the presence and absence of 10^{-7} M acetazolamide which inhibited the animal CA type II by 99 % without any effect on leaf endogenous CA (Jinushi *et al.*, 1998).

2.4 Gas Exchange Measurement

The PCA rates and fluorescence signals were simultaneously determined in the youngest fully

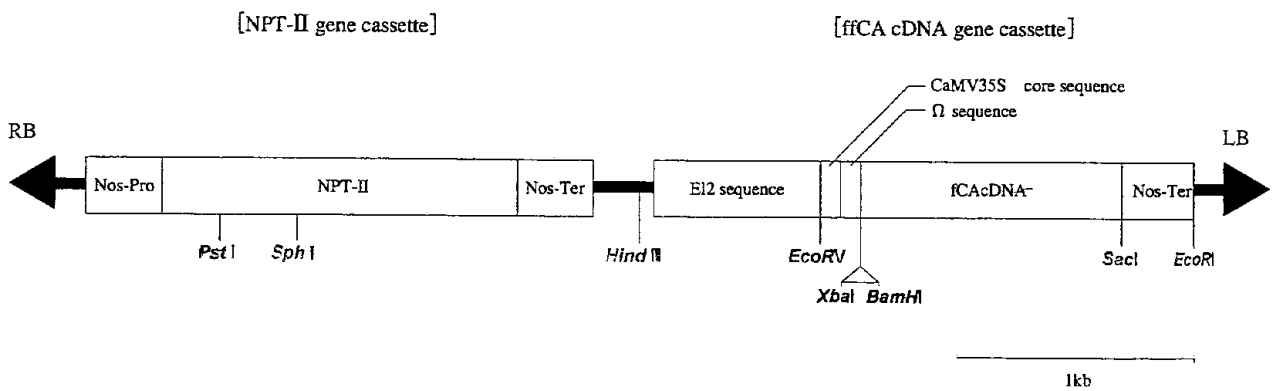

Fig.1 The vector construction of pBffCA010; the ffCA cDNA gene cassette which was designed to express the partially modified mouse CA type II protein with total size of 252 amino acids at the left border and the NPT-II gene cassette for the screening of transgenic plants at the right border.

expanded leaves. In order to confirm the unique effects of cytoplasmic foreign CA activity specifically, sample plants with similar leaf conditions were selected by leaf chlorophyll content measured by a non-destructive method. The measuring system for the PCA rate was the same as reported in the previous paper (Jinushi *et al.*, 1998; Nobel *et al.*, 1975). The ambient CO_2 concentration was sequentially changed in the order of 350, 200, 100 and 80 ppm. The light intensity, and the leaf temperature were maintained constant at 1000 μ E · m^{-2} · s^{-1} and 26 ± 1 ℃, respectively. In some measurements, PCA was also determined under 350 ppm of CO_2 at 500 μ E · m^{-2} · s^{-1} of the light intensity.

2.5 Chlorophyll Fluorescence Analysis

To estimate Rr, chlorophyll fluorescence parameters were measured with a PAM 101 modulated fluorometer (Waltz) (Genty *et al.*, 1989; Harley *et al.*, 1992; Schreiber, 1986). To measure the fluorescence signals simultaneously with PCA determination, a polyfurcated optic fiber was supported at angle of 45° to the leaf chamber to avoid the shading of the leaf light illuminating.

The measurement of Rr value is known to be rather stable under lower Ci conditions (Loreto *et al.*, 1992), thus, we compared the Rr values at Ci = 100 ppm. The calculated Rr value of Wild types of tobacco was nearly the same level as those reported in other papers which measured using the isotopic carbon method (Evans *et al.*, 1986; Evans *et al.*, 1994; von Caemmerer and Evans, 1991).

2.6 Western Blot Analysis

The RubisCO and expressed foreign CA levels in the leaves were measured by SDS-PAGE and Western blot analysis as described in the previous paper (Jinushi *et al.*, 1998).

2.7 Growth Analysis

Using non-destructive leaf area measurement with a video camera and 2-dimension pattern analysis program (ATTO, Japan), we selected transgenic and wild type seedlings of similar size at 5 weeks after sowing. They were cultured for 17 or 30 days under natural light or 50 % shaded conditions in the green house. The temperature was maintained at 25 ℃ /20 ℃ (day/night). Day time light intensity was at least 1000 μE · m^{-2} · s^{-1} and the CO_2 concentration was around 390 ppm.

3. Results and Discussion

The transgenic plants which showed a high level of foreign CA protein were confirmed by Western blot analysis of the leaf extracts of individuals of 4 strains (ffCA-1, -3, -6 and -7) among the 14 seedlings recovered from the screening antibiotic Km medium. By the PCR Southern and Northern analyses, it was confirmed that the mouse ffCA cDNA gene cassette existed in the chromosomal genome and was transcribed to messenger RNA in these 4 strains. However, two plantlets of these strains showed a strange appearance; the strain ffCA-6 was dwarf with slender leaves, probably due to somaclonal variation and the strain ffCA-7 was sterile. Therefore, T1 seeds were obtained only from two remaining strains (ffCA-1 and ffCA-3). The segregation ratios of foreign CA expressing and non-expressing phenotypes in T1 plants were 27:0 (ffCA-1) and 33:4 (ffCA-3), which indicated that at least two copies of mouse CA cDNA gene cassette were introduced into the chromosomal DNA in these transgenic plants. **Fig. 2** shows the Western blot analysis of leaf extracts in ffCA plants (ffCA-3) under the control of El2 Ω promoter and of fCA plants under the control of single CaMV35S promoter at 6 weeks after sowing. The expression level of foreign CA peptide in ffCA-3 was calculated with the 2-dimentional-density pattern analysis program to be 3.2 times higher than the highest expression among the 23 fCA plants (High CA Plant). This indicated that the El2 Ω promoter could enhance the mouse CA cDNA gene expression just like GUS gene reported before (Mitsuhara *et al.*, 1996). On the other hand, ffCA-1 showed a lower

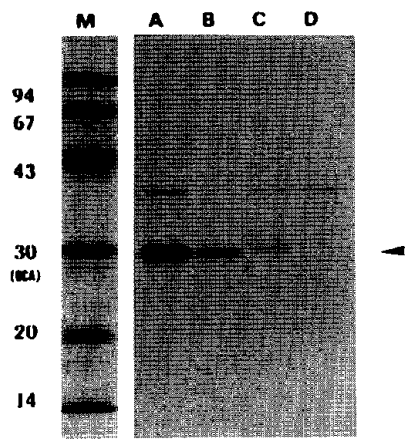

Fig. 2 Comparison of mouse CA expression level between transgenic plants containing different promoters. Lane M shows the molecular weight markers (Pharmacia) with CBB staining. The lanes A, B, C, and D, show the immuno-detection of the leaf extract in the ffCA transgenic plant (the strain ffCA-3), in the High CA Plant and Low CA Plant of the fCA transgenic plant and in Wild Type, respectively. The arrow indicates the immuno-detection band of about 29 KD.

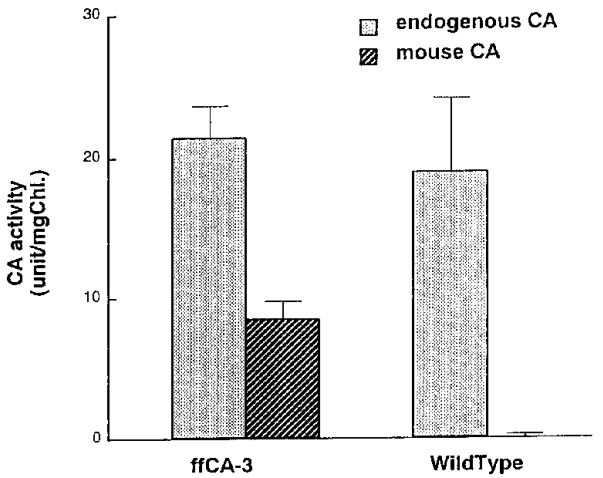

Fig. 3 Partially modified mouse CA activity in the leaf of ffCA transgenic plant (the strain ffCA-3).

expression level of foreign CA compared to ffCA-3 (data not shown). From the differential CA activity measurements with and without acetazolamide (Jinushi *et al.*, 1998), the expression levels of foreign CA activity were estimated as 8.39 ± 1.41 and −0.01 ± 0.27 WA unit · mg Chlorophyll^{-1} in the leaves of the strain ffCA-3 and wild type, respectively. Thus, foreign CA activity occupied as high as 39.2 % of total leaf CA activity in ffCA-3 (**Fig.3**). Specific activity of expressed foreign CA in ffCA plants were, nearly the same level (800 ± 133 WA unit / mg CA peptide) as those of fCA plants in the previous experiment with c.v. Xanthi (841 ± 58 and 851 ± 163, for High CA phenotype and Low CA phenotype, respectively) (Jinushi *et al.*, 1998).

At 8 weeks after sowing, the average expression level of foreign CA was 425.1 ± 15.0 ng · cm^{-2} in the T2 plant of ffCA-3 (**Table 1**). While leaf RubisCO contents in ffCA plants showed no difference from that in the wild type. The PCA rates of ffCA-3 were significantly higher than those of the wild type even at the ambient air condition (Ci = 250ppm) where little improvement could be seen in the fCA previously reported (Jinushi *et al.*, 1998) (**Table 2**). At 250 ppm of Ci, the PCA rate of the transgenic plant was 14.1 ± 0.1 μmol CO_2· m^{-2}· s^{-1}, which was 2.9 % higher than that of the wild type. Moreover, at 100 ppm of Ci, the transgenic plants showed 6.7 % higher PCA rate than that of the wild type. However, compared to fCA plants the percentages of the improvement of PCA rate at lower CO_2 concentration did not increase drastically in ffCA plants. This might indicate the existence of a certain saturation level of cytoplasmic foreign CA activity for the improvement of PCA rate in tobacco plants. Regarding this point, it was reported that Rr might not be a major limiting step in the C3 PCA process (Sasaki *et al.*, 1996). Our data showed little effects of foreign CA on the PCA rate at such a low light

Table 1. The levels of mouse CA, RubisCO and chlorophyll in the leaves of ffCA (SR − I) and fCA (Xanthi) transgenic tobacco plants.

	The leaf content of		
	mouse CA	Rubis CO	chlorophyll
	ng cm^{-2}	μ g cm^{-2}	μ g cm^{-2}
ffCA plants (n=4)	425.1 ± 15.0	201.9 ± 7.7	40.5 ± 1.0
Wild type (n=5)	---	207.7 ± 5.5	41.3 ± 1.1
Reference data from the previous report with Xanthi			
fCA plants (n=9)	277.0 ± 8.1	203.0 ± 4.8	40.4 ± 0.6
Wild type (n=8)	---	207.8 ± 4.1	41.7 ± 0.8

Table 2. The PCA rate in ffCA transgenic plants under high light condition of 1000 μ molE^{-2} S^{-1}.

	PCA rate at CO_2 concentrations of			
	Ci300ppm	Ci250ppm	Ci150ppm	Ci100ppm
	μ mol CO_2 m^{-2} s^{-1}			
ffCA plants (n=4)	16.9 ± 0.2*	14.1 ± 0.1**	8.21 ± 0.05***	4.48 ± 0.05**
Wild type (n=5)	16.5 ± 0.1	13.7 ± 0.1	7.78 ± 0.10	4.20 ± 0.08
Enhancement (ffCA/WT) x100	2.4%	2.9%	5.5%	6.7%

*, ** and ***: 5, 2.5 and 1% significance, respectively.

intensity as 500 μ E · m^{-2} · s^{-1} and a higher CO_2 concentration such as 300 ppm, namely, 6.65 ± 0.16 and 6.79 ± 0.21 μmol CO_2 · m^{-2} · s^{-1}, ffCA plants (n=4) and wild type (n=5), respectively. It can be concluded that the optimal manifestation of the cytoplasmic CA introduction effect on C3 photosynthesis is only observable under light saturated and CO_2 supply limiting conditions.

We also conducted the simultaneous measurement of chlorophyll fluorescence parameters. No significant differences in the calculated quantum yield of PS Ⅱ (Φ Ⅱ) was observed between ffCA transgenic plants and the wild type (data not shown). However, the Rr of ffCA plants was significantly lower than that of the wild type. On the other hand, we could detect no differences in stomatal resistance (Rs) and mesophyll resistance for CO_2 fixation (Rx) between ffCA and wild type **(Table 3)**. Therefore, these results indicated that the improvement of PCA rate under CO_2 limiting conditions occurrs with the introduction of foreign CA activity in mesophyll cytoplasm through the reduction of Rr. The failure to detect any significant improvement in PCA rate under lower light conditions where Rr was not a limiting factor in PCA (von Caemmerer and Farquhar, 1981) would support this hypothesis.

As to the growth observation under the natural light condition, the high - ffCA transgenic plants grew faster than the wild type plants. The seedlings of strain ffCA - 3 showed 10 % larger leaf area and 7 % heavier total dry weight than wild type plants at 7 weeks after sowing **(Table 4)**, although there was no significant change in the low - ffCA transgenic plant, ffCA - 1. In the similar experiment under low light condition with 50 % shading of the natural light intensity, ffCA - 3 did not show any growth

Table 3. Total resistance (Rt), stomatal resistance (Rs), mesophyll resistance for CO_2 transfer(Rr) and mesophyll resistance for CO_2 fization (Rx) in ffCA transgenic plants under the condition of 1000 μ molEm^{-2} s^{-1} of light intensity and Ci=250ppm.

	Rt	Rs	Rr	Rx
	molm^{-2} s^{-1}			
ffCA plants (n=4)	20.9 ± 0.2**	3.18 ± 0.10	6.20 ± 0.18**	11.5 ± 0.2
Wild types (n=5)	21.7 ± 0.2	3.44 ± 0.19	6.81 ± 0.17	11.4 ± 0.1

**: 2.5% significance.

Table 4. Leaf area, total dry weight, mouse CA and chlorophyll content in the ffCA transgenic plants under natural light condition.

	Total Leaf Area		Total Dry Weight	Leaf mouse CA	Leaf Chl.
	cm^2		g	ng · cm^{-2}	μg · cm^{-2}
	5weeks	7weeks	7weeks	7weeks	7weeks
ffCA-3 (n=12)	17.1 ± 0.5	532.4 ± 15.0**	1.69 ± 0.05*	235.4 ± 23.6	33.2 ± 0.4*
ffCA-1 (n=7)	17.0 ± 0.4	486.9 ± 18.8	1.55 ± 0.07	80.3 ± 14.3	32.7 ± 0.6
Wild type (n=10)	17.1 ± 0.4	483.9 ± 15.1	1.58 ± 0.05	–	31.7 ± 0.8

* and **: 5 and 2.5% significance, respectively.

Lable 5. Leaf area, total dry weight, mouse CA and chlorohpyll content in the leaf of ffCA trnasgenic plants under 50% shading natural light condition.

	Total Leaf Area		Total Dry Weight	Leaf mouse CA	Leaf Chl.
	cm^2		g	ng · cm^{-2}	μg · cm^{-2}
	5weeks	9weeks	9weeks	9weeks	9weeks
ffCA-3 (n=4)	10.0 ± 0.5	449.6 ± 24.6	0.97 ± 0.08	116.4 ± 9.2	27.6 ± 0.5
Wild type (n=7)	10.3 ± 0.4	502.4 ± 20.8	1.07 ± 0.05	–	27.6 ± 0.4

enhancement (**Table 5**). These findings corresponded well to those of photosynthetic analysis data and suggested the positive effects of introduced cytoplasmic CA on their growth rate under light saturated and CO_2 supply limiting conditions. However, there remain alternative possible indirect reasons induced by the introduction of foreign CA activity in the cytoplasm connected to the observed growth enhancement: 1) Leaf chlorophyll and RubisCO contents increased along with the plant size in the vegetative stage of tobacco seedlings. Hence, the transgenic plants with higher PCA activity might have relatively thick and photosynthetically active leaves at an earlier growing stage compared with the wild type plants (**Table 4**). 2) When transgenic plants grew faster for the reasons above, they might have expanded their leaves faster than wild type plants. Therefore, they might have higher light capturing efficiency at the whole plant level, which brought about further growth enhancement. 3) Under extremely high light conditions, stomatal closure would lead a decrease in the internal CO_2 concentration of the leaf bringing about photosynthetic photoinhibition. Enhancement of leaf carbon utilization efficiency by the introduced cytoplasmic CA activity might prevent mesophyll tissues from accumulating extra-reducing power. Regarding this, the reduction of photoinhibition is one of the major target points of the recent crop breeding challenge in molecular biology.

Price *et al.* (1994) carried out suppression experiments of endogenous CA activity by means of anti-sense mRNA technology and reported that the drastic reduction of tobacco leaf endogenous CA activity to 1 or 2 % of wild type made little effect on its PCA rate. Thus, it was considered that there should be too much CA activity in the mesophyll cells of C3 plants and any over-expression of CA activity in the mesophyll cells (in the chloroplasts) might not contribute to improvement of its PCA rate (Majeau *et al.*, 1994). However, the results of our previous and present paper suggested that the level of CA activity in choloroplasts in C3 tobacco appears to be saturated in nature but the specific introduction of CA activity into the cytoplasmic area of mesophyll cells would improve PCA activity through reduction of Rr even with a small increase range of the introduced CA activity.

Acknowledgments

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