A4. Photosynthetic Performance of Transgenic Rice Plants Overexpressing Maize C_4 Photosynthesis Enzymes

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옥수수 C_4 광합성효소가 고발현되는 형질전환 벼의 광합성특성

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Due to the CO_2-concentrating mechanism in the C_4 pathway of photosynthesis, C_4 plants have many desirable agronomic traits, such as high photosynthetic capacity and high mineral-use efficiency, especially under high light, high temperature, and drought condition(Hatch 1987, Ku et al 1996). On the other hand, plants that assimilate atmospheric CO_2 via the C_3 pathway, including many agronomically important species such as rice, suffer from O_2 inhibition of photosynthesis and the associated photorespiration, and thus exhibit a lower photosynthetic efficiency under these conditions. Conventional hybridization has been employed to transfer C_4 traits to C_3 plants(Brown and Bouton1993);however, epistatic interaction between the alleles suppresses the expression of C_4 traits in the progeny, and genes for kranz leaf anatomy and biochemistry of C_4 photosynthesis are not closely linked. Most importantly, no closely related C3 and C4 crops can be hybridized. Several attempts have been made in the past to transger the genes involved in C4 photosynthesis (Kogami et al 1994, Ishimaru et al 1998) in an effort to tune up their photosynthetic metabolism. However, limited physiological consequences were observed in these transgenic plants, which may be due to the low levels of expression of these genes.

Using an *Agrobacterium*-based transformation system, they have independently introduced three key C_4 photosynthesis genes from maize into rice with high levels of expression(Agarie et al 1990, Ku et al 1999). These are phospho*enol*pyruvate carboxylase(PEPC), pyruvate orthophosphate dikinase(PPDK), and NADP-malic enzyme(NADP-ME). The major objective of these research is to introduce enzymes involved in C_4 photosynthesis into C_3 plants and test their effects on photosynthesis into C_3 plants with proper

intercellular compartmentation, a limited C_4 acid metabolism may be installed for fixing atmospheric CO_2 directly via this pathway and partially concentrating CO_2 in the chloroplast.

Enhanced expression of enzymes of C_4 photosynthesis in C_3 plants may increase carbon and nitrogen metabolism in certain tissues of C_3 plants. All enzymes involved in C_4 photosynthesis are found in leaves of C_3 plants. Although they are low in activity in leaves of C_3 plants, some of them are found at high levels in reproductive tissues. For example, the cytosolic isoform of PPDK occurs at high levels in seeds of both the C_3 plant wheat(Aoyagi and Bassham 1984a,b, Aoygi and Chua 1988, Blanke and Lenz 1989)and the C_4 plant maize (Imaizumi et al 1997). Thus, PPDK may play an important role in linking carbon and nitrogen metabolism or supply of energy(e.g., release of ATP from PEP caralyzed by PPDK) in reproductive tissues, and enhanced expression of the enzyme may boost seed development and grain productivity.

Enzymes involved in C_4 photosynthesis, although low in C_3 plants, may also play important roles in plant defense responses to biotic and abiotic stress. Metabolic alterations in response to stress allow plants to adapt to adverse conditions. For example, an increase in NADP-ME by wounding, low oxygen, low temperature, salinity, and ultraviolet light has been reported in C_3 plants such as rice(Fushimi et al 1994) and bean (Walter et al 1994, Schaaf et al 1995, Pinto et al 1999) and the C_4 plant maize (Drincovich et al 1998). It is postulated that the reductant(NADPH) released from decarboxylation of malate by NADP-ME may be required for the increased synthesis of secondary metabolites for defense purposes. The biosynthesis of phenylpropanoids requires the efficient flow of carbon into phenylalanine biosynthesis. Thus, increased expression of some C_4 photosynthesis enzymes in C_3 plants could confer enhanced tolerance under stress conditions. We have independently introduced three key C_4 photosynthesis genes from maize into rice: PEPC, PPDK and NADP-ME(Agarie et al 1998, Ku et al 1999).

Rice transformation and expression of transgenes

Using the *Agrobacterium*-mediated transformation system, we have transformed two Japonica rice cultivars, Kitaake and Nipponbare, with three maize C_4 photosynthesis genes, as mentioned above (Agarie et al 1998, Ku et al 1999). Transgenic plants derived from Kitaake were used for further

characterization since Kitaake has a shorter life span and flowers more readily under growth conditions. The level of expression varies considerably among the transgenic plants derived from the same transformation. In part, this could be due to site of insertion of the transgene in the rice genome, but it is also dependent on gene construct used for transformation and gene copy. In comparison, intact maize genes with its own promoter and terminator sequences and introns and exons tend to give higher levels of expression, as compared with cDNA. In some transgenic plants harbouring the intact maize PEPC gene, the maize enzyme accounts for up to 18% of the total leaf soluble protein(Ku et al 1999). Also, the level of expression is related to gene copy number and locus number of transgene insertion. Genetic studies show that the maize genes are stably inherited in a Mendelian manner, with the genes being inserted at one or two loci into the rice genome. Immunolocalization studies show that the maize PEPC is expressed in the cytosol whereas PPDK and NADP-ME are expressed in the chloroplast, as one would expect. The maize enzymes remain active in the transgenic rice plants, and the activity is highly correlated with the enzyme protein amount(Ku et al 1999). These results suggest that the regulatory mechanisms for maintaining the activity of the maize C_4 enzymes are present in rice. In terms of organ specificity of expression, both leaf and leaf sheath of transgenic rice plants express high levels of these enzymes. A substantial amount of PEPC is also detected in the palea and remma tissues of seed in PEPC transgenic plants. In contrast, these enzymes are predominantly expressed in leaves of maize. The results suggest that the regulatory mechanisms responsible for organ-specific expression of these genes in maize is not present in C_3 rice. Except for few plants, most of the transgenic rice plants exhibit a normal phenotype and retain high fertility (85-90% as versus 90% in the untransformed plants). However, NADP-ME transgenic plants have reduced height(10cm), and the time required to flower is shortened by 4 days for PPDK transgenic plants but delayed 4-6 days in PEPC transgenic plants. Another interesting observation is that leaves of NADP-ME transgenic plants stay green even after the seeds have reached maturity. The reason for this is not clear, but may be related to the production of extra reductant via NADP-ME for chlorophyll synthesis. Maintenance of photosynthetically active leaves during grain filling further contribute to yield potential.

Photosynthetic performance of transgenic rice plants expressing maize C4 photosynthesis enzymes

PEPC transgenic rice plants

On a leaf area basis, the photosynthetic rates of the primary PEPC transgenic plants, measured under ambient conditions, are comparable or higher than those of untransformed plants(Ku et al 1999). In addition, O_2 inhibition of photosynthesis decreases progressively with increasing level of PEPC activity among the transgenic plants. Our preliminary labelling experiment with $^{14}CO_2$ shows only a small increase (4%) in atmospheric CO_2 being directly fixed by PEPC in these plants. The supply of PEP, the substrate for PEPC, may be limited in C_3 leaves. Thus, the biochemical and physiological bases of these alterations in photosynthetic trait remain unclear. Using the segregation populations from four primary transgenic lines which exhibit high levels of the maize PEPC, we have shown that the photosynthetic rates of flag leaves in most PEPC transgenic rice plants are comparable or up to 30% higher than those of untransformed plants (Ku et al 2000). Photosynthetic rate begins to decrease as the level of expression reaches very high, as one would expect. Indeed, transgenic plants with extremely high levels of PEPC have lower chlorophyll contents. Analysis of the relationship between photosynthetic rate and stomatal conductance among these plants shows a good positive correlation between the two parameters. Furthermore, stomatal conductance is highly correlated with intercellular CO_2 concentration, and the intercellular CO_2 concentration in some transgenic plants is as high as 275 $\mu l\ l^{-1}$, in comparison to 235 $\mu l\ l^{-1}$ in untransformed plants. Therefore, part of the higher photosynthetic capacity of the transgenic plants may be due to the ability of the plants to maintain a higher internal CO_2 in the leaf due to increased stomatal opening. The immediate benefit of a higher intercellular CO_2 is elevated net carbon fixation due to more CO_2 and suppression of Rubisco oxygenase and the associated photorespiration. Consistently, we also observed an upward shift in optimal temperature for photosynthesis by the transgenic plants from 26 to 28-32℃(Cho & M.S.B. Ku, unpublished data), presumably due to reduced photorespiration.

Consistent with the suggestion that PEPC transgenic rice plants have a higher stomatal conductance than untransformed wild-type plants, the $\delta^{13}C$

values for the transgenic plants are 1.5-2.5 ‰ more negative than that of untransformed plants and the value increases with increasing PEPC activity among the transgenic plants (Ku et al 2000). An increased stomatal conductance would allow more CO_2 to diffuse into the leaf and thus more ^{13}C being discriminated during photosynthesis (Winter et al 1982). However, the possibility that the lower ^{13}C content in the leaves of transgenic plants could be due to refixation of photorespiratory CO_2 by PEPC and then Rubisco again can not be ruled out. The interesting question here is how transgenic plants manage to maintain a higher stomatal conductance. The mechanism underlying this phenomenon is not quite clear. However, it is conceivable that an increased expression of PEPC in the guard cells would allow more fixation of atmospheric CO_2 into organic acids such as malate, which is stored in the vacuole. Consequently, inorganic solutes such as potassium move from subsidiary or epidermal cells into guard cells for balance of charge. The accumulation of ions in the vacuole lowers the water potential of the guard cells, thereby stimulating the osmotic uptake of water and increasing turgor for opening of stomates.

PPDK transgenic rice plants

The photosynthetic performance of PPDK transgenic plants was evaluated using the segregations populations from four primary transgenic lines that exhibit high levels of PPDK(Ku et al 2000). Most fo the PPDK transgenic plants exhibit a higher photosynthetic rate (up to 35%) than the wild-type plants, and the higher photosynthetic rates are associated with increased stomatal conductance and higher intercellular CO_2 concentration. Thus, as with PEPC transgenic plants, PPDK transgenic rice plants may also be able to maintain a higher internal CO_2 level due to increased stomatal conductance. Increased expression of PPDK in the guard cells may function to supply PEP, the substrate for PEPC, for synthesis of organic acids and stimulate stomatal opening(Schnabl 1981). How the elevated PPDK may affect carbon and nitrogen metabolism in leaves of transgenic rice plants awaits further investigation. The effects of elevated expression of maize PPDK on carbon metabolism in transgenic potatoes(C_3) has been reported recently (Ishimaru et al 1998). PPDK activities in leaves of transgenic potatoes are up to fivefold higher than those of untransformed control plants. Analysis of metabolites shows that PPDK

activity in leaves is negatively correlated with pyruvate content and positively correlated with malate content. It is suggested that elevated PPDK activity in the leaf may lead to a partial function of C_4-type carbon metabolism. However, the altered carbon metabolism dose not have any significant effect on other photosynthetic characteristics in the transgenic potatoes.

Transgenic rice plants simultaneously expressing maize PEPC and PPDK

Since PEPC catalyzes the initial fixation of atmospheric CO_2 in the C_4 pathway and PPDK catalyzes the conversion of pyruvate to PEP, overexpression of both enzymes simultaneously may enhance fixation of atmospheric CO_2 via PEPC. Using conventional cross-hybridization, we have integrated both PEPC and PPDK genes into the same plants from two independent homozygous transgenic plants. As detected by specific antibodies raised against the two maize enzymes, the amounts of the two enzymes in the F1 hybrids are about half of those in the parents(Ku et al 2000 and Ku et al 2001). The photosynthetic performance of the transgenic plants expressing varying amounts of the two maize enzymes was first evaluated in the segregation population from one of the F1 hybrids. As expected, the segregation population exhibits different combinations for the amounts of the two enzymes, with some having only the same basal amount as the wild-type plants (without the maize gene inserted) to twice the amount of the parental transgenic plants (homozygous with respect to the inserted maize gene). The activities of each enzyme are well correlated with the amounts of the protein among these plants. Hybrid transgenic rice plants expressing high levels of both PEPC and PPDK tend to have a high photosynthetic rate than untransformed plants, again due to a higher stomatal conductance and a higher intercellular CO_2. It is quite possible that overexpression of both enzymes further enhances the capability of the plants to synthesize organic acids in the guard cells and consequently the conductance of CO_2 into the leaf.

Future directions

The higher stomatal conductance exhibited by the transgenic rice plants implies that more water may be needed. However, this may not be a serious problem for paddy rice. On the other hand, an increased stomatal conductance

may help cool the leaf canopy at high temperatures, as shown in wheat (Fischer et al 1998). A preliminary, small-scale field trial shows that the grain yield is about 10-20% higher in selected PEPC and 30-35% higher in selected PPDK transgenic rice lines and the hybrids between these two transgenic lines relative to untransformed plants, in spite of a lower fertility(5%) in some of the transgenic plants(Ku et al 2000). The increased yields are mainly due to increased tiller number. More field tests on a large scale are underway to confirm this. Also, whether this trait will be stable inherited in the following generations needs to be evaluated.

As one would expect, transgenic rice plants overexpressing maize PEPC, PPDK or both may not be capable of fixing large amounts of atmospheric CO_2 directly via the C_4 pathway due to limited supply of substrates of further metabolism of reaction products. However, with the introduction of another key enzyme of the C_4 pathway, NADP-ME in the chloroplast, a limited CO_2 concentrating mechanism, as exhibited by H. *verticillata*, may be achieved. Recently, we have obtained transgenic rice plants simultaneously expressing PEPC, PPDK and NADP-ME using cross-hybridization. These plants grow more rapidly and put out more tillers than the parental transgenic lines. Whether they are capable of concentrating CO_2 in the leaf and suppress photorespiration is under investigation. Enhanced expression of other biochemical components of the C4 pathway, such as CA, NADP-malate dehydrogenase and adenylate kinase may allow the cycle to function more effectively. In this regard, the increased activities of CA and Rubisco in PEPC transgenic rice plants is worth noting; some related enzymes in the pathway may be induced or enhanced and it may not be necessary to genetically alter them simultaneously.

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