REVIEW PAPER

Lessons from engineering a single-cell C4 photosynthetic pathway into rice

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Received 15 November 2010; Revised 16 January 2011; Accepted 19 January 2011

Abstract

The transfer of C₄ plant traits into C₃ plants has long been a strategy for improving the photosynthetic performance of C₃ plants. The introduction of a pathway mimicking the C₄ photosynthetic pathway into the mesophyll cells of C₃ plants was only a realistic approach when transgenic technology was sufficiently well developed and widely adopted. Here an attempt to introduce a single-cell C₄-like pathway in which CO₂ capture and release occur in the mesophyll cell, such as the one found in the aquatic plant Hydrilla verticillata (L.f.) Royle, into rice (Oryza sativa L.) is described. Four enzymes involved in this pathway were successfully overproduced in the transgenic rice leaves, and 12 different sets of transgenic rice that overproduce these enzymes independently or in combination were produced and analysed. Although none of these transformants has yet shown dramatic improvements in photosynthesis, these studies nonetheless have important implications for the evolution of C₄ photosynthetic genes and their metabolic regulation, and have shed light on the unique aspects of rice physiology and metabolism. This article summarizes the lessons learned during these attempts to engineer single-cell C₄ rice.

Key words: C₄ photosynthesis, metabolic engineering, NADP-malate dehydrogenase, NADP-malic enzyme, phosphoenolpyruvate carboxylase, pyruvate, orthophosphate dikinase, transgenic rice.

Introduction

Many important crops, such as rice (Oryza sativa L.), wheat (Triticum aestivum L.), soybean (Glycine max L.), and potato (Solanum tuberosum L.), are classified as C₃ plants. This plant group assimilates atmospheric CO₂ directly through the C₃ photosynthetic pathway, which is also called the Calvin cycle or the photosynthetic carbon reduction cycle. In contrast, C₄ plants such as maize (Zea mays L.), sorghum [Sorghum bicolor (L.) Moench], and sugarcane (Saccharum officinarum L.) evolved from C₃ plants, acquiring the C₄ photosynthetic pathway in addition to the C₃ pathway. The C₄ pathway acts to concentrate CO₂ at the site of the reactions of the Calvin cycle, and thus inhibits photorespiration (Hatch, 1987). This CO₂-concentrating mechanism enables C₄ plants to achieve higher photosynthetic capacity and higher water- and nitrogen-use efficiency than C₃ plants. Since the discovery of C₄ photosynthesis and its agronomic advantages, the transfer of C₄ traits to C₃ plants has been one strategy for improving the photosynthetic performance of C₃ plants. This strategy was initially attempted by means of conventional hybridization between C₃ and C₄ plants (reviewed in Brown and Bouton, 1993) and more recently using transgenic techniques (reviewed in Matsuoka et al., 2001; Häusler et al., 2002; Miyao, 2003).

The C₄ pathway consists of three key steps: (i) initial fixation of CO₂ by phosphoenolpyruvate carboxylase (PEPC) to form a C₄ acid; (ii) decarboxylation of a C₄ acid to release CO₂ near the site of the Calvin cycle; and (iii) regeneration of the primary CO₂ acceptor phosphoenolpyruvate (PEP) by

Abbreviations: BSC, bundle sheath cell; GUS, β-glucuronidase; MC, mesophyll cell; NADP-MDH, NADP-malate dehydrogenase; NADP-ME, NADP-malic enzyme; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEP-CK, phosphoenolpyruvate carboxykinase; PPDK, pyruvate, orthophosphate dikinase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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pyruvate, orthophosphate dikinase (PPDK) (Hatch, 1987). In terrestrial C₄ plants, CO₂ release from C₄ acids and the resulting elevation of cellular CO₂ levels take place at a site that is physically separated from the site of the initial carboxylation. This separation occurs through the bundle sheath cells (BSCs) and mesophyll cells (MCs) in typical C₄ plants (Hatch, 1987), and through two distant subcellular compartments in the recently discovered single-cell C₄ plants (Edwards et al., 2004). Structural and biochemical features of the chloroplasts at these two sites are also different. It has been proposed that such compartmentation and chloroplast differentiation, together with structural adaptation to minimize CO₂ diffusion away from the CO₂ release site, are essential for C₄ photosynthesis (Hatch, 1987; Edwards et al., 2004). The molecular basis for such structural and chloroplast differentiation is not yet fully understood, but is a hot topic in current C₄ photosynthesis research.

Another example of C₄ photosynthesis has been found in aquatic plants, in which C₄ photosynthesis is accomplished in a single cell without any compartmentation and chloroplast differentiation (Bowes et al., 2002; Fig. 1A). At least three submerged macroalgae, all of which are freshwater monocots, perform C₄ photosynthesis in this manner (Bowes et al., 2002). Hydrilla verticillata (L.f.) Royle has been the best documented. It is a facultative C₄ plant that shifts from C₃ to C₄ photosynthesis under low CO₂ conditions without undergoing any structural modifications of its leaf cells. During the shift to C₄ photosynthesis, genes encoding the C₄-specific isozymes of PEPC, PPDK, and NADP-malic enzyme (NADP-ME) are upregulated (Rao et al., 2002, 2006; Estavillo et al., 2007).

Because of the simplicity of the Hydrilla system, a number of attempts have been made to introduce this type of C₄-like pathway into the MCs of C₃ plants (reviewed in Matsuoka et al., 2001; Häusler et al., 2002; Miyao, 2003). Other C₄-like pathways consisting of two enzymes [PEPC and phosphoenolpyruvate carboxykinase (PEP-CK); Suzuki et al., 2006; Fig. 1B] or of five enzymes [PEPC, PPDK, PEP synthetase, PEP-CK, NADP-MDH, and NADP-ME; Häusler et al., 2002] have also been proposed. The key steps in these pathways are the carboxylation of PEP by PEPC in the cytosol and decarboxylation of the resultant C₄ acid inside the chloroplast. Whether or not such pathways can operate with desirable effects for C₃ photosynthesis has been a matter of controversy (Edwards, 1999; Häusler et al., 2002; Leegood, 2002). Two major concerns have been raised. One is whether a closed cycle is created without any modification of intracellular metabolite transport. All the proposed pathways involve the import of oxaloacetate (OAA) into and the export of PEP from the chloroplasts (Fig. 1). The import of OAA has not been a subject of debate, since OAA is actively taken up by the chloroplast via the malate valve, which functions in transferring reducing equivalents from the chloroplast stroma to the cytosol (Scheibe, 2004) when chloroplasts perform photosynthesis under illumination. In addition, the envelope of the C₃ MC chloroplast has a high capacity to translocate dicarboxylic acids (Bräutigam and Weber, 2011). In contrast, it has long been argued that the export of PEP should limit this pathway, since the transport activity of the chloroplast envelope for PEP is low in the leaves of C₃ plants (e.g. see Fischer et al., 1997). The second concern is whether CO₂ released inside the chloroplast can be retained long enough to promote photosynthesis or would be lost to the atmosphere (Leegood, 2002). Mathematical modelling of the Hydrilla-type C₄-like pathway suggested that for elevation of the CO₂ concentration to occur inside the chloroplast, the activity of the C₄-like pathway must reach at least 38% of the maximal carboxylation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) under atmospheric CO₂ conditions (von Caemmerer, 2003).

Here an attempt to introduce the Hydrilla-type C₄-like pathway into the MCs of rice by overproducing C₄ photosynthetic enzymes from C₄ grasses is described. Each of the four C₄ enzymes (i.e. PEPC, PPDK, NADP-MDH, and NADP-ME) have been successfully overproduced in rice leaves (Ku et al., 1999; Fukayama et al., 2001; Tsuchida et al., 2001; Taniguchi et al., 2008). Among the 12 different sets of transgenic rice that overproduce the four enzymes, independently or in combination, only quadruple transformants that overproduce all four showed increased photosynthesis rates (Taniguchi et al., 2008). At present, however, this enhanced photosynthesis does not appear to

![Fig. 1. Introduction of the C₄-like pathway into the MCs of C₃ plants. (A) The C₄-like pathway of H. verticillata, consisting of PEPC, PPDK, NADP-MDH, and NADP-ME. Previously, the activity of NADP-MDH in the C₃ mesophyll chloroplasts was believed to be sufficiently high that the C₄-like pathway would operate if PEPC, PPDK, and NADP-ME could be overproduced. (B) The C₄-like pathway consisting of PEPC and PEP-CK (Suzuki et al., 2006). The Hydrilla pathway (A) consumes two molecules of ATP (one consumed by PPDK and the other required for the conversion of AMP produced by PPDK into ADP), whereas the PEPC+PEP-CK pathway (B) consumes one molecule of ATP. CA, carbonic anhydrase; Mal, malate; Pyr, pyruvate.](https://academic.oup.com/jxb/article-abstract/62/9/3021/472668)
result from a CO₂-concentrating function of the introduced pathway. Although these results are disappointing, nonetheless during the course of this research unexpected findings were made that have important implications for the evolution of C₄ photosynthetic genes, regulation of the activity of C₄ enzymes in C₃ plants, and metabolic engineering of rice. In this article, these findings are presented.

Lesson 1: sites of overproduction of C₄ enzymes in rice leaves

To overproduce PEPC and PPDK, MC-specific enzymes of C₄ plants, intact maize C₄-specific genes for these enzymes, each containing its own promoter and terminator sequences and exon–intron structure were introduced. This strategy was based on observations that the 5′-flanking region of the maize C₄-specific genes (–1212 to +78 bp and –1032 to +71 bp from the transcription start site for the maize PEPC and PPDK genes, respectively) drove high-level and MC-specific expression of a reporter β-glucuronidase (GUS) gene in rice leaves (Matsuoka et al., 1993, 1994). As expected, the intact maize genes were strongly expressed in the transgenic rice leaves, and the maize PEPC and PPDK proteins accounted for up to 12% and 35%, respectively, of total leaf soluble protein (Ku et al., 1999; Fukayama et al., 2001). Comparison of the PPDK protein levels among transgenic rice plants with three different introduced gene constructs (Fukayama et al., 2001) indicated that, in addition to the 5′-flanking region, one or more introns or the terminator sequence of the maize genes, or a combination of both, was necessary for high-level expression of the maize genes.

Contrary to expectations, however, immunoelectron microscopy subsequently revealed that the maize PPDK protein accumulated in both MCs and BSCs of rice leaves (Fig. 2). Based on the densities of gold particles, the levels of the maize PPDK protein in the chloroplasts do not differ greatly between the two cell types. It is likely that faint GUS staining in the BSCs was underestimated because of the much denser staining in the surrounding MCs in a previous promoter::GUS analysis using the maize PPDK gene (Matsuoka et al., 1993). However, even if the expression is not specific to the MCs, the majority of the maize PPDK protein could be located in these cells if the distribution of the chloroplasts between the MCs and BSCs is taken into account; >90% of the total number of chloroplasts are found in the MCs of rice leaves (Yoshimura et al., 2004). At present, the possibility that maize PEPC might also accumulate in both cell types of transgenic rice leaves cannot be ruled out.

Information on the expression of BSC-specific C₄ enzyme genes in the leaves of C₃ plants was unavailable when these studies began. A conventional technique for overproducing foreign proteins was therefore employed, in which a full-length cDNA was expressed under the control of the rice Cab promoter, which directed strong and light-regulated expression of a reporter gene in leaf chlorenchyma cells (Sakamoto et al., 1991). The expression of the cDNA for the maize C₄-specific NADP-ME in this way resulted in up to 2000% elevation of NADP-ME activity in rice leaves (Tsuchida et al., 2001). The rice C₃-specific NADP-ME and the sorghum NADP-MDH were overproduced in rice leaves in the same way. These foreign enzymes might be present in both MCs and BSCs of rice leaves, both of which have chloroplasts (see Fig. 2).

The evolutionary scenario inferred from the previous promoter::GUS analyses of the maize PEPC and PPDK genes was relatively simple: in this scenario, maize acquired one or more cis elements for high-level MC-specific expression of the genes, whereas mechanisms for such expression, including one or more trans factors, were already present in rice leaves (Matsuoka, 1995; Nomura et al., 2000). This scenario was considered for BSC-specific C₄ enzyme genes, since the promoter::GUS analyses of the two different genes revealed GUS expression in the BSCs: the 5′-flanking region of the C₄-specific NADP-ME gene of C₄ Flaveria bidentis (L.) Kuntze conferred preferential expression of GUS in the BSCs of transgenic F. bidentis (Marshall et al., 1997), and that of the PEP-CK gene of Zoysia japonica Steud. led to strong and specific expression of GUS in the BSCs and vascular bundles of transgenic rice (Miyao, 2003). However, subsequent analyses using rice plants (Nomura et al., 2005a, 2005b) have demonstrated that the 5′-flanking regions of BSC-specific C₄ genes follow
the expression patterns of their respective rice orthologues and do not always confer BSC-specific expression of a reporter gene in the rice leaves. Unlike the *Zosia* PEP-CK gene, GUS staining was detected in almost all tissues for the mitochondrial aspartate aminotransferase gene from *Panicum miliaceum* L. and the maize NADP-ME gene in transgenic rice leaves (Nomura *et al.*, 2005a, 2005b). These observations suggest that the one or more presumed *cis* elements are not sufficient for BSC-specific expression of *C₄* genes in the leaves of *C₃* plants.

Recent studies have made much progress in identifying *cis* elements in *C₄* photosynthetic genes (reviewed in Hibberd and Covshoff, 2010). The promoter-deletion studies of the *GLDPA* gene for the P-subunit of glycine decarboxylase, a BSC-specific gene of *C₄* *Flaveria trinervia* (Spreng.) C. Mohr (*Engelmann et al.*, 2008), identified two regions in its 5'-flanking region, one (−1571 to −1138 bp from the translation start site) functioning as a transcriptional enhancer and the other (−1138 to −926 bp) involved in ‘repression’ of gene expression in MCs. A region proximal to the translation start site (−298 to −1 bp) could be involved in BSC-specific expression and, in combination with the region for repression in MCs, it conferred BSC-specific expression of a reporter gene in *C₄* *F. bidentis*. Similar results have been reported for two *MC*-specific enzyme genes of *C₄* *Cleome gynandra* L.: the genes for PPDK and for plasma membrane-bound carbonic anhydrase (K. Kajala and J. M. Hibberd, University of Cambridge, unpublished data). One or more general transcriptional enhancer elements are present in the 5'-flanking region, whereas the 5' and 3' untranslated regions, either independently or in combination, are sufficient for the MC-specific expression of a reporter gene in *C₄* *Arabidopsis* but not in *Arabidopsis thaliana* (*Arabidopsis thaliana* L.). The latter observation suggests that post-transcriptional regulation is involved in the cell specificity of the *C₄* enzymes in *C₄* plants. All of this evidence taken together suggests that mechanisms for cell-specific expression of *C₄* photosynthetic genes are more complex than previously expected, and that not only *cis* elements but also mechanisms for strict cell specificity of gene expression must have developed during the evolution of *C₄* plants.

**Lesson 2: kinetic properties of the introduced enzymes are important**

Overproduction of maize *C₄*-specific NADP-ME, a chloroplastic enzyme, in rice leaves led to enhanced photoinhibition of photosynthesis, bleaching of leaf colour, and serious stunting, even when the activity of the maize enzyme was two or three times higher than that in non-transgenic rice (Tsuchida *et al.*, 2001). These detrimental effects were ascribed to an increase in the NADPH to NADP⁺ ratio in the chloroplast stroma and to suppression of photorespiration due to depletion of cellular malate, which must be exported from the chloroplast in exchange for 2-oxoglutarate during photorespiration. A significant reduction in the leaf malate level was observed in transgenic *Arabidopsis* in which cDNA for the maize *C₄*-specific NADP-ME was expressed under the control of the cauliflower mosaic virus 35S promoter (Fahnenstich *et al.*, 2007). In contrast to the maize NADP-ME, overproduction of the rice *C₃*-specific isozyme, at activities up to five times that in non-transgenic rice, did not affect either photosynthesis or growth of rice plants (Taniuguchi *et al.*, 2008).

Kinetic studies using recombinant maize proteins showed that a major difference between *C₃*-specific (non-photosynthetic) and *C₄*-specific NADP-MEs was found in the *Kₘ* value for NADP⁺, which was 70 and 8 µM for the *C₃*- and *C₄*-specific isozymes, respectively (*Saigo et al.*, 2004). The NADPH to (NADP⁺+NADPH) ratio in the chloroplast stroma ranges from 0.35 to 0.70, and is higher under illumination (*Fridlyand et al.*, 1998). It is likely that the *C₄*-specific isozyme’s high affinity for NADP⁺ allows it to sustain the reaction in the direction of decarboxylation of malate, even when the NADP⁺ concentration in the stroma is low under illumination. Transport of dicarboxylic acids across the chloroplast envelope of *C₃* leaves is mediated by antiporters that transport molecules along their concentration gradients (Bräutigam and Weber, 2011). Therefore, the consumption of the stromal malate by the maize enzyme leads to uptake of malate from the cytosol, though malate must be exported from the stroma as a carrier of reducing power under illumination (*Scheibe*, 2004).

Because of the detrimental effects of maize *C₄*-specific NADP-ME on rice plants, the rice *C₃*-specific isozyme was used for the introduction of the *C₄*-like pathway into rice, although the elevation of leaf NADP-ME activity was <5-fold that of non-transgenic rice (Tsuchida *et al.*, 2001). With the rice enzyme, care should be taken to shift its reaction equilibrium towards decarboxylation of malate, since a high NADPH to (NADP⁺+NADPH) ratio in the stroma under illumination promotes carbon fixation of pyruvate by the *C₃*-specific isozyme, whose affinity for NADP⁺ is low.

Studies of the NADP-ME gene family in *Arabidopsis* (*Gerrard Wheeler et al.*, 2005; *Brown et al.*, 2010) have demonstrated that, among the four isozymes, the cytosolic NADP-ME2 protein that is mainly located in leaf veins contributes >80% of total NADP-ME activity in the rosette leaves. The chloroplastic NADP-ME4, an orthologue of the *C₄*-specific enzyme, thus accounts for <20% of the total leaf activity of NADP-ME. In addition, the *NADP-ME4* gene is expressed in both MCs and veins. When cDNA for the chloroplastic isozyme is expressed under the control of the *Cab* promoter, the resulting 200% increase in leaf NADP-ME activity very likely corresponds to an increase of >1000% in the enzyme activity in the MC chloroplasts.

**Lesson 3: enzyme activities must be properly regulated**

The activity of *C₄*-specific PPDK is strictly regulated by light in *C₄* plants through protein phosphorylation by the PPDK regulatory protein, which catalyses both
phosphorylation and dephosphorylation (Burnell and Hatch, 1985). The activity of maize PPDK in rice leaves is regulated by light, as it is in C₄ leaves, and this is an indication that the rice PPDK regulatory protein recognizes and regulates the maize isozyme in the same way as the corresponding protein does in C₄ leaves (Fukayama et al., 2001; Taniguchi et al., 2008). The activity of the chloroplastic NADP-MDH is also strictly regulated by light in both C₃ and C₄ plants through the thioredoxin cascade (Migniac-Maslow et al., 2000). It was confirmed that the sorghum MDH overproduced in rice leaves was active only under illumination, although the activation by light proceeded more slowly than did the activation of endogenous MDH in non-transgenic rice (Taniguchi et al., 2008).

In contrast, maize PEPC and rice NADP-ME are active both in the light and in darkness. The activity of plant-type PEPC is regulated by two different but interactive mechanisms, one that functions through various metabolite effectors and another that involves reversible phosphorylation of a conserved serine residue at the N terminus (Vidal and Chollet, 1997). After phosphorylation, PEPC becomes less sensitive to its feedback inhibitor malate and more sensitive to the activator glucose 6-phosphate, thereby attaining higher activity (Vidal and Chollet, 1997). The activity of maize PEPC in rice leaves is regulated by protein phosphorylation, but in a manner opposite to that observed in C₄ leaves: it is phosphorylated at night and dephosphorylated during the middle part of the day, in the same way as the endogenous rice enzyme (Fukayama et al., 2003, 2006). This indicates that maize PEPC is more active at night than during the day. For chloroplastic NADP-ME, no particular regulation mechanisms have been proposed. It is likely that its activity and the direction of the reaction it catalyses are regulated by pH, substrate concentrations, and probably by the Mg²⁺ concentration in the chloroplast stroma. So far as the optimum pH is considered (7.8 for the maize non-photosynthetic isozyme; Saigo et al., 2004), NADP-ME is slightly more active in the light than in darkness.

It is desirable that the introduced enzymes be active only under illumination to minimize adverse metabolic effects. In fact, overproduction of maize PEPC and rice NADP-ME together leads to stunting of the rice plants (Fig. 3; Taniguchi et al., 2008). On this basis, it is hypothesized that the combination of PEPC with NADP-ME catalyses a futile pathway that wastes metabolic energy. Based on the phosphorylation pattern of maize PEPC, this pathway could be more active at night than during the day in rice leaves.

**Lesson 4: what is the key to driving the C₄-like pathway in rice MCs?**

To produce quadruple transformants that overproduced all four enzymes (PEPC, PPDK, NADP-MDH, and NADP-ME), transgenic lines that overproduced PEPC and PPDK together were first developed by means of conventional crossing, and then a gene construct for expression of sorghum NADP-MDH and rice NADP-ME cDNAs was introduced into the PEPC×PPDK line (Taniguchi et al., 2008). The leaf activities of PEPC and PPDK in the resulting quadruple transformants were therefore the same as in the PEPC×PPDK line (i.e. 40- to 50-fold and 4- to 5-fold the levels in the non-transformants, respectively). Unexpectedly, four homozygous lines selected from the quadruple transformants showed similar activity of NADP-MDH and NADP-ME, at 7- to 13-fold and 3- to 4-fold the levels in the non-transformants, respectively. As described above, the 400% increase in leaf NADP-ME activity corresponded to an increase to 2000% of the enzyme activity level in the MC chloroplasts.

These quadruple transformants showed a higher CO₂ assimilation rate than that of the original PEPC×PPDK cross (Taniguchi et al., 2008): the CO₂ assimilation rate, which had been lowered by the action of overproduced PEPC in the PEPC×PPDK line, was restored to levels comparable to or higher than those in non-transformants. Since triple transformants that overproduced three of the four enzymes (but not NADP-MDH) did not show enhanced CO₂ assimilation, the increased NADP-MDH activity appears to be the key to the enhanced CO₂ assimilation. This finding was unexpected, since there was no previous report of significant elevation of NADP-MDH activity as a result of a shift from C₃ to C₄ photosynthesis in Hydrilla, and it had been generally believed that the activity of NADP-MDH in C₃ MC chloroplasts was high enough for operation of the C₄-like pathway; the NADP-MDH activity was an order of magnitude higher than those of the
other three enzymes in the leaves of non-transgenic rice (Taniguchi et al., 2008). As described in Lesson 2, it is necessary to shift the equilibrium of the rice C₄-specific NADP-ME towards decarboxylation of malate under illumination. It is likely that NADP-MDH supports the decarboxylation reaction of NADP-ME by lowering the NADPH to (NADP⁺+NADPH) ratio in the stroma and by facilitating the import of OAA into the chloroplasts. Further elevation of NADP-MDH activity is expected to improve CO₂ assimilation by the quadruple transformatants.

The use of other NADP-ME isozymes with different kinetic properties might be another choice, but it is important to couple reactions by NADP-MDH and NADP-ME so as to avoid raising the stromal NADPH level.

In addition to the importance of increased NADP-MDH activity, there is no information available on the relative abundance of introduced enzymes required for operation of the C₄-like pathway. Based on the absolute activities of the introduced enzymes in leaf extracts from the quadruple transformatants (i.e. 3, 0.1, and 2–3 μmol mg⁻¹ protein min⁻¹ for PEPC, PPDK, and NADP-MDH, respectively; Taniguchi et al., 2008), PPDK activity must be further increased to drive a closed cycle. This cannot be achieved simply by increasing the PPDK protein level because of limitations on driving a closed cycle. This cannot be achieved simply by overproduction of both PEPC and NADP-ME (Lesson 3).

Other points to be considered are the kinetic properties of the introduced enzymes and PEP transport activity. At present, no experimental data are available to enable discussion of these points. A mathematical modeling and systems biology approach, rather than trial and error experiments, might be a more productive way to clarify these points.

**Lesson 5: what has been learned from rice**

Rice has been used as a model crop species in plant genomics. However, it was found that it is unique in its physiology and primary metabolism among the plant materials generally used for molecular biology and physiology research. One important difference is that rice plants preferentially use ammonium (NH₄⁺) and thrive better with NH₄⁺ than with nitrate (NO₃⁻) as their nitrogen source. Many upland crop species, such as wheat, barley (Hordeum vulgare L.), tobacco (Nicotiana tabacum L.), tomato (Lycopersicon esculentum L.), and spinach (Spinacea oleracea L.) preferentially use NO₃⁻. The waterlogged soil in paddy fields is a reducing environment, so it is therefore reasonable that rice would have evolved or been bred for adaptation to such a habitat by acquiring mechanisms for efficient utilization of NH₄⁺.

A peculiar feature that was identified for the first time was phosphorylation of PEPC at night in rice leaves (Fukayama et al., 2003). One of three rice PEPC kinases (OsPPCK3) is responsible for this nocturnal phosphorylation (Fukayama et al., 2006). It had long been considered that PEPC was phosphorylated only during the daytime in both C₃ and C₄ plants. Among the 30 plant species tested, however, PEPC was phosphorylated during the daytime in only 12 species (Fukayama et al., 2006). It was later found that the extent of the phosphorylation at night in rice leaves depended on the nitrogen source, and became less marked when rice plants were grown with NH₄⁺ (Fig. 4). Similar effects of the nitrogen source were reported for PEPC in pea (Pisum sativum L.) leaves, in which diurnal oscillation of the PEPC activity (high in the daytime and low at night) disappeared when plants grown with NO₃⁻ were transferred into a medium containing NH₄⁺ as the nitrogen source (Leport et al., 1996). By analysing the expression pattern of OsPPCK3 in rice plants grown in soil (data not shown), it was recently realized that a commercial soil mixture used was rich in NO₃⁻ even when waterlogged. This finding was surprising, but it is very important for introduction of the C₄-like pathway, since it suggests that the stunting caused by overproduction of both PEPC and NADP-ME (Lesson 6)
3) could be largely mitigated when rice plants are grown only with NH₄⁺. The nocturnal phosphorylation of PEPC was also observed in two hydrophytic weeds that are commonly found in paddy fields (Fukayama et al., 2006). It is therefore likely that the nocturnal phosphorylation plays some role in adaptation to waterlogged soil.

Another recently discovered unique feature is that rice has PEPC intrinsically located in the chloroplast, Osppc4 (Masumoto et al., 2010). The Osppc4 gene shows a high expression level in leaf MCs, and the Osppc4 protein accounts for about one-third of the total PEPC protein in the leaf blades. Genes encoding the chloroplastic PEPC have been found in cultivated and wild Oryza species, all of which are adapted to waterlogged soil. It is suggested that, in addition to glycolysis, the genus Oryza has a unique route to provide organic acids for NH₄⁺ assimilation that involves the chloroplastic PEPC, and that this route is crucial for growth with NH₄⁺ as the nitrogen source (Masumoto et al., 2010). The activity of the chloroplastic PEPC in leaf extracts of non-transgenic rice is between 0.02 and 0.03 μmol mg⁻¹ protein min⁻¹ (Masumoto et al., 2010). Although this value is lower than the activities of the introduced enzymes in the quadruple transformants (see Lesson 4), the endogenous PEPC inside the chloroplasts may shortcut the C₄-like pathway in rice MCs.

It thus appears that both the carbon and nitrogen metabolism of rice plants differ from those of upland plant species. For metabolic engineering to succeed, it will be necessary to understand these aspects of the basic metabolism of a target plant species. Unfortunately, the primary metabolism of rice plants has not yet been completely described.

The last point to be mentioned is technical, and involves how to minimize genome alterations (somaclonal variation) that can occur during the course of cell cultivation and the regeneration of plants from the cultured cells (Labra et al., 2001). It appears that somaclonal variation is inevitable when calli are used for transformation. Unfortunately, all procedures for rice transformation that are presently available use calli induced from mature seeds (Hiei et al., 1994; Toki et al., 2006), and methods similar to the floral dip transformation that is used for Arabidopsis are unavailable. To minimize genome alterations, researchers should avoid prolonged cell cultivation to prevent accumulation of undesirable phenotypes, such as stunting and morphological abnormalities, in multiple transformants. Finding an alternative to rice callus transformation would also represent a significant breakthrough in this context.

Lesson summary

The transport activity of PEP across the chloroplast envelope appears to be the most critical issue for the operation of the single-cell C₄-like pathway in rice. However, the problems that actually arose during the course of these studies of transgenic rice plants were the import of OAA into the chloroplasts and the direction of the NADP-

ME reaction. The importance of NADP-MDH was also recognized only after the initial series of experiments had been completed. This is not the only case in which transgenic plants have shown unexpected phenotypes, leading to the discovery of novel regulatory mechanisms and metabolic interactions that would not have been discovered using traditional physiological and biochemical approaches. The transgenic approach is therefore a powerful tool for basic research in plant science. These attempts to introduce the C₄-like pathway in rice have also deepened the understanding of the physiology of this species. There is little doubt that such an approach will contribute greatly in future towards the metabolic engineering of rice plants.

For engineering a single-cell C₄-like pathway into rice, the presence of the endogenous PEPC inside the MC chloroplasts has emerged as a novel problem that must be addressed. At the same time, strategies to improve the quadruple transformants have been revealed by this research. Among them, further elevation of NADP-MDH activity seems to be the most promising approach. To answer the question of whether a single-cell C₄-like pathway can improve the photosynthetic performance of rice, additional step by step research informed by the lessons described in this article will be required.

Acknowledgements

The authors are grateful to Professor Osamu Ueno, Kyushu University, Japan, for performing the immunoelectron microscopy analysis. This work was supported in part by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics for Agricultural Innovation, GPN00006) to M.M.

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