Transcriptional activation of phosphoenolpyruvate carboxylase by phosphorus deficiency in tobacco

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Abstract

Phosphoenolpyruvate carboxylase (PEPC), which catalyses the carboxylation of phosphoenolpyruvate using HCO₃⁻ to generate oxaloacetic acid, is an important enzyme in the primary metabolism of plants. Although the PEPC genes (ppc) comprise only a small gene family, the function of each gene is not clear, except for roles in C₄ photosynthesis and CAM. Three PEPC genes (Nsppc1–3) from the C₃ plant Nicotiana sylvestris were used to investigate their roles and regulation in a C₃ plant, and their regulation by phosphorus depletion in particular. First, the induction of PEPC by phosphorus depletion was confirmed. Next, Nsppc1 was determined to be mainly responsive to phosphorus deficiency at the transcriptional level. Further studies using transgenic tobacco harbouring a chimeric gene consisting of the 2.0 kb promoter region of Nsppc1 and the β-glucuronidase (GUS) reporter showed that PEPC is transcriptionally induced. It was also found that sucrose had a synergistic effect on the induction of PEPC by phosphorus deficiency. A series of transgenic tobacco containing 5¢-deletion mutants of Nsppc1 promoter::GUS fusion revealed that the –539 to –442 bp region confirmed that this region was sufficient to induce the phosphorus-deficiency response in tobacco.

Key words: Cis-acting element, Nicotiana tabacum, PEPC, phosphoenolpyruvate carboxylase, phosphorus deficiency, sucrose effect, tobacco, transcriptional regulation.

Introduction

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) catalyses the carboxylation of phosphoenolpyruvate (PEP) using HCO₃⁻ to generate oxaloacetic acid (OAA) and release Pi (Lepiniec et al., 1994; Chollet et al., 1996). Whereas PEPC plays an important role in C₄ and CAM plants as the first photosynthetic carbon-fixing enzyme, it is also commonly found in bacteria, protozoa and all plants, and is considered to play an essential role in the anaplerotic reaction to replenish C₄-dicarboxylic acids to the TCA cycle (O’Leary, 1982). In addition, various physiological roles, such as regulation of pH and osmotic pressure, regeneration of NADPH, regulation of stomatal movement, and nitrogen assimilation in roots and root nodules have been postulated for C₃ PEPC in higher plants (Latzko and Kelly, 1983). Therefore, the expression of C₃ PEPC is expected to be fairly uniform and constitutive, except for possible specific expression in some cell types.
such as guard cells and spikelets (Imaizumi et al., 1990; Michalke and Schnabl, 1990).

Recently, the expression of C3 PEPC has been shown to be activated by phosphorus deficiency in roots (Lipton et al., 1987; Pilbeam et al., 1993; Johnson et al., 1994, 1996a, b), leaves (Hoffland et al., 1992; Pilbeam et al., 1993), cultured cells (Duff et al., 1989; Nagano and Ashihara, 1993; Moraes and Plaxton, 2000), and green algae (Theodorou et al., 1991). Under phosphorus-deficient conditions, plants generally exude a greater quantity of organic acids such as citrate, malate, and succinate from roots to the rhizosphere (for review, see Raghothama, 1999). These organic acids are synthesized via C3 PEPC. This exudation of organic acids, which acidifies the rhizosphere and chelates metal ions, is thought to make plants more effective in taking up sparingly available phosphate in the soil. Therefore, it would be reasonable to expect that the expression of C3 PEPC is activated in roots under phosphorus deficiency, as seen in the roots of white lupins (Johnson et al., 1994; Neuman et al., 1999). It is possible that the exuded organic acids are synthesized not only in roots but also in leaves. Whereas C3 PEPC in white lupins is activated predominantly in roots and very slightly in leaves under phosphorus deficiency, C3 PEPC in rape plants is activated mainly in leaves, and organic acids produced in leaves are transported to the roots (Hoffland et al., 1992). In either case, organic acids like citrate and malate are synthesized to enable the plant to survive under phosphorus deficiency.

In plants, phosphorus deficiency also induces changes in metabolism. Plaxton and co-workers have proposed a ‘bypass’ model to describe metabolic changes in the glycolytic pathway (Duff et al., 1989; Plaxton, 1996). During phosphorus deficiency, some enzymes are up-regulated to release Pi, which is used for the efficient recycling of phosphate in plants, and to maintain glycolytic activity without producing ATP. Thus, C3 PEPC acts as an alternative to pyruvate kinase, in concert with malate dehydrogenase in the cytosol.

These results suggest that C3 PEPC has significant functional roles under phosphorus deficiency. While PEPC activity has been reported to be induced under phosphorus deficiency as described above, the exact mechanism of the regulation of C3 PEPC gene expression by phosphorus deficiency has not yet been clarified, due to the difficulty in dealing with the constitutive and low level expression of C3 PEPC. The genome structure of Nicotiana sylvestris, an ancestor of common tobacco, has been studied and it was found that this plant species has only three copies of the PEPC genes (Nsppec1–3) (Koizumi et al., 2002). Further characterization of the expression of Nsppec1–3 indicated that among these three Nsppe genes, Nsppec1 was the main gene that was expressed in both in phosphate-supplied and phosphate-depleted plants. Using this advantage, an Nsppec1 promoter::GUS fusion was constructed and its expression under phosphorus deficiency and the cis-element needed for phosphorus-deficiency response was characterized. Since sucrose was found to have a synergistic effect on the induction of Nsppe gene expression under phosphorus deficiency, the relationship between phosphorus deficiency and sucrose is also discussed.

**Materials and methods**

**Plant material and culture conditions**

Seeds of tobacco (Nicotiana tabacum cv. Samsun NN) were planted on a half concentration of Linsmaier–Skog agar media (Linsmaier and Skoog, 1965) with/without 2% sucrose under continuous illumination (60 μE m−2 s−1) at 25±2 °C. Eight-day-old seedlings to be used in the study were grown in ‘Agri®ots’ (transparent culture vessels, 10 cm height and 6 cm in diameter; Kirin, Japan). For phosphorus-deficiency treatment, the seeds were planted directly on KH2PO4-free medium and whole 8-d-old seedlings grown on the medium were used. KCl was added to compensate for the loss of K in KH2PO4-free medium.

**PEPC and protein assay**

About 30 seedlings (per assay) of tobacco were homogenized in 1 ml of extraction buffer (50 mM TRIS-Cl, pH 7.5, 10 mM MgCl2, 10 mM sodium ascorbate, 10 mM 2-mercaptoethanol) using an ice-cold mortar and a pestle with a small amount of sea sand. The homogenates were centrifuged at 20 000 g for 30 min at 4 °C. The supernatant was desalted with a NAP-5 column equilibrated with a column buffer (10 mM TRIS-Cl, pH 7.5, 5 mM MgCl2, 5 mM 2-mercaptoethanol) at 4 °C and then used as a crude extract for the following assay. PEPC activity was determined spectrophotometrically by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 30 °C. The standard assay solution contained 50 mM TRIS-HCl, pH 8.5, 20 mM MgCl2, 10 mM NaHCO3, 0.1 mM NADH, 3 mM PEP, malate dehydrogenase (5U, Oriental Yeast Co., Ltd., Japan), and 100 μl of PEPC crude extract in a total volume of 1 ml. The reaction was initiated by adding PEP after about 5 min of preincubation. One unit of PEPC activity was defined as the amount that catalysed the oxidation of 1 μmol NADH min−1 at 30 °C. The protein concentration was determined by the procedure of Bradford (1976) using a protein assay reagent (Bio Rad) and bovine serum albumin as a standard.

**Immunoblot analysis**

Proteins extracted from tobacco seedlings were used for immunoblot analysis of the PEPC assay. Equal amounts (3 μg) of proteins were subjected to SDS-PAGE on 10% polyacrylamide gels containing 0.1% SDS. After electrophoresis, proteins were transferred from the gel to a PVDF membrane by electroblotting. Membranes were blocked in TBS (20 mM TRIS-HCl, pH 6.8, 500 mM NaCl) containing 3% gelatin, and PEPC bands were immunochemically labelled with polyclonal antibodies against tobacco PEPC (Koizumi, 1993). Subsequent detection was performed by incubation with secondary antibodies (goat anti-rabbit IgG conjugating horseradish peroxidase, Bio-Rad) using a Renaissance® kit (NU™ Life Science Products, USA).

**Northern blot analysis**

Whole 8-d-old tobacco seedlings were collected and total RNA was extracted using the Plant RNeasy Mini Kit (Qiagen, CA). Ten micrograms of total RNA was separated on a formaldehyde-agarose gel, and transferred to Hybond N+ (Amersham Pharmacia) according
to the standard procedure (Sambrook et al., 1989). The membrane was hybridized with the full-length tobacco PEPC cDNA (Koizumi et al., 1991) probe.

**RT-PCR analysis**

Total RNA was isolated from whole 8-d-old *Nicotiana sylvestris* seedlings using the Plant RNeasy Mini Kit (Qiagen, CA). For phosphorus-deficiency-induced material, 8-d-old seedlings planted and grown on phosphorus-free medium were used, as described above. Approximately 10 μg RNA was reverse-transcribed, and then PCR-amplified (30 cycles) using primers 1 and 2, i.e. 5'-AGGCCGTCCATCAAGGGTAAAAC-3' and 5'-TCCAGCAGAATACCCCTTCCAT-3', respectively. These primers 1 and 2 were designed to hybridize at the last two exons of genes *Nsppc1*, 2 and 3 (Koizumi, 1993) and to distinguish PCR products for each *Nsppc* gene transcript by PsI digestion. PCR products were digested with PsI and transferred to a nylon membrane to detect the expression level of each gene using 5'-GATTGCTGGACACAAGGAG-3' as the probe. Three *Nsppc* gene transcripts were distinguished by the restriction fragment length because of the difference in the PsI site among the three *Nsppc* genes.

**Construction of the *Nsppc1* promoter::GUS fusion and 5' deletion clones**

A plasmid of about 6 kb including the promoter region and the first exon of the *Nsppc1* in pBlueScript II SK* (Stratagene, USA) between the EcoRI and XhoI sites was used. A promoter region of about 2.0 kb, with a coding region for the first 20 amino acids of the *Nsppc1* gene, was subcloned into plUC 19 between the SalI and KpnI sites to generate L2SK. After modification of the KpnI site into the BamHI site by linker ligation, the 2.0 kb promoter sequence was inserted into pBI101.3 (Clontech, CA, USA) between the SalI and BamHI sites to generate the translationally fused *Nsppc1* promoter::GUS construct. The identity of the nucleotide sequence was confirmed by sequencing. A series of 5'-deleted mutants of the 2.0 kb *Nsppc1* promoter was prepared by partial exonuclease III treatment. The deletion products were ligated into pBI101.3 as described above to produce 5'-deleted mutants of *Nsppc1* promoter::GUS fusion.

**Construction of the plasmids for gain-of-function analysis**

Firstly, three tandem repeats of the –539 to –442 bp region of the *Nsppc1* promoter were ligated into pBlueScript II SK* vector as follows. Using six primers designed to have a restriction site at its end, the sequences of primer pairs were 5'-AATATTAAGCTTAC-3', 5'-AGGCCATGAAGGGTAAATC-3' and 5'-TGTAATTCAGAGCAGTAAATCAGAC-3'. The identity of the nucleotide sequence was confirmed by sequencing.

**Results**

**Phosphorus deficiency increased PEPC activity and its protein amount in tobacco**

Before the molecular biological characterization of *ppc* genes was started, PEPC responses under phosphorus deficiency were examined. Since phosphorus is an essential element for plant growth and is stored in large amounts, *in vitro*-cultured seedlings were used to examine the effect of phosphorus deficiency. After a preliminary investigation, germinating seedlings showed the clearest...
responses to phosphorus deficiency, as seen in Fig. 1; phosphorus deficiency increased PEPC activity. The addition of sucrose to the medium showed that sucrose increased PEPC activity, especially under phosphorus deficiency. Since the level of PEPC activation under phosphorus deficiency in the presence of 2% sucrose was the greatest among the combination of phosphorus deficiency and sucrose treatment, the effects of phosphorus deficiency and sucrose were considered to be synergistic. Immunoblot analysis (Fig. 1) indicated that the increase in PEPC activity was due to an increase in the amount of protein, suggesting possible transcriptional activation.

**Phosphorus deficiency increased PEPC transcript**

To examine the transcriptional activation of PEPC in tobacco, Northern blotting was carried out under the same conditions as in Fig. 1. As shown in Fig. 2, the PEPC transcript level was suggested to be increased by sucrose addition and phosphorus deficiency, although the bands were somewhat difficult to distinguish because of the low expression of PEPC. Thus, it was decided to investigate the transcriptional regulation of PEPC under phosphorus deficiency with transgenic tobacco harbouring the promoter::GUS fusion.

**Among three*** Nsppc ***genes, Nsppc1 was responsible for phosphorus deficiency***

To investigate whether or not the increase in PEPC under phosphorus deficiency is regulated by gene activation, ppc gene expression was characterized in *N. sylvestris*, which is an ancestor of *N. tabacum*, and from which three *Nsppc* genes were previously isolated (Koizumi et al., 2002). Although *Nsppc1*–3 have a quite similar genome structures and sequences, they show some restriction polymorphism. Thus, this restriction enzyme polymorphism was used to identify the gene(s) expressed in *N. sylvestris*; *PstI* digestion of PCR-amplified fragments with specially designed primers 1 and 2 provided (280*+270) bp, (460*+90) bp and 550* bp fragments for *ppc1*, 2 and 3 transcripts, respectively. A specific probe was also used to identify the fragments with asterisks easily. Analysis of mRNAs isolated from 8-d-old seedlings grown on medium with/without phosphate clearly indicated that *Nsppc1* was the predominant gene expressed in tobacco under both phosphate-sufficient and -deficient conditions (Fig. 3).

**Construction of the Nsppc1 promoter::GUS fusion gene and its expression in transgenic tobacco under phosphorus deficiency**

Since *Nsppc1* was predominantly expressed in leaves, stems, roots, and cultured cells (Koizumi et al., 2002), expression of the *Nsppc1* gene was investigated further. An expression vector with a 2.0 kb promoter region of the *Nsppc1* gene was fused with the β-glucoronidase (GUS) gene and introduced into tobacco, since *N. sylvestris* and *N. tabacum* were speculated to show similar responses to phosphorus deficiency (Ren and Timko, 2001). Transgenic tobacco was selected on kanamycin-containing medium and transgene expression was confirmed by PCR. Several transgenic lines were self-pollinated, and T1 seedlings were used for further analysis. Among the transgenic tobacco plants produced, three lines with a single copy of transgene were selected based on the separation ratio (kanamycin-resistant versus kanamycin-sensitive) of 75%. These lines showed high GUS activity under phosphorus deficiency (data not shown). Examination of the effect of
phosphorus deficiency on GUS expression in transgenic tobacco carrying the *Nsppc1* promoter::GUS fusion construct clearly showed that GUS gene expression was induced by phosphorus deficiency under both sucrose-supplemented and sucrose-free conditions (Fig. 4). This experiment also confirmed that sucrose had a synergistic effect with phosphorus deficiency on the expression of the *ppc* gene. These results suggest that PEPC gene expression is transcriptionally regulated by phosphorus deficiency and sucrose.

**Characterization of the promoter sequence that responds to phosphorus deficiency**

A series of 5′-deleted mutants of *Nsppc1* promoter::GUS fusion constructs was constructed and introduced into tobacco to investigate the sequence that responds to phosphorus deficiency. GUS activities of T1 seedlings grown under phosphorus-sufficient/deficient conditions indicated that neither the 442 bp, 400 bp nor 265 bp promoter sequence was sufficient for the induction of GUS activity under phosphorus deficiency (Fig. 5). On the other hand, the 539 bp promoter sequence gave a clear response. This result suggests that the promoter region between ±539 bp and ±442 bp relative to the translation start site was essential for the phosphorus-deficiency response. To examine the importance of this sequence in response to phosphorus deficiency, a gain-of-function analysis using four expression vectors (−819 bp *Nsppc1* promoter::GUS as a positive control, gain-of-function plasmid, CaMV 35S minimal promoter::GUS as a negative control, and CaMV 35S promoter::luciferase plasmid as a bombardment control) was conducted (Fig. 6). The gain-of-function plasmid was constructed to have three tandem repeats of the ±539 bp to ±442 bp promoter region of *Nsppc1* fused to the CaMV 35S minimal promoter (−50). These plasmids were introduced into tobacco seedlings grown on phosphorus-deficient or phosphorus-supplemented medium containing 2% sucrose by particle bombardment and GUS activities were measured in comparison with luciferase activity as a reference in microbombardment. Gain-of-function plasmid showed higher activity in phosphorus-deficient seedlings than phosphorus-supplemented seedlings. While the increase in GUS activity by the gain-of-function plasmid was not large due to inefficient tissue transformation in the transient assay, it was still significantly higher than that with the positive control plasmid, and this increase was observed repeatedly in five to eight experiments. The \( t \)-values of the difference between +P and −P with the three conditions were calculated as 1.99, 4.25 and 1.04, respectively, and corresponding \( P \)-values were 0.077, 0.0029 and 0.317, respectively. The statistical values

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**Fig. 3.** RT-PCR analysis of three *Nsppc* genes in *Nicotiana sylvestris* with/without phosphorus. (A) Primers 1 and 2 were designed from around the last exon in the three *Nsppc* genes. (B) PCR-amplified fragments were digested with *Pst* I and fractionated by an agarose gel. A probe was used to distinguish the three *Nsppc* genes. Based on the variation of *Pst* I sites, 280 bp, 460 bp and 550 bp fragments should be detected if *Nsppc1*, *Nsppc2* and *Nsppc3* are expressed, respectively.

**Fig. 4.** Phosphorus deficiency and the addition of sucrose increased GUS expression in transgenic tobacco harbouring a 2 kb *Nsppc1* promoter::GUS chimeric gene. T1 seedlings grown on phosphorus-sufficient/deficient medium with/without 2% sucrose were used, as described in Materials and methods. GUS activities were calculated from three independent lines to obtain the mean values of three replicates. The bars indicate standard error of nine replicates.
calculated prove the responsiveness of the promoter element to phosphorus deficiency.

**Discussion**

Whereas C₃ PEPC is regulated by several factors, PEPC activity and the amount of its protein were both confirmed to be increased in tobacco seedlings under phosphorus-depleted conditions, as reported in other plant species (Lipton et al., 1987; Duff et al., 1989; Theodorou et al., 1991; Hoffland et al., 1992; Nagano and Ashihara, 1993; Pilbeam et al., 1993; Johnson et al., 1994, 1996a, b; Moraes and Plaxton, 2000). This investigation further clarified that this phosphorus-deficiency-induced PEPC activation was transcriptionally regulated, whereas C₄ PEPC in maize is decreased under phosphorus deficiency (Usuda and Shimogawara, 1991). This different regulation of the ppc gene by phosphorus deficiency may be due to the different functions of C₃ and C₄ PEPC, i.e. house-keeping versus photosynthetic functions. However, the induction of PEPC in tobacco occurred rather slowly under phosphorus deficiency. The induction of PEPC activity and

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**Fig. 5.** (A) Loss-of-function assay of PEPC promoter activity under phosphorus deficiency. 5'-Deleted mutants of Nsppc1 promoter::GUS chimeric genes (2 kb, −673 bp, −539 bp, −442 bp, −400 bp, and −265 bp) were introduced into tobacco (N. tabacum) and the GUS activities of these T₁ seedlings were measured under phosphorus deficiency/sufficiency with 2% sucrose. The numbers indicate the average of the fold of activation in three lines and the standard error (SE) with three replicates. (B) Nucleotide sequence of Nsppc1 promoter (−539 to +36). Putative DNA binding motifs, Dof protein binding domain (ACTTTA, Baumann et al., 1999) and a similar sequence of the phosphate-response domain of the VspB gene (CATTATTAG, Tang et al., 2001) are boxed. The nucleotide position is numbered with regard to the putative translation initiation codon (ATG) which is bold.
transcript was only obvious after 8 d of culture under phosphorus deficiency. Plants first respond to phosphorus deficiency by the induction of Pi transporter and acid phosphatase to stimulate Pi uptake by the roots. PEPC is then induced as the second step in the adaptation to phosphorus deficiency. This induction of PEPC by phosphorus deficiency in tobacco seedlings was observed in both aerial parts and roots (data not shown), suggesting that tobacco PEPC might be involved not only in organic acid excretion from the roots, but also in the reallocation of Pi during glycolysis, as proposed by Plaxton (1996), or the aerial parts might be involved in the excretion of organic acids in the roots.

The response of PEPC to Pi depletion was clear in seedlings. Tobacco plants grown hydroponically showed a very slight response to phosphorus deficiency. It is speculated that seedlings require more phosphate for rapid cell proliferation, and express more PEPC. Another reason for the lower response in developed plants is that germinating seedlings may suffer a more rapid depletion of Pi on medium without phosphate because of a limited availability of stored Pi. On the other hand, well-developed plant cells accumulate a high percentage of total phosphorus (up to 80%) in the vacuole (Schachtman et al., 1998), and this stored phosphorus may be used during phosphorus deficiency (Mimura, 1995). Interestingly, the addition of sucrose synergistically induced PEPC activity and transcription under Pi depletion, while sucrose itself also had a PEPC-inducing activity. Whereas one of the mechanisms of PEPC induction may be depletion of the intracellular phosphorus level (Sadka et al., 1994), the synergistic effects of sucrose and phosphorus suggested that sucrose itself has a different mechanism for Nsppc1 induction. However, a preliminary experiment failed to identify whether or not the sucrose-responsive element and the phosphorus-depletion element were the same.

Determination of the phosphorus-depletion-responsive element in transgenic tobacco as well as the gain-of-function experiment suggested that the promoter sequence between −539 to −442 bp is necessary and sufficient for the phosphorus-deficiency response. This nucleotide sequence from −539 to −442 bp is rich in A and T (Fig. 5), and contains a putative transcription factor binding site, i.e. Dof protein binding site (ACTTTA). Maize Dof protein is a transcriptional regulator that is involved in tissue-specific and light-regulated gene expression of the C_4 PEPC gene (Yanagisawa and Sheen, 1998). Since C_4 PEPC responds differently to phosphorus deficiency than C_3 PEPC (Usuda and Shimogawara, 1991), the importance of Dof protein in the response to phosphorus deficiency should be examined carefully. The existence of the ACTTTA sequence in the rolB oncogene promoter in N. tabacam as the binding site of NtBBF1 and the involvement of tissue-specific and auxin-regulated expression suggest that different types of Dof proteins could be found in C_3 plants (Kim et al., 1999).

On the other hand, the sequence CATTATTAG has recently been identified as the first cis-acting element in the phosphate-responsive VspB gene promoter in soybean
was also found in tomato and rape (Hofand et al). The plasmid constructed here may be useful for dissecting the Nsppc1 gene. Determination of the signal transduction for microbombardment and Pi-depleted culture of seedlings. A similar induction of Nsppc1 was induced under phosphorus deficiency in both shoots and roots (data not shown), which enabled a transient assay using microbombardment. A similar induction of ppc in whole plants was also found in tomato and rape (Hofand et al., 1992; Pilbeam et al., 1993), whereas PEPC activity in lupin was increased only in roots, suggesting that the regulation of transcriptional factor(s) might be different among plant species. Furthermore, phosphorus deficiency is modulated by several factors. Cytokinins, as well as sucrose, have been reported to regulate the phosphate responsiveness in Arabidopsis (Martin et al., 2000). The deletion expression plasmid constructed here may be useful for dissecting the regulatory mechanism of ppc in combination with microbombardment and Pi-depleted culture of seedlings. Determination of the signal transduction for ppc should contribute to the understanding of the complicated physiological function of ppc genes.

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References


