Coordinate Regulation of Phosphoenolpyruvate Carboxylase and Phosphoenolpyruvate Carboxykinase by Light and CO₂ during C₄ Photosynthesis[^OA]

Karen J. Bailey, Julie E. Gray, Robert P. Walker, and Richard C. Leegood*

Robert Hill Institute and Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, United Kingdom

The aim of this study was to investigate the relationship between the phosphorylation and activation states of phosphoenolpyruvate carboxylase (PEPC) and to investigate how the phosphorylation states of PEPC and phosphoenolpyruvate carboxykinase (PEPCK) are coordinated in response to light intensity and CO₂ concentration during photosynthesis in leaves of the C₄ plant Guinea grass (*Panicum maximum*). There was a linear, reciprocal relationship between the phosphorylation state of PEPC and its activation state, determined in a selective assay that distinguishes phosphorylated from nonphosphorylated forms of the enzyme. At high photon flux density and high CO₂ (750 μmol quanta m⁻² s⁻¹) were reached. After illumination at lower light intensities and CO₂ concentrations, the overall change in phosphorylation state was smaller and it took longer for the change in phosphorylation state to occur. Phosphorylation states of PEPC and PEPCK showed a strikingly similar, but inverse, pattern in relation to changes in light and CO₂.

Phosphorylation of PEPC appears to be regulated solely by transcriptional control and protein turnover (Nimmo, 2003; Izui et al., 2004). Changes in PEPCK phosphorylation state lead to diurnal changes in its sensitivity to regulation by adenylates, which are likely to lead to its activation in illuminated leaves (Walker and Leegood, 1996). Other than diurnal measurements of its activation state (Walker et al., 2002), it is not known

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how the activation state of PEPCK is related to changes in the phosphorylation state or how changes in CO₂ concentration and light intensity influence the activation state of PEPCK in the leaves of C₄ plants.

A major deficiency in our understanding of the regulation of C₄ photosynthesis is how carboxylation processes in the mesophyll and decarboxylation processes in the bundle sheath are coordinated. PEPC and PEPCK are both cytoplasmic enzymes that are active in the light. In some C₄ plants, such as Guinea grass, light activation of PEPC occurs by phosphorylation and light activation of PEPCK occurs by dephosphorylation. The aim of this study was to investigate whether the phosphorylation states of PEPC and PEPCK are coordinated in response to light intensity and CO₂ concentration during photosynthesis in leaves of Guinea grass.

RESULTS

Sequence Analysis of Guinea Grass PEPCK

PEPCK cDNA from Guinea grass was sequenced (GenBank accession no. AAQ10076). The molecular mass predicted from the amino acid sequence is 70,682 D. Figure 1 shows a comparison of the derived N-terminal regulatory sequence with those of U. panicoides (GenBank accession no. S52988), tomato (AAQ10078), and cucumber (S52637). Black shading indicates identical residues; gray shading indicates similarity between residues. The putative phosphorylated Ser is indicated by ▼.

Effect of Light and CO₂ on Phosphorylation State of PEPC and PEPCK

The phosphorylation states of PEPC and PEPCK were measured by illuminating leaves supplied with [³²P]inorganic phosphate (Pi). PEPC (103 kD) and PEPCK (71 kD) proteins were identified on gels by immunoblotting, which were then compared with autoradiographs (Fig. 2A). There were reciprocal changes in the phosphorylation of PEPC, which increased during illumination, and the phosphorylation of PEPCK, which decreased during illumination (Fig. 2, A and B). The bands on the autoradiographs were then quantified by densitometry.

The relationship between the phosphorylation states of PEPC and PEPCK and the rate of photosynthesis in relation to light intensity is shown in Figure 3. Phosphorylation of PEPC and PEPCK showed a similar, but inverse, pattern (Fig. 3C). Like the rate of CO₂ assimilation (Fig. 3A), the phosphorylation state of both enzymes did not saturate until high photon flux densities (PFDs; about 1,400 µmol quanta m⁻² s⁻¹) were reached. The relative activity (activation state) of PEPCK was tomato (Lycopersicon esculentum), in which PEPCK is known to be phosphorylated. Like the cucumber and tomato enzymes, PEPCK from Guinea grass contains a similar sequence (RKRS, residues 40–43), including a putative target Ser (indicated by the arrow) that is recognized by plant Ca²⁺-dependent protein kinase and mammalian cAMP-dependent protein kinases, but absent from PEPCK from U. panicoides (Leegood and Walker, 2003).
determined in the carboxylation direction, but at a low concentration of PEP and by adding ATP, in addition to ADP, which distinguishes phosphorylated from nonphosphorylated forms of the enzyme (Walker et al., 2002). There was a linear, reciprocal relationship between the phosphorylation state and the activation state of PEPCK (Fig. 3B).

The effects of illumination at three different light intensities on the phosphorylation states of PEPC and PEPCK are shown in Figure 4. During the preceding dark pretreatment, PEPC was dephosphorylated and PEPCK was phosphorylated. The phosphorylation state at the end of the dark period was taken as the reference point for determination of the relative phosphorylation state. At high PFD, PEPC was maximally phosphorylated and PEPCK maximally dephosphorylated within 1 h of illumination. At lower PFDs, the overall change in phosphorylation state was smaller and it took longer for the change in phosphorylation state to occur.

The effects of illumination at three CO₂ concentrations on the phosphorylation states of PEPC and PEPCK are shown in Figure 5. As for Figure 4, PEPC was dephosphorylated and PEPCK was phosphorylated during the preceding dark pretreatment. At a high CO₂ concentration (750 µL L⁻¹, approximately twice ambient CO₂ concentration), PEPC was maximally phosphorylated within 1 h of illumination and PEPCK maximally dephosphorylated within 3 h. At ambient (378 ppm) and below ambient (83 ppm) CO₂, the overall change in phosphorylation state of both enzymes was smaller and it took longer for the change in phosphorylation state to occur. After illumination at different CO₂ concentrations for differing periods, the PEPCK activation state showed a similar inverse relationship to the phosphorylation state as seen in Figure 3.

Influence of Dithiothreitol and Inhibitors on Phosphorylation and Activation States of PEPC and PEPCK

The effects of dithiothreitol (DTT) and inhibitors on the phosphorylation state and activation states of PEPC and PEPCK were investigated by feeding them directly to the cut ends of leaves via the transpiration stream (Table I). This means that they were supplied directly to the bundle-sheath cells from the xylem. Access to the mesophyll cells would then occur after diffusion through the bundle-sheath cells. The phosphorylation state is expressed as a percentage of the maximum in the water control in the dark for PEPCK or in the light for PEPC. The activation state of PEPC was measured by comparing its activity in the presence and absence of 1 mM malate, which inhibits the dephosphorylated, dark form of the enzyme to a greater degree (Echevarria et al., 1994).

Okadaic acid, which inhibits the activity of protein phosphatase1 (PP1) and PP2A (Cohen et al., 1990), significantly increased phosphorylation of PEPCK above the water control in both light and dark and thereby decreased the activation state of PEPCK. Okadaic acid significantly increased phosphorylation of PEPC in the dark, thereby increasing its activation state. There was also a marginal increase in the PEPC activation state in the light, but this was not accompanied...
by an increase in the phosphorylation state. Feeding an inhibitor of protein synthesis, cycloheximide, significantly decreased phosphorylation and the activation state of PEPC in the light and decreased phosphorylation and increased the activation state of PEPC in the dark. Feeding DTT significantly decreased the phosphorylation and activation states of PEPC in the light (Table I).

**DISCUSSION**

Phosphorylation of PEPCK is a regulatory mechanism that contributes to its inactivation in the dark in certain C4 and Crassulacean acid metabolism plants (Leegood and Walker, 2003). The data in Figures 3 and 5 establish that the phosphorylation and activation states of PEPCK are strongly inversely related and that

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**Figure 4.** Responses of the phosphorylation states of PEPC and PEPCK to changes in PFD during illumination of Guinea grass leaves. Leaves were preincubated with [33P] P as described in "Materials and Methods." Samples were taken, leaves darkened for 18 h, then illuminated at the light intensities indicated (μmol quanta m⁻² s⁻¹) for up to a further 6 h.

**Figure 5.** Responses of the phosphorylation states of PEPC and PEPCK and the activation state of PEPCK to changes in CO₂ concentration during illumination of Guinea grass leaves. Leaves were preincubated with [33P] P as described in "Materials and Methods." Samples were taken, leaves darkened for 18 h, then illuminated at the CO₂ concentrations indicated (μmol quanta m⁻² s⁻¹) for up to a further 6 h. The PFD was 400 μmol quanta m⁻² s⁻¹. The activation state of PEPCK was measured using a selective assay, as described in "Materials and Methods."
the activation state of PEPCK can be used as a measure of the phosphorylation state in vivo. It is also clear from a comparison of the data in Table I that there is a positive correlation between the activation and phosphorylation states of PEPC.

These results show that regulation of PEPC and PEPCK by phosphorylation in vivo is a factor determining their activity that can operate over a wide range of CO₂ concentrations and light intensities up to full sunlight. There is strong correlation between phosphorylation/activation and the rate of photosynthetic CO₂ fixation. Phosphorylation of these two enzymes is not, therefore, merely acting as an on-off switch between light and dark. The response of phosphorylation to changes in light intensity or CO₂ concentration takes from minutes to hours and is therefore likely to complement regulation of the activity of these enzymes by metabolites. Moreover, the rapidity of the response depends upon the magnitude of the change in light intensity or CO₂ concentration. Previous measurements of the change in malate sensitivity of PEPC as a function of light intensity in another C₄ plant, sorghum, are similar to those in Figure 4, with both the amplitude and initial velocity of activation varying with incident light (Bakrim et al., 1992). Somewhat surprisingly, light and CO₂ response curves have not been measured for many enzymes of C₄ photosynthesis or simultaneously for multiple enzymes, although previous measurements of the kinetics of activation of PEPC in the C₄ plants maize (Zea mays), sorghum, and Salsola soda suggest similar kinetics of light activation and dark inactivation, with each process taking 1 to 2 h (Karabourniotis et al., 1983; Nimmo et al., 1987; Jiao and Chollet, 1988; Bakrim et al., 1992). This slow activation of PEPC and PEPCK by light compares with the very short times (5–10 min) required to fully activate NADP-malate dehydrogenase via thioredoxin (Johnson and Hatch, 1970) or pyruvate, Pi dikinase, by phosphorylation (Burnell and Hatch, 1985) in maize leaves or comparably short times to activate thioredoxin-linked enzymes and Rubisco in the leaves of C₃ plants (e.g. Sassenrath-Cole and Pearcy, 1994).

DTT and inhibitors were fed to leaves to ascertain how these affected the phosphorylation or activation states of PEPC and PEPCK. The effect of DTT was examined because sulphhydryl groups may play a role in the regulation of PEPC in vitro (Walker et al., 1997) and of PEPCK in isolated C₄ mesophyll cells (Pierre et al., 2004). Feeding DTT to leaves had no significant effect on PEPC phosphorylation, but it did significantly decrease both the phosphorylation state and the activation state of PEPCK. Although it has been suggested that PEPCK kinase from maize and Flaveria trinervia may be redox activated by thioredoxin (Saże et al., 2001; Tsuchida et al., 2001), this would be expected to lead to activation of PEPC rather than the observed inactivation.

Cycloheximide and okadaic acid both had a profound effect on the phosphorylation of PEPC and PEPCK. Okadaic acid is an inhibitor of PP2A, which dephosphorylates PEPC and PEPCK (Walker and Leegood, 1995; Nimmo, 2003). Treatment with this inhibitor therefore promoted the phosphorylation of both enzymes. Cycloheximide is an inhibitor of protein synthesis, which prevents the synthesis of PEPC kinase, which phosphorylates PEPCK (Jiao et al., 1991; Bakrim et al., 1992). Accordingly, it blocked the activation of PEPC. A similar strong inhibition of the dark phosphorylation of PEPCK by cycloheximide suggests that the PEPCK kinase might also be regulated by protein turnover.

Overall, the data show that there was a tight reciprocal relationship between the phosphorylation states of PEPC and PEPCK in relation to both light intensity and CO₂ concentration. Light saturation curves for the phosphorylation state of both enzymes were similar, as was the extent of phosphorylation/dephosphorylation at different CO₂ concentrations. These data suggest that the factors that regulate the phosphorylation of both enzymes are similar. PEPCK kinase is the smallest known protein kinase with no regulatory domains, but is under exquisite transcriptional control and is also controlled by protein turnover (Nimmo, 2003). There is also little evidence to suggest that PEPC

### Table 1. Effects of DTT and inhibitors on the phosphorylation and activation states of PEPC and PEPCK in intact leaves of Guinea grass

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phosphorylation State</th>
<th>Activation State</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Light 2 h</td>
<td>Light 2 h</td>
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<tr>
<td></td>
<td>Dark 2 h</td>
<td>Dark 2 h</td>
</tr>
<tr>
<td></td>
<td>% water control</td>
<td>% maximum</td>
</tr>
<tr>
<td>Control</td>
<td>31 ± 11</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>10 mM DTT</td>
<td>26 ± 7</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>250 μM cycloheximide</td>
<td>116 ± 55</td>
<td>44 ± 6</td>
</tr>
<tr>
<td>50 nM okadaic acid</td>
<td>165 ± 16*</td>
<td>16 ± 2*</td>
</tr>
</tbody>
</table>

For measurements of the phosphorylation state, leaves were preincubated with [³²P] Pi, as described in “Materials and Methods.” Data are means ± si of three samples. The phosphorylation state for the control (supplied only with water) is shown as 100% in the light for PEPC and 100% in the dark for PEPCK. Activation state is shown as a percentage of the maximal enzyme activity measured. *, P is significantly different (at 5% level) when compared to the control.
phosphatase activity is regulated by metabolites (Nimmo, 2003). However, there is evidence from in vitro studies that metabolites, such as malate and G6P, can affect the susceptibility of PEPC to phosphorylation (Bakrim et al., 1992; Wang and Chollet, 1993; Echevarria et al., 1994). Coregulation of phosphorylation of PEPC and dephosphorylation of PEPPCK would therefore have to occur either at this level or at the level of transcription of the respective kinases and/or phosphatases. Previous studies have implied that the process of PEPC phosphorylation is coordinated with Calvin cycle activity. It has been suggested that such coordination could occur either through changes in metabolites that accompany changes in the rate of C4 photosynthesis or through changes in energy charge (Bakrim et al., 1992; Jiao and Chollet, 1992; Giglioli-Guivarc’h et al., 1996). Giglioli-Guivarc’h et al. (1996) suggested that glycerate-3-P from the bundle sheath might act as a message that triggers cytosolic acidification in the mesophyll cells, which leads to a signaling cascade that activates PEPC kinase (see also Bakrim et al., 2001; Osuna et al., 2004). However, evidence contrary to the involvement of the Calvin cycle comes from studies of a maize mutant that lacks a functional Calvin cycle, but nevertheless retains effective light regulation of PEPC and its kinase (Smith et al., 1998).

We attempted to investigate the regulation, by metabolites, of the phosphorylation status of PEPPCK in bundle-sheath strands isolated by the method of Hatch and Kagawa (1976). However, in these, PEPPCK was readily proteolytically cleaved, which removes the regulatory N-terminal phosphorylation site (Walker et al., 1997) and therefore precludes measurements of its phosphorylation state. Rapid deproteinization of PEPPCK in crude extracts and the inability of protease inhibitors to prevent it (Walker et al., 1997) also render measurement of PEPPCK kinase activity extremely difficult.

We also fed metabolites to leaves. The major influences on the phosphorylation/activation states of both enzymes were the C3 metabolites, dihydroxyacetone-P, glycerate-3-P, and PEP, with dihydroxyacetone-P significantly decreasing the activation/phosphorylation of PEPPCK in the light, while these metabolites all significantly decreased the activation state of PEPPCK in the light (data not shown). However, it is not known how much metabolite interconversion occurred in these experiments or what the cytosolic concentrations of potentially regulatory metabolites were in the mesophyll and bundle sheath.

Clearly, further studies are needed of the effects that metabolites have on the expression and activity of the kinases and phosphatases that can regulate PEPC. The kinase and phosphatase for PEPC also need to be identified and characterized and regulation of their activity and expression by metabolites studied. This would then give clues as to how these enzymes are coregulated, not only in Guinea grass, but also in some Crassulacean acid metabolism plants and in many other tissues of C3 plants in which both enzymes occur in the cytosol of the same cells and in which they are both regulated by phosphorylation (Walker and Leegood, 1996; Leegood and Walker, 2003). In addition, the regulatory properties of intact, unproteolyzed PEPPCK (Burnell, 1986; Chen et al., 2002) need to be compared in C4 plants in which it is phosphorylated, such as Guinea grass, and in other C3 plants, such as U. panicoides, that lack PEPC phosphorylation and in which coordination of PEPC and PEPPCK must still occur.

MATERIALS AND METHODS

Plant Material

Seeds of Guinea grass (Panicum maximum; Herbiseed) were grown in a plant growth chamber at a light intensity of 1,500 μmol quanta m−2 s−1 with a 12-h photoperiod (30°C day, 20°C night, 60% humidity). Nutrient solution was applied once weekly in the form of Miracle-Gro (ICI). The third or fourth leaf was harvested at midday from plants at 5 weeks of age. For photosynthesis measurements, attached leaves were used.

cDNA Sequence Determination

Total RNA was purified from 40-d-old Guinea grass using an RNeasy plant mini kit (Qiagen). Two sets of degenerate primers were designed complementary to regions highly conserved between maize (Zea mays), Urochloa panicoides, and Arabidopsis (Arabidopsis thaliana) PEPPCK genes: forward 1, GCCGTCATGCACACTTCAAT and reverse 1, CACGGCAGGGCTTGAT; forward 2, GCAGTACGGCTGAGGAG and reverse 2, CCTGTACGCC-GCCCTTGT. Reverse transcription-PCR was carried out using a SuperScriptII RNase H− reverse transcriptase kit (Gibco-BRL). PCR products of expected sizes were gel extracted using the QiAgquick gel extraction kit (Qiagen) and sequenced.

To obtain the full-length cDNA sequence, 5′ and 3′ RACE-PCR was carried out using a FirstChoice RLM-RACE kit (Ambion) and primers GGA-CCACGGCTGTGTTGACAA and CGTAGCAACCTCCCTCAA as 5′ RACE outer and inner primers, and forward 2 and forward 1 (above) as outer and inner primers for 3′ RACE. PCR fragments were sequenced and the full-length cDNA amplified. The complete sequence was submitted to GenBank (accession no. AAP10076) and compared to databases using BLASTs and BLASTNs algorithms and homologous sequences aligned using BioEdit (Hall, 1999).

In Vivo Phosphorylation Assay

For the experiments in which light intensity and CO2 concentration were changed, terminal 4-cm portions of Guinea grass were excised, placed in a 1-mL cuvette containing 160 μL of water and 40 μL of 33P Pi (specific activity 148 TBq mmol−1, ICN Biomedicals) and illuminated (1,600 μmol mol−1 s−1) at 25°C to 30°C for 2 h, at which point about 90% of the solution had been taken up. A further 160 μL of water and 4 μL of [33P] Pi were then added and the above procedure repeated. Leaf portions were then supplied with 400 μL of water and incubated in the dark overnight (18 h). For varying light intensity, one leaf portion was placed at each light intensity for up to 6 h. For varying CO2 concentrations, leaf portions were incubated in a glass chamber at a light intensity of 400 μmol quanta m−2 s−1 for up to 6 h. After incubation, a 2-cm portion of the basal leaf was cut and frozen in liquid N2. Each complete experiment in Figures 3 to 5 was repeated at least three times on different days, yielding similar results.

For feeding experiments, a similar feeding procedure for [33P] Pi was followed. One-centimeter pieces of leaves were excised and placed in a multiwell plate (Corning), each well containing 160 μL of water and 4 μL of 40 μCi of [33P] Pi and illuminated for 2 h, after which a further 160 μL of water were added and the above procedure repeated. Leaf portions were then supplied with 200 μL of water and incubated overnight (18 h) in the dark. Leaf portions were transferred to a new multiwell plate containing 160 μL of a solution containing DTT or inhibitors (at pH 7.0; in triplicate) and illuminated
at 1.600 μmol quanta m⁻² s⁻¹ for 2 h, or illuminated for 2 h and then darkened for 2 h. The leaf pieces were then transferred immediately into liquid N₂. Tissue was homogenized with 5 volumes of ice-cold 200 mM Bicine-KOH (pH 9.8), 50 mM DTT, and then centrifuged at 20,000 g for 3 min. Supernatants were added to an equal volume of SDS-PAGE solubilization buffer (62.5 mM Tris-HCl [pH 6.8], 10% [v/v] glycerol, 5% [v/v] SDS, 5% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue), placed at 100°C for 3 min, centrifuged at 20,000 g for 3 min, and supernatants analyzed by SDS-PAGE.

SDS-PAGE, Autoradiography, and Immunoblotting

SDS-PAGE was performed using a 4.7% T/2.7% C stacking gel and a 10.5% T/2.7% C resolving gel. After electrophoresis, polypeptides were fixed in gels by immersion in 50% (v/v) methanol and 12% acetic acid. Polypeptides were visualized by colloidal Coomassie Blue G-250 (Sigma). For immunoblotting, transfer of polypeptides from an SDS-PAGE gel to Immobilon P membrane (Sigma) was done in a Pharmacia Multiphor apparatus. Immunoreactive polypeptides were visualized using an antisem raised to purified Guinea grass PEPC or Amaranthus edulis PEPC in conjunction with an ECL kit (Amersham). Autoradiography of dried gels was performed at −80°C using Kodak X-ray film MR B/W (RX), using intensifying screens for 96 to 168 h. Dried films were quantified by a densitometer (Vilber Lourmat). Comparison of autoradiographs and immunoblots enabled the identification of bands at 103 kD for PEPC and at 71 kD for PEPCK in Guinea grass (Walker et al., 1997). The degree of phosphorylation of PEPC or PEPC in treatments was expressed as a percentage of the phosphorylation of the overnight dark sample for all experiments other than the substrate feeding experiments in which water was used as the control.

Activation States of PEPC and PECK

For measurement of PEPC and PECK activity, leaf samples were extracted in 5 volumes of ice-cold 200 mM Bicine-KOH (pH 9.8), 50 mM DTT. The carboxylation activity of PEPC was measured in a continuous assay at 25°C, including 100 mM HEPES (pH 7.0), 100 mM KCl, 90 mM KHCO₃, 0.5 mM PEP, 1.0 mM ADP, 5 μM MnCl₂, 4 mM MgCl₂, 0.14 mM NADH, 6 units mL⁻¹ of malate dehydrogenase for optimum activity, and with 0.8 mM ADP and 0.2 mM ATP instead of 1.0 mM ADP to estimate its activation state (Walker et al., 2002). The activity of PEPC was measured in a continuous assay at 25°C containing 100 mM HEPES (pH 7.3), 0.8 mM PEP, 5 mM MgCl₂, 4.8 mM KHCO₃, 0.35 mM NADH, 5 units mL⁻¹ malate dehydrogenase. For estimation of the PEPC activation state, 1 mL malate was included in the assay (Echevarria et al., 1994). Activation state was expressed as a percentage of the maximal enzyme activity measured. Activities in the assays ranged between 7.8 and 13.3 units g⁻¹ fresh weight for PEPC and 15.1 and 19.5 units g⁻¹ fresh weight for PEPC.

Photosynthesis Measurements

Steady-state rates of photosynthesis were measured using a portable infrared gas analyzer (LCA4; Analytical Development Co.). Light was supplied through fiber optics by a Schott KL 1500 lamp (H, Wala). The maximal PFD achievable at the surface of the leaf was 1800 μmol quanta m⁻² s⁻¹. The leaf temperature was measured with a copper-constantan thermocouple secured on the underside of the leaf (supplied with the infrared gas analyzer). For all measurements, the leaf was illuminated in the chamber until the maximal rate of photosynthetic assimilation was attained.

PEPC sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AAQ10076, S52988, AAQ01894, and S52637. Received November 16, 2006; accepted February 1, 2007; published March 2, 2007.

LITERATURE CITED


