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Research note

Dynamic protein expressions of phospho*enol*pyruvate carboxylase in developing rice seeds

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The enzyme phospho*enol*pyruvate carboxylase (EC4.1.1.31, PEPCase) catalyzes a reaction to change phospho*enol*pyruvate and bicarbonate into oxaloacetate and phosphate (O'Leary et al., 2011). PEPCase is thought to be involved in the carbon partitioning in developing seeds (Plaxton and Podestá, 2006; Andre et al., 2007; Junker et al., 2007). The produced oxaloacetate is consumed to provide carbon skeletons of the TCA cycle members, which are used in many biosynthetic reactions such as amino acid synthesis and fatty acid synthesis. Several studies of seeds support the contribution of PEPCase to the accumulation of seed storage compounds such as storage proteins and lipids (Sugimoto et al., 1989, 1997; González et al., 1998; Sebei et al., 2006; Gennidakis et al., 2007; Radchuk et al., 2007; Fan et al., 2013; Yamamoto et al., 2014).

Rice is one of the most fully characterized cereals regarding PEPCase (Matsuoka and Hata, 1987; Sugimto et al., 1997; Sánchez and Cejudo, 2003; Lin et al., 2004; Masumoto et al., 2010; Yamamoto et al., 2014). Developing rice seeds have high PEPCase activity (approx. 1 U/g fresh weight) (Sugimoto et al., 1997). In six rice cultivars, PEPCase activity was observed in response to nitrogen fertilization, and it was correlated with the response of nitrogen content in mature seeds (Sugimoto et al., 1997). As the gene candidates for PEPCase, we isolated two cDNA clones of PEPCase (*Osppc1* and *Osppc3*) in developing rice seeds (Yamamoto et al., 2014). *Osppc1* was highly expressed in developing seeds at 7 and 10 days after flowering (DAF), when storage protein synthesis was active, at which stages the PEPCase activity was also high (Sugimoto et al., 1997). However, to our knowledge, there is no report about the temporal patterns of PEPCase proteins in developing rice seeds throughout grain-filling stages.

In this Research Note, we describe the monitoring and dissection of multiple PEPCase proteins in developing rice seeds. We found that the accumulation of PEPCase proteins was correlated to PEPCase activity in grain-filling stages. We determined the protein patterns by performing the separation of layered tissues (pericarp and aleurone layer) and inner tissues (embryo and starchy endosperm) of developing ovules. Two-dimensional electrophoresis immunoblotting revealed the occurrence of plural PEPCase proteins showing different mobility shifts from each other in developing seeds, suggesting their different isoelectric points.

Developing rice seeds (cv. *Nipponbare*) grown in the field of Kobe University in 2001 were subjected to soluble protein extractions. The samples were collected and frozen in liquid nitrogen immediately. For PEPCase extraction, each seed sample was thawed on ice to be homogenized with 0.1 M Tris–HCl (pH 7.8), 1 mM EDTA, 1 mM 2-ME, and 20% glycerol with Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) at the triple quantity of fresh weight of the sample by using a pre-cooled motor and pestle at

Abbreviations: PEPCase, phospho*enol*pyruvate carboxylase; the TCA cycle, the tricarboxylic acid cycle; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; DAF, days after flowering; SDS-PAGE, sodium-dodecyl-sulphate polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

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0 °C. Instantly, the homogenate was centrifuged at 16,000 g for 15 min at 0 °C, and the supernatant was collected for the PEPCase assay and electrophoresis in quick. PEPCase activity was determined as described by Yamamoto et al. (2014). As shown in Fig. 1a, PEPCase activity was the highest at 5 DAF and decreased gradually toward maturation (25 DAF). The supernatants were electrophoresed by SDS-PAGE by the method of Laemmli (1970) and analyzed by western blotting by using a polyclonal antibody of soybean seed PEPCase (Sugimoto et al., 1992). The antibody was confirmed to cross-react with Osppc1 recombinant protein and proteins of the purified PEPCase from developing rice seeds (Yamamoto et al., 2014).

Consequently, four PEPCase proteins (116, 112, 109 and 105-kDa proteins) were observed near the expected molecular weight range (105–116 kDa), which was mentioned in the rice PEPCase gene information in the MSU Rice Genome Annotation project (Kawahara et al., 2013). Although the 112- and 109-kDa proteins were detected in all of the samples, the 116- and 105-kDa proteins were detected only at 5 to 17 DAF and 5 to 13 DAF samples, respectively. We quantified the intensities of all of the protein bands using ImageJ software (National Institutes of Health, USA), and we used the sum of intensities for each stage to compare their PEPCase activity. A strong positive correlation was observed at the Pearson correlation coefficient 0.977 ($P = 7.87\ E^{-4}$) (Fig. 1c). This result suggests that the translation level of PEPCase proteins is a main factor to determine PEPCase activity during grain-filling stages in rice seeds. The data at 25 DAF may indicate accumulation of inactivated PEPCase proteins in mature seeds.

We next conducted a western blot analysis in the layered tissues (pericarp and aleurone layer) and the inner tissues (embryo and starchy endosperm) of the developing ovules to analyze the patterns of proteins (Fig. 2). Notably, the patterns of the protein bands

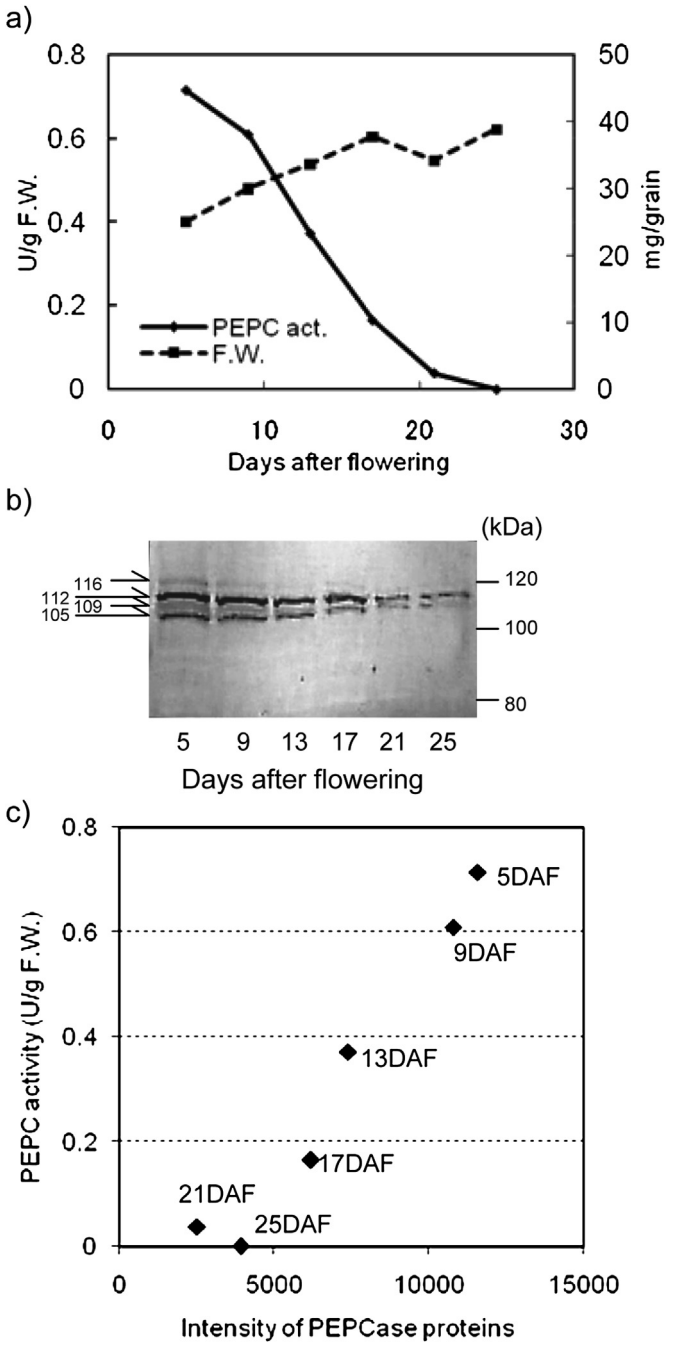

Fig. 1. Monitoring of PEPCase activity and proteins in developing rice seeds. a: PEPCase activity and fresh weight. Horizontal axis represents DAF. Left vertical axis: the PEPCase activity per seed fresh weight. Right vertical axis: the fresh weight of a seed. b: Western blotting of PEPCase in developing rice seeds using a polyclonal antibody of soybean seed PEPCase. The same amount of the crude extract was loaded into each lane of SDS-PAGE (8.5% of polyacrylamide). Arrows represent detected proteins (116, 112, 109 and 105 kDa, respectively). The molecular weight marker was indicated in the right side of the Fig. 1b. c: Correlation of accumulation between the amount of PEPCase protein and PEPCase activity. Horizontal axis: the amount of PEPCase protein. Vertical axis: PEPCase activity.

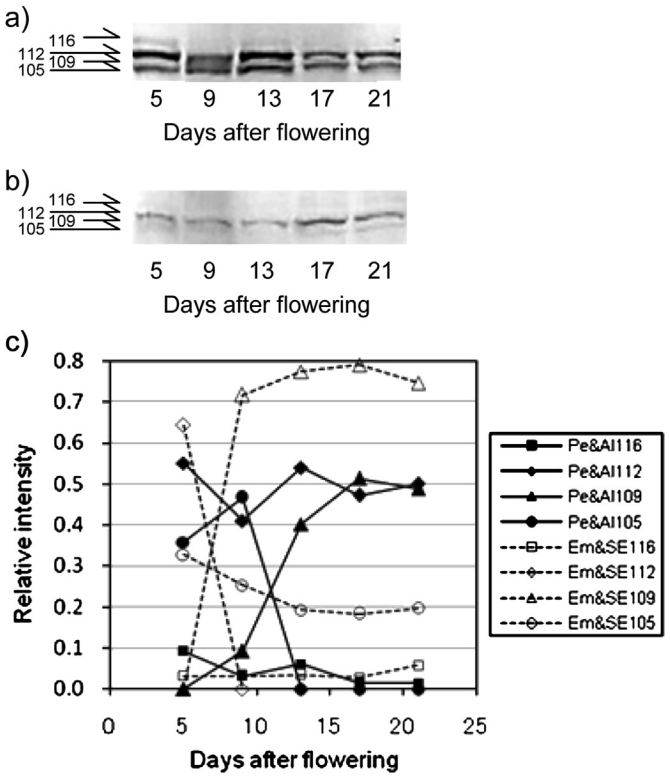

Fig. 2. Western blot analysis of PEPCase proteins in layered tissues (pericarp and aleurone layer) and inner tissues (embryo and starchy endosperm) of developing rice seeds. The same amount of soluble protein (20 μg each), determined by the method of Bradford (1976), was loaded for SDS-PAGE (8.5% of polyacrylamide). Arrows represent detected proteins (116, 112, 109 and 105-kDa, respectively). a: layered tissues. b: inner tissues (embryo and starchy endosperm). **c**: Expression pattern of PEPCase proteins in layered and inner tissues. Horizontal axis: DAF. Vertical axis: relative intensity of each PEPCase protein to those of the four proteins in each sample. Pe&AL116, Pe&AL112, Pe&AL109 and Pe&AL105 show 116, 112, 109 and 105-kDa proteins in the layered tissues. Em&SE116, Em&SE112, Em&SE109 and Em&SE105 show 116, 112, 109 and 105-kDa proteins in the inner tissues.