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Original article

Changes in redox regulation during transition from C_3 to single cell C_4 photosynthesis in *Bienertia sinuspersici*

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ABSTRACT

Bienertia sinuspersici performs single cell C_4 photosynthesis without Kranz anatomy. Peripheral and central cytoplasmic compartments in a single chlorenchyma cell act as mesophyll cells and bundle sheath cells. Development of this specialized mechanism is gradual during plant development. Young leaves perform C_3 photosynthesis, while mature leaves have complete C_4 cycle. The aim of this work was to investigate changes in redox regulation and antioxidant defence during transition from C_3 to single cell C_4 photosynthesis in *B. sinuspersici* leaves. First, we confirmed gradual development of C_4 with protein blot and qRT-PCR analysis of C_4 enzymes. After this activities and isoenzymes of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and H_2O_2 and TBARS and glutathione pool and redox status (GSH/GSSG) were determined in young, developing and mature leaves during transition from C_3 to single cell C_4 photosynthesis. Activities of SOD, APX and POX decrease, while GR and DHAR were increased. However, most striking results were the changes in isoenzyme patterns of SOD, CAT and GR which were gradual through transition to C_4 photosynthesis.

1. Introduction

Terrestrial plants have three different types of basic photosynthetic mechanisms; C_3, C_4 and CAM, each of these photosynthetic pathways are specialized for different environmental conditions (West-Eberhard et al., 2011). C_4 plants, which are adapted to hot and arid climates, employ a mechanism that increases the CO_2 concentration around ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to prevent its oxygenation activity. To concentrate CO_2 around RuBisCO, C_4 plants use two different types of photosynthetic cells, mesophyll and bundle sheet cells arranged in a specific manner that is called Kranz anatomy (Sharpe and Offermann, 2014). Atmospheric CO_2 is fixed in mesophyll cells by phosphoenolpyruvate carboxylase (PEPC) and the product (4 carbon organic acid) is transported to bundle sheath cells. After its decarboxylation in the bundle sheath cells, it releases CO_2, released CO_2 is fixed again by RuBisCO in bundle sheath cells and is used in the Calvin cycle (Wang et al., 2011). Pyruvate produced during decarboxylation is moved back to mesophyll cells and is regenerated back to phosphoenolpyruvate by pyruvate phosphate dikinase (PPDK) for a new cycle (Edwards and Voznesenskaya, 2010). This CO_2 concentration mechanism can be only achieved by cell type specific expression of photosynthetic enzymes. Utilization of this mechanism prevents photorespiration, which is a futile process that decreases plant productivity under conditions such as high temperatures and water deficiency that favour it (Sage, 2002; Chaves et al., 2003).

For years, Kranz anatomy was considered as an essential structural requirement for C_4 plants. However, early in 2000′s, three species in Chenopodiaceae, *Suaeda aralocaspica, Bienertia cycloptera* and *Bienertia sinuspersici* were found to be capable of using C_4 photosynthesis in a single chlorenchyma cell without requirement of Kranz anatomy (Voznesenskaya et al., 2001, 2003, 2005; Akhani et al., 2005). Thus, the absolute necessity of the Kranz anatomy for C_4 photosynthesis has lost its validity. In *Bienertia* species, there are two separate compartments within a cell that contain biochemically and structurally different chloroplasts (Offermann et al., 2011). These are peripheral compartment chloroplasts and central cytoplasmic compartment chloroplasts, which are separated from each other by a complex cytoskeletal network containing channels for metabolite transfer between two compartments. When compared to Kranz type C_4, peripheral compartment acts as an analog to mesophyll cells, while central compartment acts as an analog to bundle sheet cells (Voznesenskaya et al., 2005; Park et al., 2009).

This unique cellular arrangement for single cell C_4 photosynthesis is gradually formed during leaf development (Lara et al., 2008). Young leaves of *B. sinuspersici* cannot use C_4 photosynthesis due to lack of cellular compartmentalization and C_4 photosynthesis enzymes such as

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PEPC and PPDK, are not expressed. Thus young leaves perform C_3 photosynthesis. In further developmental stages of the leaves of *B. sinuspersici*, first cellular compartmentalization is formed and in the following stages C_4 cycle enzymes are specifically expressed in peripheral and central cytoplasmic compartment chloroplasts, forming the complete single cell C_4 cycle (Offermann et al., 2011).

Discovery of this unique photosynthetic pathway draw attention of scientists that are working to convert C_3 plants to C_4 to increase plant productivity with the ultimate aim of increasing yield of crop plants. A well-known example for this is the C_4 rice consortium (Karki et al., 2013). One of the most challenging problems during this conversion is the formation of Kranz anatomy which is lacked by C_3 plants. However, possibility of engineering C_4 photosynthesis into a single cell without need for Kranz anatomy is a feasible and attractive alternative to overcome this challenge (Langdale, 2011).

During engineering of C_4 pathway to a C_3 plant, it is expected that other metabolic pathways will also need to be adjusted besides the photosynthetic metabolism. One of the most intimately related processes with the photosynthesis are the redox reactions and especially the redox status of the chloroplasts (Uzilday et al., 2012). Redox balance is the regulation of reduction/oxidation reactions in the cell by non-enzymatic and enzymatic components and maintenance of their equilibrium (Foyer and Noctor, 2013). Non-enzymatic antioxidant include ascorbate, glutathione (GSH) and other low molecular weight antioxidants, while enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and enzymes that regenerate non-enzymatic antioxidants such as glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Mittler et al., 2004). Cellular redox in plants is related to control of the photosynthesis, stomatal closure, signal transduction, hormone signalling, vegetative and reproductive growth and stress related gene expression (Potters et al., 2010). Also, another important relationship between photosynthesis and oxidative load is the photorespiration. During photorespiration a great amount of H_2O_2 is produced in C_3 plants due to oxidation of glycolate to glyoxylate in peroxisomes, however, in C_4 plants this process is nearly completely inhibited. In our previous works, we have elucidated differences in redox regulation of C_3 and Kranz type C_4 plants of *Cleome* and *Flaveria* (Uzilday et al., 2012; Uzilday et al., 2014). We have demonstrated that there are correlations in activities of different antioxidant enzymes through transition from C_3 to C_4 photosynthesis. However, these findings are valid for Kranz type C_4 plants and there is no information available in the literature about redox regulation in a single cell C_4 plant. Hence, gradual development from C_3 to single cell C_4 photosynthesis in the leaves of *B. sinuspersici* offers a unique model to investigate this phenomenon.

Questions we tried to answer in this work are (i) how specific redox needs of C_3 and single cell C_4 chlorenchyma cells of *Bienertia sinuspersici* change and (ii) how this transition occurs in terms of antioxidant isoenzyme patterns. There are two reasons that lead us to ask these questions. First, in young leaves, chloroplasts appear monomorphic and are in a C_3 state (Voznesenskaya et al., 2005). However, as the cells develop, distinct cytoplasmic compartments are observed and dimorphic chloroplasts that function in C_4 pathway are formed (Lara et al., 2008). This inevitably changes light reactions, which is the primary driver of cellular redox during photosynthesis. Secondly, suppression of photorespiration during single cell C_4 changes the oxidative load in the cell, again which requires an adaptive response in terms of cellular redox regulation. To answer these questions, in the present study, we have investigated activities and isoenzyme patterns of antioxidant enzymes SOD, CAT, APX, POX, PRXQ, GR and DHAR and H_2O_2 and glutathione levels along with protein blot and qRT-PCR analysis of C_4 enzymes and in young, developing and mature leaves of *B. sinuspersici* to obtain a snapshot of redox regulatory mechanism during each phase of single cell C_4 transition.

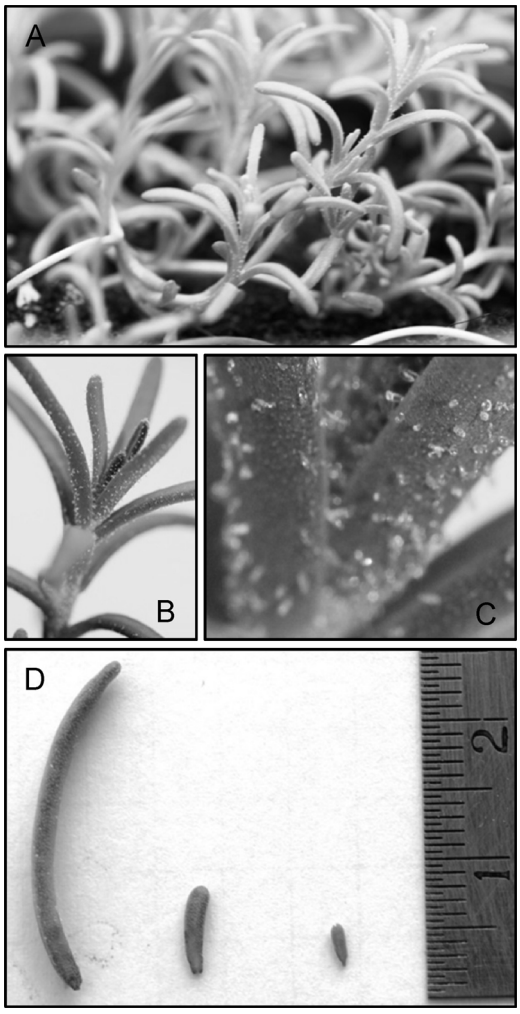

Fig. 1. Pictures of *Bienertia sinuspersici* plants and leaves. (A) Three months-old *B. sinuspersici* plant. (B) A close up photo of shoots. (C) Salt crystals extruded by salt glands of *B. sinuspersici* leaves. (D) Young (0.1–0.3 cm), developing (0.5–0.7 cm) and mature (> 2 cm) leaves.

2. Material and methods

2.1. Plant material

B. sinuspersici Akhani seeds were germinated on filter paper with sterile water for 1 week and then germinated seedlings were transferred to pots containing growth medium (7: 2: 1 peat moss: vermiculite: sand). Plants were grown in a plant growth chamber at 25 °C with 250 μE light intensity (14/10 h light/dark) and 60% RH for 3 months (Fig. 1A–C). Plants were watered with water every second day and were fertilized once a week with 1 g L^{-1} Peters Professional (20:20:20). Also, plants were watered with 150 mM NaCl once a week; otherwise growth was inhibited due to halophyte nature of the plant. After the growth period plants were used to harvest young (0.1-0.3 cm), developing (0.5-0.7 cm) and mature (> 2 cm) leaves (Fig. 1D).

2.2. Enzyme extractions

Enzyme extractions were performed at 4 °C. Samples (0·1 g) were ground to a fine powder in liquid nitrogen and then homogenized in 500 μL of 50 mM Tris-HCl, pH 7.8, containing 0.1 mM EDTA, 0.1% (w/v) Triton-X 100, 1 mM phenylmethanesulfonyl fluoride (PMSF) and polyvinylpyrrolidone (PVP; 1%, w/v). For APX activity determination, 5 mM ascorbate was added to the homogenization buffer. Samples were