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Comparative proteomic analysis of amaranth mesophyll and bundle sheath chloroplasts and their adaptation to salt stress

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ABSTRACT

The effect of salt stress was analyzed in chloroplasts of *Amaranthus cruentus* var. Amaranteca, a plant NAD-malic enzyme (NAD-ME) type. Morphology of chloroplasts from bundle sheath (BSC) and mesophyll (MC) was observed by transmission electron microscopy (TEM). BSC and MC from control plants showed similar morphology, however under stress, changes in BSC were observed. The presence of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) was confirmed by immunohistochemical staining in both types of chloroplasts. Proteomic profiles of thylakoid protein complexes from BSC and MC, and their changes induced by salt stress were analyzed by blue-native polyacrylamide gel electrophoresis followed by SDS-PAGE (2-D BN/SDS-PAGE). Differentially accumulated protein spots were analyzed by LC–MS/MS. Although *A. cruentus* photosynthetic tissue showed the Kranz anatomy, the thylakoid proteins showed some differences at photosystem structure level. Our results suggest that *A. cruentus* var. Amaranteca could be better classified as a C_3–C_4 photosynthetic plant.

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Introduction

A renewed interest in C_4 photosynthesis research is stimulated by the fact that C_4 plants make a significant contribution to the global carbon budget, with potential use of C_4 plants as a source of biofuels (Carpita and McCann, 2008), but most important, C_4 crops such as maize and sorghum are pivotal to current and future global food security (Ghannoum, 2009). Due to these facts, ambitious projects have been proposed with the aim to increase crop yields by introducing C_4 traits into C_3 plants (Covshoff and Hibberd, 2012).

It is widely accepted that the C_4 photosynthetic pathway has evolved recently in terms of angiosperm phylogeny and that there is considerable anatomical and biochemical variation associated with this trait (Sage et al., 2012). C_4 photosynthesis is observed as an adaptation to deleteriously high levels of photorespiration (Kadereit et al., 2012). Plants exhibiting C_4 photosynthesis are found in 17 different families of angiosperms, including monocots and dicots. At least 23 species of plants have been reported to exhibit characteristics of photosynthesis and photorespiration intermediate between C_3- and C_4-type plants (Monson and Moore, 1989), suggesting that these species could be good models to understand the paths taken during C_4 evolution. The term C_3–C_4 intermediate denotes a syndrome of physiological, anatomical, and biochemical traits that are between to the more widely occurring C_3 and C_4 syndromes (Griffiths et al., 2013). Many C_3–C_4 intermediaries grow in arid or saline zones, for example intermediate species of *Heliotropium*, *Salsola*, *Neurachne*, *Alternanthera* (*Amaranthaceae*), and *Flaveria*. In *Flaveria* all intermediates that have been investigated, both ribulose bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoenol pyruvate (PEP) carboxylase are not entirely compartmentalized between the mesophyll (M) and bundle-sheath cells (BS), as in fully-developed C_4 plants (Sage et al., 2012). In the *Amaranthaceae*, C_4 photosynthesis has evolved multiple times but the C_4 evolutionary lineages are unclear because the photosynthetic pathway is unknown for most species of the family.

Abbreviations: BN, blue native; BS, bundle sheath cells; BSC, bundle sheath chloroplasts; M, mesophyll cells; MC, mesophyll chloroplasts; NAD-ME, NAD-malic enzyme; PEP, phosphoenol pyruvate; PPDK, pyruvate phosphate dikinase; PSI, photosystem I; PSII, photosystem II; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; TEM, transmission electron microscopy.

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Amaranths (*Amaranthus* spp.), are dicotyledonous stress-tolerant plants that resist drought and soil salinity better than wheat, maize, sorghum or cotton (Kauffman and Weber, 1990). But the interest in amaranth is increasing because the plant produces high-protein seeds with an excellent balance of essential amino acids and has unique starch and oil characteristic (Pavlík, 2012). Amaranth seeds also contain biopeptides with antihypertensive, cancer preventive, and antidiabetic properties (Silva-Sánchez et al., 2008; Velarde-Salcedo et al., 2013), and several secondary metabolites with claimed medicinal properties (Barba de la Rosa et al., 2009).

However, there are a number of physiological, structural, and developmental characteristics that distinguish the amaranth C_4 photosynthetic pathway from that of maize and other C_4 monocots. For example, amaranth uses a mitochondrial NAD-malic enzyme (NAD-ME) for malate decarboxylation in BS, whereas maize uses a chloroplastic NADP-ME for this function (Ueno, 2001). In addition, amaranth has bundle sheath chloroplasts (BSC) with well-developed grana and normal photosystem II (PSII) activity, while in maize BSC are agranal and lack many polypeptides associated with PSII (Gutierrez et al., 1974). Because of the many variations between C_4 dicots and monocots, it might be expected to find differences between these two groups in the morphogenesis of Kranz anatomy, the development of C_4 capacity, and the regulation of genes encoding C_4 photosynthetic enzymes (Wang et al., 1992).

Chloroplasts are of particular interest for plant biologists, they are the largest metabolically active compartments of the mature leaf cell (Baginsky and Gruissem, 2004; Behrens et al., 2013). Analysis of data regarding the effects of environmental stress in arabidopsis revealed a total of 279 proteins claimed as unique organelle stress responsive, 73% of these belonging to the chloroplast. The predominance of chloroplast proteins is likely a consequence of its specific sensitivity to environmental stress (Taylor et al., 2009). In amaranth leaves, most of the abiotic stress-responsive proteins also belong to chloroplast (Huerta-Ocampo et al., 2009). Therefore, information of chloroplast proteins will provide new insights of key mechanisms underlying amaranth tolerance to abiotic stress.

Thylakoid membranes in chloroplasts contain protein complexes that carry out oxygenic photosynthesis. Photosynthesis is among the primary processes to be affected by water or salt stress (Chaves et al., 2009). The composition of the four protein complexes: photosystem I (PSI), PSII, ATP-synthase and cytochrome $b_{6/f}$ ($CYTb_{6/f}$) complexes are well known. Two-dimensional electrophoresis (2-DE) has been used for membrane protein separation but the isoelectric focusing (IEF) step is especially problematic. Blue native (BN) gel electrophoresis, as an alternative first dimension, has proven to be a good method to allow complete separation of each protein by SDS-PAGE in the second dimension (Andaluz et al., 2006; Behrens et al., 2013). The high mass accuracy, sensitivity, and high-throughput possibilities of mass spectrometry (MS), together with the availability of genomes allow the rapid identification of large sets of proteins (Aebersold and Mann, 2003). The available data of proteomes from arabidopsis and rice chloroplasts are available at http://ppdb.tc.cornell.edu/. The availability of the grain amaranth transcriptome database facilitates the amaranth proteomic studies (Délano-Frier et al., 2011).

The aim of this work was to characterize, for the first time, the proteomic profiles of thylakoid protein complexes from BSC and mesophyll chloroplasts (MC) isolated from amaranth leaves and their changes induced in response to salt stress. Thylakoid protein complexes were analyzed using BN/SDS-PAGE and LC–MS/MS. Amaranth leaves fine cuts were analyzed by transmission electron microscopy (TEM) and RuBisCO, as chloroplastic protein marker, was detected by immunohistochemical stain and in protein gel blot analysis.

Materials and methods

Plant growth conditions

Amaranthus cruentus L. var. Amaranteca seeds were germinated on sterile soil (Berger BM2, Berger Peat Moss, Saint/Modest, Québec, CA). Seedlings with three to four true leaves were transplanted into plastic pots containing sterile soil and kept in greenhouse under regular watering with 1/2 strength nutrient solution (Hydro-Environment, México, EC 2.0–2.2 ds m^{-1}). For salt stress imposition, plants were watered with 150 mL/day of 300 mM NaCl solution for 4 days. At 24 h post-treatment (the fifth day), fully developed leaves from the middle part of the plant were collected and chloroplasts were immediately isolated. Intact leaves samples were kept for optical and TEM analysis.

Optical and transmission electron microscopy (TEM)

Leaf samples were fixed for 24 h with 0.1 M sodium cacodylate solution pH 7.7 containing 3% glutaraldehyde. After fixation, the samples were washed with 0.2 M sodium cacodylate and then with 0.1 M cacodylate buffer containing 2% osmium tetroxide and finally samples were dehydrated with 2 washes in 10% ethanol for 10 min, 2 times in 96% ethanol for 10 min, and 2 times in absolute ethanol for 15 min.

Before inclusion, the samples were washed twice for 30 min each with propylene oxide:resine (1:1). Samples were included in fresh resin Epon 812 (Electron Microscopy Sciences, Hatfield, PA, USA) and incubated at 60 °C for 36 h for resin polymerization. Semi and ultra-fine cuts were obtained with an ultramicrotome (MTX, RMC, Tucson, AR, USA). Semi-fine cuts were observed using a light microscope Axio Imager.A2 M (Carl Zeiss Microscopy GmbH, Göttingen, GER) and images were used to measure the area of bundle-sheath cells (BS) and mesophyll cells (M), as well the areas of bundle sheath chloroplasts (BSC) and mesophyll chloroplasts (MC) using the software AxioVision 4.8.2.0 (Carl Zeiss MicroImaging). Data were processed with GraphPad Prism, v 5.01 (La Jolla, CA, USA). Ultrafine cuts were observed with TEM (JEM-1010, JEOL, Peabody, MA, USA).

Immunohistochemical staining

Plant tissues were fixed in 4% of paraformaldehyde and embedded in paraffin and histological cuts were obtained. Cuts were deparaffinized as follows: 2 times for 3 min in xylene, 1 wash for 3 min in xylene:absolute ethanol (1:1), 2 times for 3 min in absolute ethanol, then sample rehydration was carried out by 3 washes for 3 min each in ethanol solutions: 95%, 70%, and 50%. Finally, cuts were washed with distilled water.

Enzymatic antigen retrieval was carried out by incubating the sample at 37 °C with 0.05% trypsin solution containing 0.1% of $CaCl_2$ at pH 8.0 for 15 min, after that, the enzyme solution was washed with distilled water. Immunohistochemical staining was done as previously reported (Walker et al., 1999) with few modifications. Sections (10 μm) were deparaffinized and incubated at 37 °C for 30 min in blocking buffer containing 1X TBS (50 mM Tris–HCl, 150 mM NaCl) pH 7.4, 0.1% Tween-20, 1% BSA, and 1.5% gelatin. Sections were then incubated for 2 h at 37 °C with primary antibody rabbit (IgG) anti-RbcL (Agrisera AB, Vännas, Sweden) diluted 1:300 in blocking buffer, washed, and incubated with secondary antibody (anti-rabbit IgG-alkaline phosphatase conjugate, Sigma–Aldrich, St. Louis, MO, USA) diluted 1:2000 in blocking buffer. After washing 2 times with TBS-T (1× TBS pH 9.5 + 0.1% Tween-20) phosphatase activity was visualized by incubating in NBT-BCIP solution (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 50 mg/L BCIP, 340 mg/L NBT) for 20 min. The reaction was stopped with distilled water and

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