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Proteomic analysis of changes in the *Kandelia candel* chloroplast proteins reveals pathways associated with salt tolerance

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ABSTRACT

The plant chloroplast is one of the most sensitive organelles in response to salt stress. Chloroplast proteins extracted from seedling leaves were separated by two-dimensional gel electrophoresis (2-DE). More than 600 protein spots could be distinguished on each gel. Fifty-eight differentially expressed protein spots were detected, of which 46 could be identified through matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). These proteins were found to be involved in multiple aspects of chloroplast metabolism pathways such as photosynthesis, ATP synthesis, detoxification and antioxidation processes, nitrogen assimilation and fixation, protein metabolism, and tetrapyrrole biosynthesis. The results indicated that *K. candel* could withstand up to 500 mM NaCl stress for a measured period of 3 days, by maintaining normal or high photosynthetic electron transfer efficiency and an only slightly stimulated Calvin cycle. Meanwhile, we found that ROS scavenging, nitrogen assimilation, protein degradation and chaperone function in chloroplasts were also of importance for salt tolerance of *K. candel*. The ultrastructural and physiological data agree with chloroplast proteome results. These findings allow further exploration of our knowledge on salt adaptation in woody halophytes and may contribute to the development of more salt-tolerant plants in the future.

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Aαs, ATP synthase CF1 alpha subunit; Aβs, ATP synthase beta subunit; Cpn, chaperonin; Cyt*b6f*, cytochrome *b6f*; DHAP, dihydroxyacetone phosphate; DHAR, dehydroascorbate reductase; EF-Tu, Elongation factor Tu; FNR, ferredoxin-NADP(H) oxidoreductase; FtsH, Cell division protein ftsH; G3P, glyceraldehydes-3-phosphate; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GR, glutathione reductase; GS, glutamine synthetase; GSH, reduced glutathione; GSP, glutamine synthetase precursor; GSSH, oxidized glutathione; GST, glutathione S-transferase; HET-P, 4-methyl-5-(2-hydroxyethyl) thiazole phosphate; HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; MSP, chloroplast manganese stabilizing protein; $NADP^+$/NADPH, nicotinamide adenine dinucleotide phosphate; OEE, oxygen evolving enhancer protein; PC, plastocyanin; PGA, 3-phosphoglycerate; PGK, phosphoglycerate kinase; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; RCA, ribulose-1,5-bisphosphate carboxylase/oxygenase activase; RISP, Rieske iron-sulfur protein; Ru5P, ribulose-5-phosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; SBP, sedoheptulose-1,7-bisphosphate; SBPase, sedoheptulose-1,7-bisphosphatase; SOD, superoxide dismutase; THI, thiazole biosynthetic enzyme; TRI, triosephosphate isomerase; VSP, vegetative storage protein; WOC, chloroplast photosynthetic water oxidation complex 33 kDa subunit precursor.

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1. Introduction

Salt contaminated areas are an increasing problem in agriculture [1–5], with nearly a half of all irrigated land (20% of the earth's total landmass) being affected by salinity [6,7]. High salt or salinity is detrimental to plant growth for various reasons, including a reduction in photosynthesis, ion imbalance, hyperosmotic stress, oxidative damage and nutrient deficiency, all of which lead to growth inhibition [8]. To better cope with the dynamics and stability of biological systems under salt stress, a deeper insight in the responses of plants to saline environments will be required.

The plant chloroplasts like most organelles do not respond well to salinity. Studies on chloroplasts are complicated by their semi-autonomous protein synthesis. Only about 100 of an estimated 2000–3000 proteins are encoded by the individual chloroplast's genome, while the remaining proteins are encoded by the plant nuclear genome, translated in the cytosol, and then transferred to the plastid [9,10]. Consequently, alterations in gene expression are difficult to relate to actual protein changes in chloroplasts. However, isolation of a specific organelle followed by proteome analysis provides a way to define the protein complement, facilitating functional studies as well as the prediction of protein localization [11]. Proteome analysis of chloroplasts in response to salt stress has mainly focused on herbaceous plants like maize [12], wheat [13,14] and *Salicornia europaea* [8]. Far less attention has been paid to chloroplasts from xylophytes (woody plants) under salt stress. Within the xylophytes, especially mangroves are capable of adapting to wide ranges of salinity, making them excellent woody halophytic models [15,16]. Mangroves are found growing along coasts of (sub) tropical areas. *Kandelia candel* (Rhizophoraceae) is a dominant, non-salt-secreting, coastal mangrove species in China capable of adapting to salinity levels up to 450 mM NaCl [17]. It is therefore one of the major species for rehabilitation of ecological mangrove systems along the South-Chinese coast. The combined features of high salt tolerance and ecological dominance make *K. candel* gain more and more attention as a woody plant model for studies on salt tolerance mechanisms.

Several studies have addressed mangrove salinity response mechanisms by focusing on the photosynthetic performance at the whole-plant level [18,19], the expression and identification of genes that might contribute to high salinity tolerance [20–22], as well as changes in protein abundance of leaves under long term salt stress (12 d and 45 d, respectively) [23,24]. However, except for our initial report examining the *K. candel* chloroplast proteome at various salt conditions [25], no reports exist that focus on investigating the effects of salt stress on the chloroplasts proteome of salt-tolerant xylophytes. In the previous study, we found that photosynthetic activity in *K. candel* was maintained at 400 mM salt stress as indicated by induction of proteins from the light-dependent reactions and the Calvin cycle, but photosynthetic activity decreased at 600 mM salt stress as was shown by down-regulation of Calvin cycle enzymes. In spite of some recent progress, further efforts are still needed to fully uncover the regulatory mechanisms of salt tolerance networks in *K. candel*, especially for the chloroplast proteins involved in long-term salt stress, as so far the previous study focused on short-term response of salt stress [25]. In this study, we chose 500 mM NaCl for a maximum period of 6-days as the treatment conditions to further study the specific metabolic pathways and regulatory mechanisms of *K. candel* chloroplasts under high salt stress underlying our previous report [25].

2. Materials and methods

2.1. Plant material and experimental treatment

Equal-sized mature hypocotyls of *K. candel* were collected from the estuary of Luoyang River (24°58′N, 118°39′E), Quanzhou City, Fujian Province, China. Plant culture and salt treatments were performed according to Wang et al. [25]. Briefly, hypocotyls were planted in plastic pots containing washed river sand and grown in Hoagland solution until the first four leaves had fully expanded. Subsequently, seedlings were salt-treated with Hoagland solution including 500 mM NaCl for 0 days (control), 3 days, or 6 days, respectively. Leaves were harvested after salt treatment for chloroplast isolation and physiological analyses. Unless mentioned otherwise, three biological replicates were prepared for each salt treatment.

2.2. Photosynthesis parameter measurements

The net photosynthetic rate (Pn), stomatal conductance for water vapor (Gs), and intercellular CO_2 concentration (Ci) of seedlings grown as described above were determined at a constant photon density (1000 μmol m^{-2} s^{-1}) and an ambient CO_2 concentration of 380 μmol mol^{-1} using a portable photosynthesis system CIRAS-2 (CIRAS-2, England). The chlorophyll (Chl) fluorescence emission was recorded by using a Handy PEA chlorophyll fluorimeter (Hansatech, England) [26].

2.3. Chlorophyll and proline content analysis

Chlorophyll (Chl) contents of leaves were measured after extraction through washing with distilled water, extraction in 5 mL 80% v/v acetone, adjusting to a final volume of 25 mL and filtration. Absorbance of extracts was measured at 470, 645 and 663 nm according to the method as described [27]. Proline content was determined using ninhydrin reactions [28]. Six replicates were measured for each treatment.

2.4. SOD, GAD, GS and POD, activity assay

For superoxide dismutase (SOD) and peroxidase (POD) activity measurements, 1 g of leaves was ground to a fine powder in liquid N_2 and homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% (w/v) PVPP. SOD activity was determined by measuring inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm [29]. One unit of SOD activity was defined as 50% inhibition of NBT photoreduction. The activity of POD was determined by the guaiacol method at 470 nm [30]. One unit of POD activity was defined as an increase in the absorbance at 470 nm by 0.01 units per minute per gram fresh weight. The glutamate decarboxylase (GAD) activity was determined using previous methods. For glutamine synthetase (GS), 1 g of leaf tissue was ground on ice in 10 mL buffer (50 mM Tris–HCl (pH 8.0), 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM DTT, 400 mM sucrose). GS activities are expressed as the formation of the γ-glutamylhydroxamate/$FeCl_3$-complex at 540 nm [31].

2.5. Na^+ and K^+ determination

For ion analysis, leaf samples were weighed and dried at 80 °C until a constant weight was obtained. Dried samples were powdered and 0.3 g was digested in concentrated H_2SO_4. Next, the Na^+ and K^+ contents were analyzed using a flame photometer (FP 640, Shanghai, China) according to our previous report [17].