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Phosphoproteomics unveils stable energy supply as key to flooding tolerance in *Kandelia candel*

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ABSTRACT

The mangrove *Kandelia candel* (L.) Druce experiences daily flooding cycles. To explore the molecular mechanism underlying the physiological adaptation of *K. candel* to flooding, the potential role of protein phosphorylation in flooding responses was investigated by a large-scale quantitative phosphoproteomic analysis using isobaric tag for relative and absolute quantitation. Total 2141 unique phosphopeptides and 2603 non-redundant phosphorylation sites were identified from 1516 phosphoproteins in *K. candel* leaves. In addition to known phosphorylation motifs, three new motifs [GSP], [GxxSP] and [RSxS] were discovered. The phosphorylation levels of 96 differentially expressed phosphoproteins, including those involved in pyruvate metabolism and energy production, were identified in response to flooding. The physiological parameters and transcriptional levels relevant to flooding responses including photosynthesis, pyruvate metabolism, and ROS production were investigated and all were found to be robust under flooding conditions. The consistent results of the phosphoproteomic, physiological analyses and transcriptional levels reinforce each other to demonstrate that *K. candel* adapts to flooding through maintaining sufficient photosynthesis activities, achieving effective anaerobic respiration and increasing pentose phosphate pathway flux. Protein phosphorylation is likely to play a major role in the regulation of these pathways which together contribute to stable energy supply that enhances flooding tolerance in *K. candel*.

Biological significance: Flooding stress is one of the major environmental stresses. The woody mangrove *Kandelia candel* experiences daily flooding cycles in its natural habitat. Protein phosphorylation is a crucial regulatory mechanism in plants' responses to both biotic and abiotic stresses. To analyze phosphorylation levels in critical enzymes involved in key metabolic pathways, we employed phosphoproteomic approach to dissect the adaptive mechanism of *K. candel* to flooding conditions. To our knowledge, this is the first large-scale quantitative phosphoproteomic analyses of *K. candel*'s flooding responses. Multiplex iTRAQ-based quantitative proteomic and Nano-LC–MS/MS methods were used to construct the phosphorproteome. Our results indicate that *K. candel* is able to acquire stable energy supply under flooding by maintaining sufficient photosynthesis activities, enhancing effective anaerobic respiration and increasing pentose phosphate pathway (PPP) flux. The protein phosphorylation found in photosynthesis, anaerobic respiration and PPP is likely to play important roles in the flooding tolerance of *K. candel*.

1. Introduction

Global warming is the cause of increasingly frequent flooding in many countries of the world [1,2]. Flooding stress is one of the major environmental stresses under which plants experience low-oxygen and low-light conditions [3]. Low oxygen, or hypoxia, causes plants to shift from aerobic to anaerobic respiration; the latter is less efficient in ATP production. Under hypoxia, metabolic fluxes are altered in order to fulfill the housekeeping activities for plant survival. In the absence of oxygen, or anoxia, aerobic respiration is shut down, ATP production is

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solely dependent on glycolysis, and the plant experiences energy crisis [4,5]. Plants have evolved both rapid and long-term responses such as chloroplast streaming towards epidermis and leaf hyponastic growth, respectively, to low-light conditions [6–8]. In response to complete flooding, leaf acclimations facilitate underwater photosynthesis as well as the internal diffusion of O_2 from the floodwater [8].

In the last decade, transcriptomic studies on flooding and hypoxic stresses have identified hundreds of low-oxygen responsive genes in *Arabidopsis* (*Arabidopsis thaliana*) [9–12], rice (*Oryza sativa*) [13,14], soybean (*Glycine max*) [15], maize (*Zea mays*) [16], cotton (*Gossypium hirsutum*) [17], citrus (*Citrus flavedo*) [18] and gray poplar (*Populus* × *canescens*) [19]. These differentially expressed genes are involved in the processes of energy metabolism and photosynthesis, carbohydrate mobilization and sucrose catabolism, amino acid metabolism, stress response and defense, protein synthesis and destination, cell structure, phytohormone biosynthesis and signal transduction under flooding or oxygen-deprivation conditions [20–22].

In addition to transcriptional regulation, post-translation modifications, including phosphorylation, acetylation, glycosylation and methylation, also play important roles in regulating plant metabolism [23]. Protein phosphorylation is crucial in plants' responses to both biotic and abiotic stresses [24]. For example, initiation factor 4E is phosphorylated in oxygen-deprived maize roots [25], initiation factor 4G and 5A2 are dephosphorylated in flooded roots of soybean [26,27], and ribosomal proteins are dephosphorylated in maize roots under oxygen deprivation conditions [28]. Sucrose phosphate synthase (SPS) 1F in soybean and sucrose synthase (SuSy) in maize are phosphorylated in hypoxia conditions [27,28]. Nanjo et al. [22] suggested that the energy-demanding anabolic pathways might be regulated by protein phosphorylation under flooding treatments based on phosphoprotein analyses. Although protein phosphorylation has been shown to be important in transducing hypoxia signals, attention has been focused on crop plants, most of which are herbaceous [29]. Little is known about the dynamics of protein phosphorylation in woody plants under flooding conditions.

Many plant species survive flooding stress as a result of long-term adaptive evolution [30]. Mangroves are woody plants found in tropical and subtropical intertidal regions that experience periodical flooding, and are regarded as woody halophytes resistant to flooding [31]. *Kandelia candel* (L.) Druce is a dominant mangrove species of the southeastern coast of China, where *K. candel* is flooded daily for 4- to 6-h during high-tides [32,33]. Previous studies in mangrove plants reported physiological and biochemical changes in response to flooding stress [31,32]. Regulation through the fast and reversible protein phosphorylation has been shown to occur in herbaceous plants under flooding conditions. Well-coordinated protein phosphorylation may have been evolved in woody halophytes such as *K. candel* for adaptation to daily flooding in nature. In this study, we performed global analyses of protein phosphorylation in addition to physiological and transcriptional observations to investigate the potential roles in the maintenance of energy homeostasis in *K. candel* during flooding.

2. Materials and methods

2.1. Plant materials and treatments

Healthy (without wormholes and mechanical damages) and mature (with length range from 17 to 22 cm) *K. candel*'s hypocotyls were collected from Zhangjiang River Estuary in Yunxiao County, Zhangzhou City, Fujian Province of China (23°55′N, 117°26′ E). The seawater salinity is between 12‰ and 26‰ with an average of 17‰. The sea tide is not a regular semidiurnal tide; rather the flooding lasts for an average of 397 min (6 h and 37 min) and the ebb lasts for an average of 315 min (5 h and 15 min). Since *K. candel* is a salt-tolerant species naturally grown best in sea water with a salt concentration of approximately 12‰ salinity [34], the chosen hypocotyls were cultivated in Hoagland solution containing 12‰ of artificial sea salt (Fuzhou Xinrong Chemical Co. Ltd., Fuzhou, China) in rectangular plastic trays (30 cm × 42 cm × 14 cm). Tap water was added periodically to maintain the salt concentration at 12‰. Fresh salinated-Hoagland solution was changed every three days until two leaves had fully expanded. All seedlings were grown in the greenhouse with air temperature of 27–32 °C, and under natural sunlight for 40 days. Subsequently, five plastic trays (namely five biological replicates) with each tray containing 20 seedlings, were treated without (control group) and with flooding for 6 h (flooding group) in artificial seawater, respectively. In the flooding treatment, seedlings were submerged 5 cm underneath the water surface. After flooding treatments, the first pair of leaves were harvested and immediately frozen in liquid nitrogen and stored at −80 °C. Leaves from three of the five biological replicates were used for phosphoproteomic and transcriptional analyses, and the remaining two replicates were used for physiological and biochemical analyses.

2.2. Protein extraction and digestion

One g of each leaf sample was ground into fine powder in liquid nitrogen for protein extraction as described by Wang et al. [34]. After concentration determination, each protein sample was digested by filter aided sample preparation method [35]. 200 μg protein from each sample was resuspended in 30 μL SDT buffer [4% (w/v) sodium dodecyl sulfate, 100 mM dithiothreitol, 150 mM Tris-HCl pH 8.0], washed three times with 100 μL UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) and then twice with 100 μL DS buffer (50 mM triethylammonium bicarbonate pH 8.5). The protein pellet was resuspended in 40 μL DS buffer containing 4 μg trypsin (V5113, Promega, Madison, WI, USA) and digested overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptides were desalted on C18 Cartridges [Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma Corporation, St. Louis, MO, USA], concentrated by vacuum centrifugation and reconstituted in 40 μL of 0.1% (v/v) formic acid. The peptide concentration was estimated by ultraviolet light spectral density at 280 nm using an extinction coefficient of 1.1 in 0.1 g/L solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins [35].

2.3. Peptide labeling and phosphopeptide enrichment

Equal amount (160 μg) of the tryptic digested peptide from each sample was labeled using the isobaric Tags for Relative and Absolute Quantitation (iTRAQ) Reagent-8 plex Multiplex kit according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The control samples were labeled with tags 113, 114, 115 and flooding samples with tags 116, 117, 118. The labeled peptide mixtures were freeze-dried in a vacuum concentrator, then dissolved in DHB buffer [0.6% (w/v) 2,5-dihydroxybenzoic acid (DHB) in 16% (v/v) acetonitrile (ACN) and 0.1% (v/v) trifluoroacetic acid (TFA)]. Total of 400 μg TiO_2 beads (GL Sciences, Inc., Tokyo, Japan) were added to the mixture, agitated for 40 min, and centrifuged at 5000*g* for 1 min [36]. The bead collection was washed three times each with washing buffer I [30% (v/v) ACN, 3% (v/v) TFA] and washing buffer II [80% (v/v) ACN, 0.3% (v/v) TFA]. The phosphopeptides were eluted with 50 μL of elution buffer [40% (v/v) ACN, 15% (v/v) NH_4OH]. The eluate was concentrated and reconstituted in 30 μL 0.1% (v/v) formic acid (FA) for mass spectrometry (MS) analysis.

2.4. Nano-liquid chromatography and tandem MS analysis

Each phosphopeptide mixture was separated using nano-liquid chromatography system Easy nLC1000 (Thermo Fisher Scientific, San Jose, CA, USA). Briefly, each sample was loaded onto a Thermo scientific EASY column (20 mm × 100 μm, 5 μm-C18, Applied Flow Technology, Inc., Colorado Springs, CO, USA) in buffer A [0.1% (v/v)