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Proteomic identification of differentially expressed proteins in the anoxic rice coleoptile

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ABSTRACT

Rice is the staple food for more than fifty percent of the world's population, and is therefore an important crop. However, its production is hindered by several biotic and abiotic stresses. Although rice is the only crop that can germinate even in the complete absence of oxygen (i.e. anoxia), flooding (low oxygen) is one of the major causes of reduced rice production. Rice germination under anoxia is characterized by the elongation of the coleoptile, but leaf growth is hampered. In this work, a comparative proteomic approach was used to detect and identify differentially expressed proteins in the anoxic rice coleoptile compared to the aerobic coleoptile. Thirty-one spots were successfully identified by MALDI-TOF MS analysis. The majority of the identified proteins were related to stress responses and redox metabolism. The expression levels of twenty-three proteins and their respective mRNAs were analyzed in a time course experiment.

Introduction

With the growth in world population, there has been an increasing demand for food. However, due to the environmental stresses that negatively affect plant growth and productivity, crop production is not increasing at the same rate. Environmental stress means economic hardship for farmers and may be life-threatening for regions that depend on subsistence farming. In order to increase crop yields and to expand cultivated areas, increased plant tolerance to environmental stress is therefore essential. Plants are sessile organisms that must develop different defense strategies against unfavorable conditions. These strategies are often mediated by changes in gene expression leading to the production of proteins that are thought to be possibly involved in tolerance (Timperio et al., 2008). One of the most important stresses in crops is flooding, which decreases oxygen and light supply to the submerged parts of plants, negatively affecting crop productivity (Bailey-Serres and Voesenek, 2010). Because gas diffusion in water is very slow, the submerged plant experiences drastic changes in oxygen, CO_2 availability as well as ethylene entrapment (Bailey-Serres and Voesenek, 2010). Oxygen diffuses ten thousand times slower in water compared to air (Armstrong, 1980). In submerged plants, leaves, as a result of photosynthesis, may have relatively high oxygen content, while roots are often very hypoxic (Bailey-Serres and Voesenek, 2010).

Rice (*Oryza sativa*) is the staple food of more than 50% of the world's population. The ability of rice seeds to germinate under anoxic conditions allows the direct sowing of rice, which is more economical that transplanting (Perata and Alpi, 1993; Magneschi and Perata, 2009). Indeed, in the tropics, rice sowing is usually performed by distributing seeds in paddy fields that are submerged by water (Yamauchi et al., 2000).

During rice germination, the coleoptile grows much faster when submerged compared to the aerobic coleoptile, enabling the seedling to reach the water surface and thus escape from the unfavorable low oxygen environment (Magneschi and Perata, 2009). This makes study of the rice coleoptile of interest for both plant biology and agronomy. No root and primary leaf growth is observed in rice seedlings germinated under anoxia, a trait that helps to save energy before the coleoptile tip reaches the water surface. Under low oxygen stress, a metabolic adjustment takes place to

Abbreviations: 2-DE, two-dimensional gel electrophoresis; ADF4, actin depolymerising factor 4; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ANPs, anaerobic proteins; APX, ascorbate peroxidise; CBB, Comassie brilliant blue; CBL, Calcineurin B-like protein; CIPKs, CBL-interacting protein kinases; GSTU, glutathione S-transferase; IEF, isoelectric focusing; IPG, immobilized pH gradient; MS, mass spectrometry; PDC, pyruvate decarboxylase; ROS, reactive oxygen species; sHSPs, small heat shock proteins; SOD, superoxide dismutase; TUBA1, tubulin α-1 chain; Usp, universal stress protein.

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cope with this unfavorable condition. One of the major changes is in the carbohydrate metabolism (Geigenberger, 2003; Licausi and Perata, 2009). Rice has the ability to degrade starch even under anoxia, due to the presence of amylolytic enzyme activities, which are either absent or inactive in other cereals such as wheat and barley (anoxic intolerant cereals) (Perata et al., 1998). These results in the use of the starchy reserves, with concomitant ATP production through the fermentative pathway, thus enabling rice grains to germinate even in the complete absence of oxygen (Guglielminetti et al., 1995).

A microarray analysis of gene expression in anoxic rice coleoptiles revealed a wide range of differentially expressed genes (Lasanthi-Kudahettige et al., 2007), indicating that anoxia could involve the cross-talk of pathways involved in different stresses from anoxia, such as heat. Indeed, heat acclimation can increase the anoxia tolerance of *Arabidopsis* seedlings (Banti et al., 2010). These studies shed light on some of the processes activated during low oxygen stress, but do not answer all of the pertinent questions.

Proteomic studies on plants exposed to low oxygen stress have shown that, in addition to the expression of classical anaerobic proteins (ANPs), there is also expression of proteins related to other mechanisms, such as reactive oxygen species (ROS) scavengers and protein synthesis in wheat (Kong et al., 2010), heat stress response in soybean (Hashiguchi et al., 2009), intracellular trafficking in maize (Chang et al., 2000), gibberellin biosynthesis, heme biosynthesis, and cell wall degradation in tomatoes (Ahsan et al., 2007).

Despite the extensive knowledge available on the effects of anoxia at a transcriptomic level, no adequate work has yet been done at a proteomic level in rice, with the exception of three reports by Mujer et al. (1993), Millar et al. (2004), and Huang et al. (2005). In the study by Mujer et al. (1993), rice seedlings were grown under anoxia immediately after seed imbibition; however, their identification was based on the immunoblotting of a very limited number of known ANPs. Millar et al. (2004) restricted their study to mitochondrial proteins extracted from rice seedlings grown for six days under anoxia and then transferred to air for one day, while Huang et al. (2005) grew seedlings in air and then exposed them to anoxia.

In this work, rice seeds were germinated in anoxia and the anoxic coleoptile proteome was compared to the aerobic coleoptile. We used anoxia because, under these experimentally controlled conditions, it is possible to identify changes that are due to the lack of oxygen only, uncoupling the metabolic and molecular adjustment in the coleoptile in response to anoxia from other consequences of submergence, such as ethylene entrapment by water (Bailey-Serres and Voesenek, 2010). Our aim was to identify differentially expressed proteins under anoxia and to follow their expression levels at different stages of coleoptile elongation.

Materials and methods

Seeds sterilization and growth

Rice (*Oryza Sativa*, cv. Arborio) seeds were surface sterilized using a diluted sodium hypochlorite solution (1.7%, v/v) and washed with distilled sterile water several times. Seeds were germinated at 28 °C in the dark in Petri dishes containing five sterile filter paper disks, soaked with 8 mL of distilled sterile water. For the anoxic treatments, seeds were germinated inside an anoxic chamber (Anaerobic System model 1025; Forma Scientific) after seed imbibition as described by Lasanthi-Kudahettige et al. (2007). The primary leaf and root did not elongate under anoxia. We selected four-day-old seedlings to perform the initial protein profiling, since at this growth stage both the aerobic and anoxic coleoptiles were large enough to enable them to be rapidly dissected from the seedlings. In the time course experiment, coleoptiles were collected from 3-, 4-, 5- and 6-day-old grown rice seedlings. Coleoptiles were dissected and immediately frozen in liquid nitrogen, and stored at −80 °C for protein and total RNA extraction.

Protein extraction and quantification

Soluble proteins were extracted from rice coleoptiles according to Yang et al. (2007), with some modifications. Coleoptiles were ground in liquid nitrogen and homogenized with 1 mL of extraction buffer (5 M urea, 2 M thiourea, 40 mM Tris–HCl, 2%, CHAPS, 50 mM DTT). The homogenates were centrifuged for 15 min at 15,000 × *g*. Supernatants were precipitated using TCA (15%, v/v) containing 0.007% β-mercaptoethanol in acetone at −20 °C for 2 h and then at 4 °C for a minimum of 2 h. Samples were centrifuged at 4 °C for 15 min at 14,000 × *g*, supernatants were discarded and pellets were washed twice with ice cold acetone containing 0.007% β-mercaptoethanol. Pellets were dissolved in a rehydration buffer (5 M urea, 2 M thiourea, 4%, CHAPS, 40 mM DTT). Protein quantification was performed using a Bradford-based assay kit assay (Bio Rad Hercules, CA), using bovine serum albumin as a standard.

2D electrophoresis

Isoelectric focusing (IEF) of total proteins was performed using 18 cm-long immobilized pH gradient (IPG) strips, pH 3–10 non linear and pH 4–7. The protein sample was mixed with a rehydration buffer, 0.5% IPG buffer (v/v) of respective pH range and 0.002% bromophenol blue to a final volume of 340 μL and loaded onto the IEF strips. Samples were loaded onto IPG strips by passive rehydration. For analytical gels, performed to obtain the silver-stained protein maps, 100 μg of the protein sample was loaded. Instead a 500 μg sample was loaded for the preparative gels stained with mass spectrometry (MS) compatible silver nitrate and 1 mg of the sample was loaded for Coomassie brilliant blue (CBB) staining. IEF was carried out at 200 V for 3 h, 1000 V for 1 h, 2000 V for 1 h, 3500 V for 1 h and 35 kV h using the Multiphore II system (Amersham Pharmacia Biotech). Before running the second dimension SDS-PAGE, IPG strips were equilibrated twice in an equilibration buffer (6 M urea, 30% glycerol (v/v), 50 mM Tris–HCl, 2%) SDS for 15 min. The first equilibration was done using 1.2% DTT (w/v) in an equilibration buffer, while in the second equilibration, DTT was replaced by 1.5% iodoacetamide (w/v). SDS-PAGE was performed using 12.5% polyacrylamide gels at 15 °C using a BioRad Protean II XI (20 cm × 20 cm) vertical gel electrophoresis chamber. After completion of the electrophoresis, gels were fixed and stained.

The analytical gels were stained for image analysis with silver nitrate as described by Oakley et al. (1980), while for the MS analysis, the preparative gels were stained with CBB according to the manufacturer's instructions, and MS compatible silver nitrate staining was performed as described by Mortz et al. (2001). Three independent biological replicates, each with four technical replicates were run for the analytical gels. In the time course experiment, three independent biological replicates were run, each with two technical replicates.

Gel image and statistical analysis

The silver stained analytical gels were scanned at 300 dpi resolution and saved as TIF images for image analysis. The two best gels were selected out of each biological replicate set of four. Spot detection and quantification were performed using Nonlinear Progenesis Same Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK, version 4.0). Images were subjected to manual as well as automatic alignment. Both aerobic and anoxic pairwise comparisons were performed and fold values as well as p-values for all spots