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Two aquaporins of *Jatropha* are regulated differentially during drought stress and subsequent recovery

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ABSTRACT

Jatropha has potential to be an important bio-fuel crop due to such advantages as high seed oil content and the ability to grow well on marginal lands less suited for food crops. Despite its ability to grow on marginal land, *Jatropha* is still susceptible to high salt and drought stresses, which can significantly reduce plant growth, stomatal conductance, sap-flow rate, and plant sap volume. This study was undertaken to collect basic knowledge of the physiological and molecular aspects of *Jatropha* response to salt and drought stresses, and to elucidate how *Jatropha* recovers from stress. From these studies we identified candidate genes that may be useful for the development of *Jatropha* cultivars that will grow efficiently in arid and barren lands. Of particular interest, two plasma membrane intrinsic proteins were identified: *Jatropha* plasma membrane intrinsic protein 1 (JcPIP1) and *Jatropha* plasma membrane intrinsic protein 2 (JcPIP2). The expression levels of *JcPIP1* were dramatically increased in roots, stems, and leaves during the recovery from stress, whereas the *JcPIP2* gene transcripts levels were induced in roots and stems during the water deficit stress. The protein levels of *JcPIP1* and *JcPIP2* were consistent with the gene expression patterns. Based on these results, we hypothesized that *JcPIP1* plays a role in the recovery events from water stresses, while *JcPIP2* is important in early responses to water stress. Virus induced gene silencing technology revealed that both *JcPIP1* and *JcPIP2* have positive roles in response to water deficit stresses, but have antagonistic functions at the recovery stage. We suggest that both *JcPIP1* and *JcPIP2* may play important roles in responses to water deficit conditions and both have potential as targets for genetic engineering.

Introduction

Jatropha belongs to the Euphorbiaceae family and grows widely in many tropical and sub-tropical areas such as Central and South America, Africa, India, and Southeast Asia. Due to its high seed oil content (50–60%), *Jatropha* has attracted significant attention as a potential bio-fuel crop. The seed oil composition consists of approximately 26% saturated fatty acids and 74% unsaturated fatty acids with high content of oleic acids (42–49%), making it suitable as a bio-fuel (Openshaw, 2000). Recent studies of this plant have focused on oil extraction, manipulation of oil composition, detoxification of seed cake, and removal of the carcinogen, curcin (Bouaid et al., 2012; Jang et al., 2012; Nithiyanantham et al., 2013). Through this genetic and molecular engineering, improvements have been made in *Jatropha*'s traits for higher yields and for improved oil composition to support a high rate of combustion as a biofuel (Fu-Li et al., 2008; Kajikawa et al., 2012; Qu et al., 2012).

For several decades, responses to salt and drought stresses in plants have been intensively investigated (Carmen Martínez-Ballesta et al., 2003; Chen et al., 2005; Yu et al., 2005). Under salt and drought stresses, plants show delayed growth rates from germination through development to mature plants (Chen et al., 2003; Kreps et al., 2002). Plants have evolved several ways to cope with from the harmful effects of stresses, such as the accumulation of compatible osmolytes to protect cells (Shinozaki and Yamaguchi-Shinozaki, 1996). Studies of water stress signaling have mostly focused on the signaling of salt stress, primarily because mechanisms of plant responses to salt stress and drought stress

Abbreviations: d, day; PIP, plasma membrane intrinsic protein; PM, plasma membrane; RT-PCR, reverse transcription-polymerase chain reaction; RWC, relative water content; VIGS, virus-induced gene silencing.

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are closely related and overlap (Xiong et al., 2002; Zhu, 2002). The signaling of salt and drought stresses can be classified into three functional categories: (1) ionic and osmotic stress signaling for the re-establishment of cellular homeostasis, (2) detoxification signaling to repair stress damage, and (3) signaling to coordinate cell division and expansion under particular stress conditions (Shinozaki et al., 2003).

The root is the most important organ for water absorption in plants. The uptake of water from the soil and its delivery to the xylem relies on the rate of water transport across living root tissues (Santoni et al., 2003). Water channel paths have been revealed that contribute to more than 50% of water uptake in the roots of a large variety of plant species (Javot et al., 2003). It has also been proposed that these paths might regulate the physiological events of the root in response to environmental stimuli, such as drought stress and salt stress (Zhang et al., 2007).

Plants have many genes encoding a variety of aquaporins. Aquaporins are present as multiple isoforms in gene families that may be ubiquitous in flowering plants. Aquaporins form water-selective channels that mediate a rapid trans-membrane water flow during growth and developmental processes (Eisenbarth and Weig, 2005). The physiologically indispensible relation between plants and water highlights the importance of these proteins. Aquaporins are crucial for the regulation of water balance and for the modifications that take place in water flux, including the responses to drought or salt stresses. These proteins are also implicated in many other physiological processes that include seed germination, cell elongation, stomatal movement, phloem loading and unloading, and reproductive growth, as well as stress responses. Furthermore, aquaporins are involved in plant adaptation and tolerance against water deficits (Luu and Maurel, 2004).

Thirty-five aquaporin homologs have been identified in the genome of *Arabidopsis* (Quigley et al., 2002). The plant aquaporin family is classified into four homologous groups based on their amino acid sequences and subcellular localizations: (1) plasma membrane intrinsic proteins (PIPs), (2) tonoplast membrane intrinsic proteins (TIPs), (3) nodulin 26-like intrinsic proteins (NIPs), and (4) small basic intrinsic proteins (SIPs) (Quigley et al., 2002). Recently, an additional subfamily has been identified in several dicotyledonous plants such as grapevine and named the X intrinsic proteins (XIPs) (Danielson and Johanson, 2008). The most abundant aquaporins in the vacuolar and plasma membranes (PMs) belong to the TIP and the PIP classes (Javot and Maurel, 2002). The PIP family includes 13 genes in *Arabidopsis*. Phylogenetic analysis supports the separation of the PIPs into two subgroups: PIP1 and PIP2 (Tyerman et al., 2002). In *Arabidopsis*, the PIP1 group has five members (PIP1;1–PIP1;5) and the PIP2 group has eight members (PIP2;1–PIP2;8). However, the issue of whether PIPs function as positive or negative regulators in the recovery from water deficits has not been clearly revealed. For instance, in *Arabidopsis*, PM aquaporins play an important role during recovery from water deficits. Martre et al. (2002) showed that transgenic plants expressing double antisense (dAS) constructs against *AtPIP1* and that *AtPIP2* aquaporins recovered more slowly from water deficit stress and their hydraulic conductance and transpiration rates were much slower than those of the control plants. It was also found that the leaf water potential was significantly decreased in dAS-PIP1 and dAS-PIP2 plants. Moreover, the over-expression of a heterologous *PIP1* gene in tobacco plants compromises their drought tolerance (Aharon et al., 2003).

While Zhang et al. (2007) recently reported that the *JcPIP2* gene is involved in drought response in *Jatropha*, studies on the effect of salt and drought stresses related to *PIPs* in *Jatropha* are still rudimentary. A whole genome sequence analysis was recently established for *Jatropha* (Sato et al., 2011) which will prove valuable in the study of the aquaporin gene family in *Jatropha*. Based on the high homology of *JcPIP1* to other *PIP1* genes, we speculated that the gene may have a role in the drought resistance of *Jatropha*. In this study, we have focused on the roles of these two aquaporins (JcPIP1 and JcPIP2) in drought-stress and recovery from stress in *Jatropha*, as an exploration of the possibilities for molecular breeding for drought-stress resistant traits in *Jatropha*. To discover the relationship between the aquaporins and the recovery of *Jatropha* from water deficit stress, we investigated the effect of salt and drought stresses on several aspects of physiological responses. We examined the expression of *JcPIP1* and *JcPIP2* during salt and drought stress at the transcriptional and translational levels. Additionally, we performed virus-induced gene silencing by VIGS-mediated gene knock down to examine the role of *JcPIP1* and *JcPIP2* in the recovery from water deficit stress.

Materials and methods

Plant material, growing condition and treatments

Jatropha curcas L. cv. 'Biji Jarak' seeds were germinated and grown in a plastic box containing vermiculite and water. Plants were grown in a controlled-environment chamber with a 16 h day (d)/8 h night cycle, and 60 μM m^{-2} s^{-1} light during the experiment. The d/night temperatures were set at 22 ± 1 °C and relative humidity was maintained at 65%. Twenty-d-old seedlings were transferred into a half-strength Hoagland solution for 7 d. The modified nutrient solution contained 1.25 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.5 mM KH_2PO_4, 75 μM FeEDTA, 50 μM H_3BO_3, 10 μM MnCl, 2 μM $ZnSO_4$, 1.5 μM $CuSO_4$, and 0.075 μM $(NH_4)_6Mo_7O_{24}$. Solutions were changed once every 3 d to avoid the excessive depletion of any particular ion. Four-week-old seedlings of uniform size were selected and transferred into Hoagland solution with NaCl or polyethylene glycol (PEG 3500) solutions for 6 d. The stress treatments were NaCl at 100 and 200 mM; or PEG at 20 and 30% (m/v). After salt or drought stress, the plants were transferred to normal conditions such as 1× Hoagland solution at 6 d for recovery. At harvest, seedlings were carefully washed and divided into four parts (root, stem and leaves). Each sample was immediately frozen with liquid nitrogen and stored at −70 °C until use.

Measurements of diverse physiological responses

Leaf stomatal conductance (mol m^{-2} s^{-1}) was measured using a Steady State Diffusion Porometer (Decagon Devices, USA). The porometer was attached to the true first leaf of each plant and measurement was taken from each plant between 11:00 AM to 12:00 AM daily. A heat-balance sap-flow gauge (Dynamax, Houston, TX) was used to measure sap flow rate through the main stems of *Jatropha*. A 10 mm stem-gauge was attached to the stem immediately above the cotyledons. Gauge signals were recorded using a data logger (Campbell Scientific, USA) and were collected at 1 min intervals and averaged over each 15 min interval to give hourly mean values. Moreover, we collected the sap of plants for 10 min at 3, 6, 9, and 12 d after treatments, to ascertain sap volume. Also, water loss from *Jatropha* was determined as relative water content (RWC) using values obtained for fresh weight and dry weight of tissue samples. RWC was measured at the heading stage for all genotypes. Three different flag leaves were randomly sampled and recorded. The fresh weight (FW) was immediately recorded after leaf excision. The leaves were incubated in distilled water for 24 h at 4 °C in darkness and the turgid weight (TW) was recorded. The dry weight (DW) was then measured after 48 h at 80 °C. The RWC was calculated according to Barrs and Weatherley (1962) as RWC (%) = [(FW − DW)/(TW − DW)] × 100.