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Research paper

Ethephon improved drought tolerance in maize seedlings by modulating cuticular wax biosynthesis and membrane stability

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ABSTRACT

Cuticular wax is the outermost thin hydrophobic layer covering the surface of aerial plant parts, which provides a primary waterproof barrier and protection against different environmental stresses. The aim of the present study was to investigate the role of ethephon, as an ethylene-releasing compound, in counteracting drought stress by modulating cuticular wax biosynthesis, water balance, and antioxidant regulation in maize seedlings. Our results showed that ethephon significantly increased the ethylene evolution rate, regulate the expression of cuticular wax synthesis regulatory gene *ZmERE* and the wax biosynthetic genes *ZmGL1*, *ZmGL15*, *ZmFDH1*, and *ZmFAE1*, and promote cuticular wax accumulation in maize seedlings under normal or drought stress conditions. Moreover, ethephon was shown to might markedly reduce water loss and chlorophyll leaching in leaves, and maintain higher relative water content and leaf water potential under drought stress. Ethephon significantly decreased malondialdehyde and hydrogen peroxide concentrations and electrolyte leakage, but increased the accumulation of proline and the activities of SOD, POD, and CAT. In addition, ethephon resulted in an increase in the ratio of root and shoot under drought stress. These results indicated that ethephon could improve maize performance under drought stress by modulating cuticular wax synthesis to maintain water status and membrane stability for plant growth.

1. Introduction

Drought is one of the most serious problems for sustainable agriculture because it affects plant growth and reduces crop yield worldwide. Meanwhile, plants have evolved versatile mechanisms for responses to drought stress, such as osmotic adjustment, cell membrane stability, and epidermal conductance (Seki et al., 2007; Mouillon et al., 2008). Notably, cuticular wax is the outermost thin hydrophobic layer covering the surface of aerial plant parts and is involved in controlling non-stomatal water loss and gas exchange (Riederer and Schreiber, 2001), preventing pathogen infection and insect attack (Kunst and Samuels, 2009), and resisting UV radiation (Solovchenko and Merzlyak, 2003). Among the multiple functions of cuticular wax, its most pivotal function is to provide a diffusion barrier against the uncontrolled loss or uptake of water and gases (Kerstiens, 2006). Moreover, increasing evidence has demonstrated that cuticular wax production is also closely associated with drought tolerance in plants (Aharoni et al., 2004; Zhang et al., 2007; Kosma et al., 2009). This indicates that cuticular wax accumulation is an important stress adaptation strategy to minimize cellular and organismal dehydration under drought stress conditions in plants (Kosma et al., 2009). Therefore, it is possible to regulate wax biosynthesis to enhance cuticular wax production using genetic engineering and agronomic management for improving drought tolerance in crops.

Cuticular wax consists mainly of very-long-chain fatty acids (VLCFAs, C20–C34) and their derivatives, such as aldehydes, alkanes, secondary alcohols, ketones, primary alcohols, and wax esters, and its biosynthesis occurs in epidermal cells (Kunst and Samuels, 2009). The C16 and C18 fatty acids synthesized in the plastids are further elongated up to C34 fatty acids by fatty acid elongase complexes located in the endoplasmic reticulum, and these enzyme complexes include β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase (KCR), β-hydroxyacyl-CoA dehydratase (HCD), and enoyl-CoA reductase (ECR), which respectively catalyze the sequential reactions,

Abbreviations: CAT, catalase; ERF, ethylene response factor; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase activity; ROS, reactive oxygen species; RWC, relative water content; SOD, superoxide dismutase; VLCFAs, very-long-chain fatty acids

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condensation, reduction, dehydration, and a second reduction (Kunst and Samuels, 2009; Haslam and Kunst, 2013; Lee and Suh, 2013). Moreover, the major cuticular wax biosynthetic steps have been established using *Arabidopsis* or wax-deficent mutants of crops, and their function has been proposed based on the phenotype of the corresponding mutants (Sturaro et al., 2005; Rowland et al., 2006; Lu et al., 2009). Among these genes, FATB, FAE1, KCS1, CUT1, FDH and GL8 are involved in the wax biosynthetic enzymes (Millar et al., 1999; Todd et al., 1999; Pruitt et al., 2000; Dietrich et al., 2005), others encode regulatory proteins, such as WIN1/SHN1, WXP1, OsWR1, and MYB 96 (Aharoni et al., 2004; Broun et al., 2004; Zhang et al., 2005; Seo et al., 2011; Wang et al., 2012).

The biosynthesis of plant cuticular wax, and its loading to the plant surface, represents a very complicated but actively regulated process (Lee and Suh, 2013). Transcriptional, post-transcriptional, and post-translational regulations are involved in cuticular wax biosynthesis, and transcriptional regulation of wax biosynthesis is thought to be the major regulatory mechanism for determining the total wax production in plants (Lee and Suh, 2015). For example, the ethylene response factor (ERF) WIN1/SHN1 can activate the wax biosynthesis by up-regulation of *KCS1*, *CER1*, and *CER2* genes in *Arabidopsis*, and over-expression of *WIN1/SHN1* improves drought tolerance (Aharoni et al., 2004; Broun et al., 2004). In addition, ERF protein OsWR1, a homolog of *Arabidopsis* WIN1/SHN1, activates cuticular wax biosynthesis through the up-regulation of *OsLACS1* and *OsFAE1-L* genes by binding directly to DRE and GCC cis-elements in their gene promoters (Wang et al., 2012). Furthermore, overexpression of genes encoding transcriptional activators, including *Arabidopsis* MYB96 and *Medicago truncatula* WXP1 and WXP2 that encode AP2/ERF-type transcription factors, can enhance cuticular wax biosynthesis and improve drought tolerance in transgenic *Arabidopsis* and alfalfa (Zhang et al., 2005; Seo et al., 2011; Lee et al., 2014). Therefore, transcription factors play an important role in regulating cuticular wax biosynthesis, and modulating transcription factors involved in this process would be an important strategy to enhance the adaptation to environmental stresses.

The phytohormone ethylene is a key signaling molecule in plants for regulating multiple developmental processes and stress responses. Ethylene production induced by drought stress has been shown to contribute to the drought response in rice (Wan et al., 2011). In addition, ethylene can enhance accumulation of compatible solutes and decrease oxidative stress to improve drought tolerance in *Arabidopsis* (Watkins et al., 2014; Cui et al., 2015). Lin et al. (2012) reported that ethylene decreases reactive oxygen species (ROS) accumulation induced by high salinity and enhances plant tolerance to salt stress. Moreover, ethylene promotes stomatal closure by promoting NADPH oxidase-mediated ROS production in stomatal guard cells (Desikan et al., 2006). In addition, transcriptional modulation is a pivotal process controlling the ethylene synthesis and signaling pathway, and ERF proteins are responsible for regulating the transcription of ethylene-regulated genes, which play vital roles in mediating plant growth, development, and response to stresses (Pirrello et al., 2012; Rashid et al., 2012). For instance, overexpression of genes encoding ERF protein, such as WIN1/SHN1, WXP1, and OsWR1, could modulate the expression of genes involved in wax biosynthesis and enhance drought tolerance in *Arabidopsis*, alfalfa, and rice (Aharoni et al., 2004; Zhang et al., 2005; Wang et al., 2012). However, it is unclear whether ethylene could induce the expression of ERF protein to modulate wax biosynthesis for plant responses to drought stress.

Maize is a globally important cereal crop due to its high yield potential, industrial uses, and suitability as livestock feed (Campos et al., 2004). Most maize-growing areas are rainfed, and the crops are often subjected to periodic water deficits resulting in decreasing yields (Boyer and Westgate, 2004). Moreover, maize is especially sensitive to drought stress due to its relatively sparse root system (Laboski et al., 1998), and this sensitivity can result in dramatic fluctuations in yield caused by frequent drought and poor irrigation managements. Ethephon, 2-chloroethyl phosphonic acid, is a synthetic bioregulator that favorably absorbs and directly releases ethylene into plant tissues, which has been most successful and used worldwide to control plant canopy size in maize production (Shekoofa and Emam, 2008). Accumulating evidence indicates that ethylene signaling plays an important role in the plant response to drought stress (Aharoni et al., 2004; Wan et al., 2011; Wang et al., 2012). Accordingly, exploring whether ethephon could improve drought tolerance in maize production is essential.

In this study, we investigated the role of ethephon application in counteracting drought stress in maize. It was hypothesized that ethephon improved drought tolerance in maize by modulating wax biosynthesis and membrane stability, leading to a higher plant water status for maintaining plant growth under drought stress conditions. To investigate this, we studied the effect of ethephon regulation on the expression of wax biosynthesis genes and cuticular wax accumulation, and analyzed plant water status in maize subjected to drought stress. In addition, antioxidant systems and membrane stability were investigated to further explore the role of ethephon in plant responses to drought stress.

2. Material and methods

2.1. Plant material and treatments

Maize seeds (*Zea may* L. cv. Zhengdan 958) were surface-sterilized for 10 min with 75% (v/v) ethanol solution and rinsed with sterilized distilled water five times. Four seeds were then sown in pots (15 × 15 × 20 cm deep) containing a mixture of vermiculite and commercial garden soil (1:1; v/v) and were grown in a greenhouse under 30 °C/20 °C and 14 h/10 h day/night conditions. After the seedlings reached the first true leaf stage, they were thinned to two plants per pot. Water was supplied sufficiently throughout and, thus, drought stress was avoided.

The seedlings were randomly divided into four treatments comprising two regulator treatments, including deionized water as control and ethephon (1.0 mM based on our preliminary experiment), and two water treatments, including normal water and drought stress. Deionized water and 1.0 mM ethephon (fresh dispensing) plus Tween 20 surfactant at 0.01% (v/v) were separately applied by foliar spray at the V_5 stage in maize seedlings. After that, drought stress treatments commenced by withholding water from seedlings until soil relative water content declined about 30%, and then supplementing daily water to maintain a relatively stable level of water stress. Drought stress was applied for 18 d, and the seedlings for well-watered treatments remained under normal water conditions during this period. There were 120 seedlings per treatment, and each treatment had three replications (40 seedlings per replication).

The fifth expanded leaf was labeled to sample for physiological and biochemical measurements after ethephon and water treatments. The samples were measured and collected after 6, 12, and 18 d of drought stress. At harvest, the plant was separated into shoots and roots, and then all samples were kept at 105 °C for 30 min and dried at 70 °C to determine the shoot and root dry weight. Fresh samples of all treatments were used for immediate assays or frozen in liquid nitrogen and stored at −80 °C for physiological and biochemical analysis.

2.2. Scanning electron microscopy

Scanning electron microscopy was used to study wax crystals on the abaxial surfaces of labeled leaves. Fragments of leaves were first fixed in 2.5% glutaraldehyde and mounted on stubs. Samples were coated with grain-size gold particles for 15 min. Coated samples were transferred to the scanning electron microscope (S-570; Hitachi, Japan) for examination.