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Water deficit stress tolerance in maize conferred by expression of an isopentenyltransferase (*IPT*) gene driven by a stress- and maturation-induced promoter

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ABSTRACT

Senescence can be delayed in transgenic plants overexpressing the enzyme isopentenyltransferase (*IPT*) due to stress-induced increased levels of endogenous cytokinins. This trait leads to sustained photosynthetic activity and improved tolerance to abiotic stress. The aim of this study was to generate and characterize transgenic plants of maize (*Zea mays* L.) transformed with the *IPT* gene sequence under the regulation of SARK promoter (protein kinase receptor-associated senescence). Three independent transgenic events and their segregating null controls were evaluated in two watering regimes (WW: well watered; WD: water deficit) imposed for two weeks around anthesis. Our results show that the WD treatment induced *IPT* expression with the concomitant increase in cytokinin levels, which prolonged the persistence of total green leaf area, and maintained normal photosynthetic rate and stomatal conductance. These trends were accompanied by a minor decrease in number of grains per plant, individual grain weight and plant grain yield as compared to WW plants. Plants expressing the *IPT* gene under WD had PGR, anthesis and silking dates and biomass levels similar to WW plants. Our results demonstrate that expression of the *IPT* gene under the regulation of the *SARK* promoter helps improve productivity under WD conditions in C_4 plants like maize.

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1. Introduction

Grain yield of most crops is predominantly limited by water availability. The stress caused by water deficit is a major environmental constraint to plant productivity due to its detrimental effects on plant growth and development. Given the current trends towards higher global temperatures and more pronounced regional and seasonal climatic changes in some areas, together with a tougher competition for other uses of available water (Vörösmarty et al., 2010), the development of crop varieties with enhanced tolerance to water deficit stress and higher water use efficiency has become a high priority for plant breeders and genetic engineers in order to meet future food demands.

In general, plants present a variety of strategies to cope with water deficit stress, which can have different impacts on yield, depending on the particular drought circumstances (Tardieu, 2012). On one hand, there are water conservation strategies aimed to decrease cumulative transpiration in order to prolonged water availability for plants, which include low stomatal conductance and different means of reducing leaf evaporative area, such as slow leaf growth rate and early leaf senescence. These strategies can have beneficial effects under severe drought conditions, but because they limit the capacity for photosynthesis and thus reduce biomass accumulation, they may impose high yield penalties under mild to moderate water deficit. On the other hand, strategies that promote high stomatal conductance and maintenance of high photosynthesis are more advantageous under mild to moderate water deficits, since they allow sustaining growth capacity during and after the stress. Moreover, maintaining high stomatal conductance helps to reduce leaf temperature, decreasing the deleterious effects of heat

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stress that usually accompany drought (Lopes et al., 2011; Tardieu, 2012).

Water deficit stress is known to alter hormone homeostasis in plants (Pospíšilová et al., 2003; Peleg et al., 2011), producing changes in gene expression necessary for their acclimation to the stress conditions. One of the first responses to drought is the accumulation of abscisic acid (ABA), which causes stomatal closure, thereby reducing water loss via transpiration and restricting cell expansion, and limiting CO_2 fixation (Wilkinson and Davies, 2002). Furthermore, several papers postulated jasmonic acid (JA) as a possible signaling molecule mediating the response to water stress in plants (Creelman and Mullet, 1995; Fujita et al., 2006). These studies concluded that there is a transient accumulation of JA associated with progressive accumulation of ABA under drought conditions. This trend, coupled with the fact that both ABA and JA share various transcription factors associated with responses to abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 2007), suggests that there is a cross-talk between ABA and JA in the signaling cascade that is triggered in water-stressed plants. Cytokinins (CKs), which participate in several aspects of plant development (e.g., seed germination, vascular development, meristem function, stimulation of photosynthesis and of sink strength) and counteract leaf senescence, are often considered as antagonists to ABA (Hare et al., 1999). In general, application of CKs to the leaf epidermis reverses ABA-induced stomatal closure and maintains normal transpiration rates in many plants (Ha et al., 2012; Pospíšilová, 2003). In maize, this phenomenon has been reported to occur in both young and old leaves (Blackman and Davies, 1985).

Water stress causes a restriction in the biosynthesis of CKs and a decrease in their concentration in the xylem sap (Chernyad'ev, 2005). These low CKs levels were associated with inhibition of growth, a decline in source/sink relationships and onset of senescence (Roitsch and Ehneß, 2000; Peleg et al., 2011). Consistent with a role of CKs in maintaining leaf health, it is well documented that application of exogenous CKs can delay leaf senescence (Chernyad'ev, 2005) and at the same time increase stomatal apertures and transpiration in many plants (Ha et al., 2012).

The first committed and rate-limiting step in the biosynthesis of cytokinins is catalyzed by the enzyme isopentenyltransferase (*IPT*). Previous work with transgenic tobacco has shown that the controlled expression of the *IPT* gene under the regulation of p*SARK*, a senescence- and stress-induced promoter from *Phaseolus vulgaris* (Delatorre et al., 2012), resulted in maintenance of high levels of CKs under water deficit stress, which ultimately led to improved drought tolerance (Rivero et al., 2007). Transgenic plants carrying p*SARK:IPT* showed enhanced photorespiration that helped protect the photosynthetic system under conditions of water stress (Rivero et al., 2010, 2009). These observations were later extended to other crop species such as rice (Peleg et al., 2011), peanut (Qin et al., 2011) and cotton (Kuppu et al., 2013), all representative of the C3 photosynthetic metabolism. Robson et al. (2004) studied the behavior of maize plants transformed with the *IPT* gene under the regulation of the *SEE1* promoter. *SEE1* encodes a maize senescence-enhanced protease (Griffiths et al., 1997). In their study they demonstrated a prolonged greenness of the transgenic plants that was accompanied by a delay in the loss of photosynthetic capacity with leaf age (Robson et al., 2004). However, they did not study the responses of these *IPT* transgenic plants under water deficit stress.

Maize is the cereal crop with highest worldwide production in terms of annual metric tons (FAO, 2014), and the majority of its cultivation is rain-fed, with limited possibilities for alleviating water deficit stress. Therefore, there is a need to develop drought-tolerant varieties either by conventional breeding or by genetic engineering. Maize is most susceptible to drought during flowering time, with the most severe reductions in yield occurring in the 3-week period bracketing male (anthesis) and female (silking) flowering events (Hall et al., 1982). Typically, maize plants exhibit protandry (i.e., anticipated anthesis respect to silking), and the main effect of water deficits that take place immediately before anthesis is to enhance the anthesis-silking interval (ASI), with the concomitant negative effects on ovary pollination (Hall et al., 1982; Bolaños and Edmeades, 1993). In addition, when water deficits persist during silking, significant abortion of fertilized ovaries takes place due to reduced assimilate availability for successful kernel set (Anderson et al., 2004). In this species, the mobilization of recently fixed carbon is an important determinant of plant and ear growth rates, because reserves stored before silking do not contribute to alleviate a reduction in current assimilate availability (Bruce et al., 2002) and to lessen kernel abortion (Schussler and Westgate, 1994). Thus, maintenance of functional source leaves during drought episodes seems crucial for minimizing the negative effects of this constraint on final grain yield and global productivity.

In the present study, we extend previous research on the protective effect of the controlled synthesis of cytokinins during water deficit stress to maize, a C_4 plant, and we scaled up from metabolic traits to final grain yield. We compared maize plants transformed with *pSARK:IPT* and their non-transgenic counterparts in terms of their photosynthetic capacity, growth rates and reproductive behavior under conditions of moderate water deficit.

2. Materials and methods

2.1. Plant material and transformation protocol

Maize embryogenic type II calluses (Décima Oneto et al., 2011) were initiated from immature embryos of the Hi-II genotype (Gordon-Kamm et al., 2002). Immature embryos were aseptically isolated from Hi-II seeds, 10 to 12 days post pollination. Embryos were cultured in the dark at 28 °C on 2,4-D N6 culture medium (Chu, 1975). Highly embryogenic calluses were sub-cultured to fresh N6 medium every two weeks.

The vectors *pSARK::IPT* and *pDM302* were used for maize transformation. The *pSARK:IPT* vector contains the cassette *SARK:IPT:NOS* which carries the *IPT* gene under the regulation of the *SARK* promoter and the nopaline synthase (*NOS*) terminator (Rivero et al., 2007). The *SARK:IPT:NOS* cassette was introduced as a Hind III/XhoI fragment into bases 276–373 of the TOPO TA cloning vector (Invitrogen). The co-transformation vector *pDM 302* contains the sequence of the *BAR* gene (phosphinothricin acetyltransferase PAT), under control of the *UBI* (maize ubiquitin) promoter and *NOS* terminator (Cao et al., 1992).

Ten-day subculture calluses were used for transformation assays. Four hours before co-bombardment assays, calluses were placed in osmotic medium (Oneto et al., 2010).

730 embryogenic calluses were co-bombarded using a high helium pressure particle gun device. The transformation protocol used was as described by Décima Oneto et al. (2010) and the parameters used were: 650 psi helium pressure, 6 cm explant target distance and −0.9 bar vacuum pressure. The DNA for co-bombardment was precipitated onto gold microparticles in a 1:3 ratio (based in number of base pairs) of *pSARK: IPT* and *pDM302* vectors, respectively. The calluses remained in osmotic medium for 16 h post-bombardment and were then placed in N6 medium for 10 days. Transgenic calluses were grown differentially in N6 selection medium containing increasing concentrations (3, 6 and 9 mg l^{-1}) of ammonium gluphosinate. Somatic embryos were developed in the transgenic calluses and cultivated in flasks containing MS medium without growth regulators. Seedlings were acclimated to move to the biosafety greenhouse until they completed their growth and reached maturity.

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