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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 pSARK, 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 pSARK: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.

The plants obtained by *In vitro* culture were grown in soil (3:1 fertile soil:peat) under growth chamber controlled conditions (500 μmol of photons m^{-2} s^{-1}, 16-h photoperiod, 26 °C) during 20 days (until the V3 stage).

BC2 seeds of three transgenic lines (SI2, SI4 and SI6) were sown and grown in soil (3:1 fertile soil:peat) using 12-liter pots under greenhouse controlled condition (650 μmol of photons m^{-2} sec^{-1},16 h photoperiod, 30–20 °C day/night). Plants from all germinated seeds were analyzed at the V4 stage by PCR to identify transgenic individuals from the segregating nulls.

2.2. Description of experiments

Three experiments were performed in consecutive years (2011, 2012, and 2013). BC2 seeds of SI2, SI4 and SI6 and their segregating nulls (N-SI2, N-SI4 and N-SI6) were sown in speedlings and grown in a greenhouse at 16 h photoperiod and 25 °C air temperature (Décima Oneto et al., 2010). In each experiment, seedlings of each type (transgenic and null) were transferred to 12 l pots filled with soil after germination, and were grown under 650 mmol of photons m^{-2} s^{-1}, 16 h photoperiod, 28–30/23–25 °C day/night air temperature. We evaluated the (i) SI6 genotype in 2011 (60 plants), (ii) SI2, SI4 and SI6 genotypes in 2012 (20 plants each), and (iii) SI4 (14 plants) and SI6 (10 plants) genotypes in 2013. During 2011, the experiments were performed only with line SI6 in order to adjust the multiple procedures and methods. During 2012, three lines of transgenic were used (e.g., SI12, SI14 and SI16) in order to compare 3 independent events. During 2013, two lines that showed the best performances were compared (e.g., SI14 and SI16).

Plants were watered daily (320 ml plant^{-1} day^{-1}) to keep the soil near 100% field capacity (FC) until 70 days after germination (pre-flowering stage). On that date they were sorted in equal number to two contrasting water regimes, one a control that was irrigated daily to keep soil at 100% FC and the other a severe water deficit. Plants in the second group were watered to keep soil at 30% FC for ten days, and subsequently at 10% FC for another ten days. After 20 days of water deficit, the pots were watered up to FC and plants kept under normal irrigation until final harvest. Soil moisture determinations were made gravimetrically during 2011 and 2012 experiments and by means of soil humidity sensors connected to dataloggers (Cavadevices.com, Buenos Aires, Argentina) during the 2013 experiment.

2.3. Measurements

In all experiments we measured: levels of *IPT* transgene expression, number of copies of the *IPT* transgene, chlorophyll content, photosynthesis rate, stomatal conductance, plant grain yield, grain number per plant and individual grain weight. Additional measurements were: (i) CKs, ABA and JA content (2012), and (ii) plant height, green leaf area and senescence parameters, anthesis–silking interval, plant and ear growth rates, biomass production and partitioning (2013). In order to simplify the Tables, when the results obtained with the nulls were similar, these results were averaged. We reported the null results individually when were significantly different.

2.4. PCR and Southern blotting

Total genomic DNA was extracted from −80 °C frozen maize leaves using the Dellaporta method (Dellaporta et al., 1983) for PCR assays and cetyltrimethyl-ammonium bromide (CTAB) (Saghai-Maroof et al., 1984) method for Southern blot assays. PCR was perfomed with de *ipt-forward* (5′CACGGGCCACTGGAAGC3′) and *ipt-reverse* (5′TGGCGTAACCTAATACATTCCG3′) pairs of primers. The conditions were: 35 cycles or 94 °C for 1 min, 59.5 °C for 1 min

and 72 °C for 1 min. About 20 μg of genomic DNA were digested with BamHI, resolved by electrophoresis in 1% agarose gel and transferred onto a nylon membrane (Hybond N+) as described previously (De León, 1994). The membrane was pre-hybridized for 3 h in hybridization buffer containing 0.2% SDS, 0.1% Sodium *N*-lauryl sarcosinate, 5X SSC, 1% blocker (Roche) and denatured herring sperm DNA (Promega). A 354 bp probe labeled with digoxigenin-11-deoxyuridine 5′triphososphate (Dig-dUTP) was prepared by PCR using *ipt354-forward* (5′CAACAAGTTACCCGACCAAG3′) and *ipt354-reverse* (5′TACATTCCGACCAAG3′) pairs of primers. The PCR conditions were: 35 cycles or 94 °C for 1 min, 56.5 °C for 1 min and 72 °C for 1 min. The membrane was hybridized overnight at 65 °C. The hybridized membrane was washed 3 times with 2 ×, 0.2 × and 0.1 × of SSC (pH 7.2) respectively and 0.1% m/v of sodiumdodecyl sulfate (SDS) at 60 °C. The hybridization signals were detected on film (Nitrocellulose) and UVP SYNGENE® system.

The expression pattern of the *IPT* gene in transgenic maize was measured using qPCR at three time points during the assay: before drought (normal watering), during drought (WD plants at 10% of normal watering) and after recovery from drought (rewatering of WD plants). For quantitative RT-PCR, total RNA was extracted from leaf tissues with Trizol (Invitrogen, USA). The RNAs were treated with RQ1 RNase-free DNase (Promega, USA). First strand complementary DNA (cDNA) was synthesized with oligo (dT) 18 as a primer and SuperScript III Reverse Transcriptase (Invitrogen, USA).

An Icycler IQ Real—Time Detection System (BioRad, USA) was used, with the 13S ribosomal protein as a reference gene (Joanin et al., 1993). The qPCR primers were designed using the Beacon Designer® 7.6 (PREMIER Biosoft International) software. The 13S transcript (100 pb) was amplified with the primers *13S-forward* (5′AAGGCGGTGGCGATAA3′) and *13S-reverse* (5′CTCTCCACAAGAATGAG 3′). The detection of products was performed using the IQ SuperMix PCR kit (BioRad, USA).

The PCR cycling condition comprised one cycle at 95 °C for 5 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 40 s. A melting curve was generated by the equipment to assure the specificity of the amplification reaction. For each sample the reaction was carried out with three replicates. The statistical analyses of the results were performed with the Relative Expression Software Tool REST© (Pfaffl et al., 2002).

2.5. Gas exchange measurements

To characterize the effects of water deficit stress on transpiration and photosynthetic performance, gas exchange measurements were performed on fully expanded leaves of transgenic plants and their corresponding nulls before, during and after applying the water regime treatments. Light saturated CO_2 assimilation rates and stomatal conductance were determined on intact leaves with a portable gas exchange system equipped with a blue-red light-emitting diode light source (LI6400 and LED 6400-02B, respectively; Licor Inc., Lincoln, Nebraska) fitted on the standard 6 cm^2 clamp-on leaf chamber. The photosynthetically active photon flux density (PPFD) necessary for saturating photosynthesis was previously examined in control plants by recording CO_2 assimilation rates at PPFDs varying from 0 to 1500 μmol m^{-2} s^{-1}. Assimilation rates reached saturation at approximately 600 μmol m^{-2} s^{-1}, consistent with growth in a greenhouse having a maximum PPFD of 650 μmol m^{-2} s^{-1} at canopy level, measured at noon on a sunny day. Measurements of gas exchange were performed between 10 am and 4 pm, varying the order in which individual combinations of plant, genotype and treatment were sampled to reduce the bias due to time of day. Before each measurement, the leaves were equilibrated in darkness in the gas exchange chamber for a minimum of 5 min until CO_2 assimilation and stomatal conductance reached a stable value.

Sample concentration of CO_2 (Ca) and leaf temperature were kept constant at 400 μmol mol^{-1} and 25 $^\circ$C, respectively. Flow rate was 500 μmol s^{-1}. To avoid interference of the natural process of leaf senescence during the sampling period, gas exchange was assessed on the youngest possible fully expanded leaf, which corresponded to leaves 10 or 11, and exceptionally to leaves 9 or 12.

2.6. Determination of chlorophyll content

Chlorophyll content was determined on leaves 7 and 9. The determinations were performed by weekly readings with a portable chlorophyll meter (Minolta SPAD-502) from pre-anthesis until harvest. The SPAD readings were taken as an average from measurements obtained at three points of the leaf (proximal, middle and distal). The SPAD data were converted to total chlorophyll mass per unit area by comparison with a curve constructed by quantifying the total chlorophyll content of 20 mm^2 leaf disks extracted overnight with N,N dimethylformamide and determined spectrophotometrically using the equations of Wellburn (1994).

2.7. Quantification of cytokinins, abscisic and jasmonic acid

Leaf tissue from the SI6 and SI4 transgenic lines were collected during the 2012 experiment at different times along the water regime treatments (before, during and after treatment), and were immediately freeze dried. Extraction and determination of CK, ABA and JA contents from 100 mg of freeze-dried tissue were done by using a liquid chromatography–tandem mass chromatography system (model 2695; Quattro Ultima Pt; Waters) as described by Dobrev and Kamiínek, 2002.

2.8. Plant development, growth, grain yield determination and source/sink relationships

Transgenic plants and their segregating null plants were tagged at V3 for plant phenotyping. During 2013, green leaf area (GLA) of each fully expanded leaf was estimated weekly between V4 and R4 for all plants. For this purpose, the area of individual leaves was computed as the product between maximum length, maximum width and 0.75 (Dwyer et al., 1991). For each observation date, the total GLA of each plant (TGLA) was obtained as the sum of all its GLAs.

Anthesis (first anther visible in the tassel) and silking (R1, first silk visible in the ear) dates were registered in all experiments for each tagged plant and the anthesis-silking interval (ASI) was computed. Maximum plant height from the base of the stalk to the base of the tassel (2012 experiment) or to the last ligulated leaf (2013 experiment) was measured on all plants as indicative of treatment effects on tissue expansion capacity. During the 2013 experiment, the biomass of all tagged plants was estimated immediately before tasseling (i.e. before the start of water regime treatments), R1 (during treatment) and R2 (after treatment) by means of an allometric model based on the relationship between plant biomass and morphometric variables. This approach has been widely used to describe biomass of hybrids and inbreds growing under different abiotic stress conditions (D'Andrea et al., 2006; Echarte and Tollenaar, 2006; D'Andrea et al., 2009; Cicchino et al., 2010). In order to build the allometric models, 15 plants of varying size (i.e., plant height and stem base diameter) were collected along the cycle of each line, 9 of them between R1 and R2. Morphometric measurements included (i) stem diameter at the base of the stalk (as an average between maximum and minimum size) and plant height from ground level to the ligule of the last expanded leaf, and (ii) maximum diameter and length of the ear (only when it was present in R1 and R2). The plants were processed and dried at 60 $^\circ$C in an oven until constant weight to determine ear (when

present) and plant biomass. Established relationships between ear biomass and ear volume, and plant biomass (without ear) and stem volume, were used to estimate the corresponding biomass of all tagged plants grown in the greenhouse, on which we performed the same stem and ear measurements on the dates of interest. For these plants we estimated shoot growth rate (PGR, g plant^{-1} day^{-1}) and ear growth rate (EGR, in g plant^{-1} day^{-1}) between (i) the initiation of drought (immediately before tasseling) and silking (R1), and (ii) R1 and silking + 15 days (R2).

In all experiments, all tagged plants were harvested at physiological maturity (R6) for the determination of plant grain yield (PGY, g plant^{-1}), grain number per plant (GNP), and individual grain weight (GW, in mg; as the quotient between PGY and GNP). Shoot biomass at harvest was measured only during 2013. In this experiment, biomass partitioning to reproductive organs was obtained (i) for the treatment period, as the quotient between EGR and PGR, and (ii) for the whole cycle, as the quotient between PGY and total plant biomass at R6. The latter is known as harvest index (HI), and is considered an important trait in breeding of grain crops (Hay, 1995).

The source/sink relationship was determined as the ratio between the leaf green area at the end of the WD period and the number of kernels per plant.

2.9. Statistical analysis

The Sigma Stat program (Di Rienzo et al., 2011) was used for all statistical analysis. The normality of the data was analyzed by the Kolmogorov–Smirnov test. An ANOVA was carried out in cases where the data had normal distribution. The differences ($p < 0.001$) found after conducting a two-way ANOVA test, were analyzed by Tukey's test ($\alpha = 0.05$). In cases where the results did not show a normal distribution, a non-parametric analysis (Kruskal Wallis) was performed.

3. Results

3.1. Generation of SARK:IPT transgenic plants

From 5 bombardment experiments, 26 calli were obtained with differential growth in selective medium. The presence of the *IPT* transgene was confirmed in 19 of them by PCR. Fertile T$_0$ transgenic plants were obtained and crossed with inbred BLS14 (pollinator) in order to ensure the descent of transgenic plants. The two subsequent generations were obtained by backcrossing (BC1 and BC2), always in a biosafety greenhouse. BC1 seeds from 10 *SARK:IPT* events were obtained, and the BC2 generation advanced from BC1 plants of the events SI2, SI6 and SI4.

3.2. Evolution of soil water content

Measurements of soil water potential showed that the soil of water-stressed plants dried similarly among the different experiments (2011, 2012 and 2013) (Fig. 1), reaching its lowest value 15 days after irrigation was withheld and recovered to near FC after re-watering in all the experiments. Not significant differences between substrates of transgenic and null plants, at the same water regime, were found ($p = 0,765$).

3.3. IPT gene expression pattern and number of copies

While low levels of expression were seen before drought, *IPT* expression increased significantly during the water stress period and reached a maximum during the last day of stress (Fig. 2). At this stage, the transgenic plants did not display stress symptoms. Southern blot analysis fully confirmed the PCR results and showed
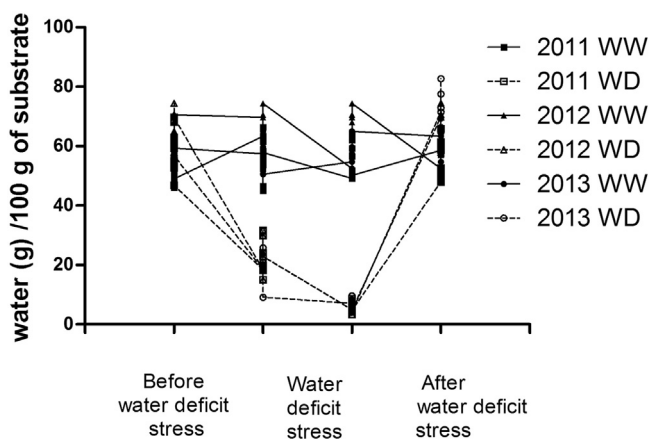
Fig. 1. Soil water potential in well watered (WW) and water deficit (WD) treatments in 2011, 2012 and 2013 experiments. Values are the mean (nWW$_{2011,2012and2013}$ = 15, nWD$_{2011,2012and2013}$ = 15).
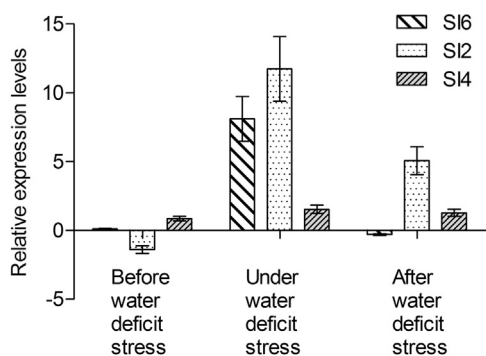

Fig. 2. Relative *IPT* expression levels before water deficit (WD), during WD and rewatering periods in SI2, SI4 and SI6 P$_{SARK-IPT}$ transgenic plants. Values are the mean ± SE (nSI2 = 20, nSI4 = 34, nSI6 = 45).

that the number of inserted copies varied across the BC2 generation of the transgenic lines from 12 to aproximately 20 per genome (Fig. 3) (Table 1). Those events which had lower number of *IPT* transgene copies were selected to water deficit stress experiments (SI2,

Table 1
Number of *IPT* inserted copies in SI2, SI4 and SI6 P$_{SARK-IPT}$ transgenic plants. [1]T$_0$: first generation of P$_{SARK-IPT}$ transgenic plants. [2]BC$_2$: subsequent generation of P$_{SARK-IPT}$ transgenic plants obtained by backcrossing BC$_1$ with inbred BLS14 (pollinator).

Transgenic event	Number of *IPT* inserted copies	
	T$_0$[1]	BC$_2$[2]
SI2	20	12
SI4	12	9
SI6	6	4

SI4 and SI6) (Fig. 3). These plants were derived from a BC2 and are not genetically settled.

3.4. Cytokinin and ABA contents related to IPT expression

For the hormone level analysis we evaluate the two events which showed higher *IPT* transgene expression (SI2 and SI6). During water deficit treatment, there was a significant increase (p <0.001) in active CKs, especially transzeatin. Transzeatin concentrations increased 50% with respect to null plants under water deficit and there were no differences with respect to well watered null plants (Table 2). Increases (up to 35%) in the concentrations of the N- and O-glycosylated forms were found in transgenic plants during water deficit (Table 2). ABA accumulation (up to 3 fold) was detected in null and SI2 transgenic plants under water deficit, but not in SI6 transgenic plants (Table 2). SI6 transgenic plants during stress had ABA levels similar to that of well watered plants. The level of JA increased only among null plants after the WD period (Table 2). CK production, however, was not affected by ABA or JA levels, and there were no clear differences between the ABA content of the null and *pSARK:IPT* transgenic plants. These trends indicated that changes in ABA were not associated with the drought tolerant phenotype displayed by the transgenic plants.

3.5. Effects of pSARK:IPT on photosynthesis and stomatal conductance

Maximum carbon assimilation rates were similar in well watered transgenic plants and their respective null plants, ranging from 15 μmol m^{-2} s^{-1} in event SI2 to 22 and 24 μmol m^{-2} s^{-1}
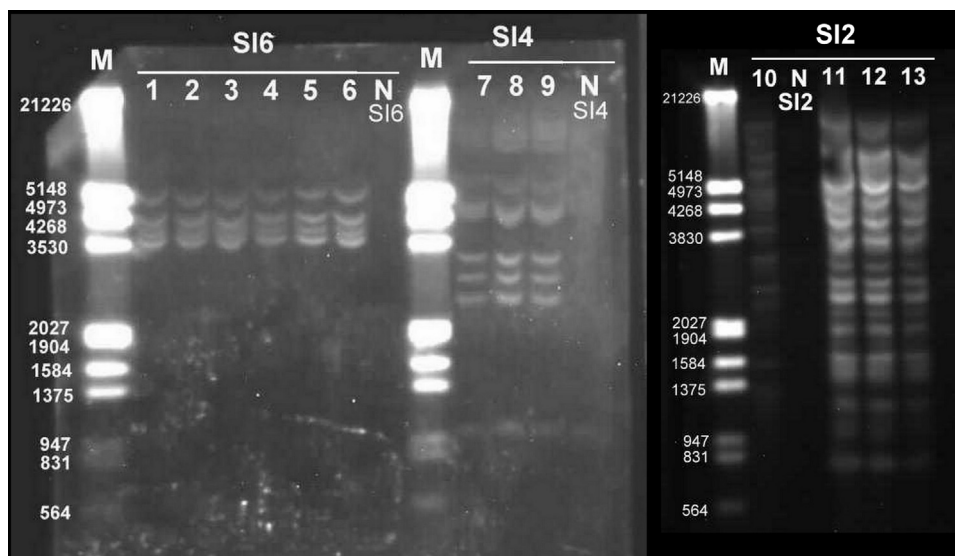
Fig. 3. Southern blot analysis of 6 plants BC2 SI6 (wells 1 to 6), 3 plants BC2 SI4 (wells 7 to 9) and 4 plants BC2 SI2 (wells 10 to 13). The genomic DNA was digested with BamHI; each band is considered an insertion site. All plants of the same event have the same gene insertion pattern, as expected. M: DNA molecular weight marker III, Digoxigenin labeled; NSI6 = null plant of SI6, NSI4 = null plant of SI4 and NSI2 = null plant of SI2.

Table 2
Treatments effect on the concentration of evaluated hormones (experiment 2012). TZ = transzeatin, N-glc = N-glucoside, O-glc = O-glucoside, ABA = abscisic acid, JA = Jasmonic acid. [1] WW: well watered (plants permanently irrigated). WD: water deficit (plants water-stressed during the drought period). [2] Null: Indicates an average of NSI2 and NSI6. Values are the mean (nNULL = 40, nSI2 = 20, nSI6 = 20). Asterisks indicate significant differences (*$p < 0,05$ **$p < 0,01$ ***$p < 0,001$) between transgenic WD and null WD.

Period	Water regime	Genotype	TZ	N-glc	O-glc	ABA	JA
			p mol g^{-1} fresh weight				
Pre water deficit	WW[1]	Null[2]	1.86	2.84	11.1	44.71	8.01
		SI2	1.46	2.63	13.23	29.15	12.44
		SI6	1.91	2.55	11.68	39.5	9.85
Water deficit	WW	Null	1.93	2.9	14.12	36.71	5.77
		SI2	1.65	3.08	13.19	32.5	6.31
		SI6	1.89	2.97	12.47	22.8	8.34
	WD	Null	0.66	2.53	12.99	150.8	17.42
		SI2	1.22*	4.2*	15.75	179.78	8.88*
		SI6	1.58*	3.59*	16.41	47.6*	7.01*
Post water deficit	WW	Null	3.14	3.03	15.33	31.55	10.07
		SI2	2.38	2.95	17.78	24.12	9.7
		SI6	3.17	2.6	17.15	32.78	6.97
	WD	Null	3.06	2.21	10.7	36.7	16.32
		SI2	2.86	4.08**	18.58**	60.5*	8.89***
		SI6	7.58***	2.55	27.72**	37.6	7.37***

Table 3
Treatments effect on gas exchange and chlorophyll content at pre-water stress, under water stress and post-water stress period.

Period	Treatment	Genotype	Stomatal Conductance			Photosynthesis			Chlorophyll[a]		
			2011	2012	2013	2011	2012	2013	2011	2012	2013
			mmol H$_2$O m^{-2} s^{-1}			µmol CO$_2$ m^{-2} s^{-1}			mg g^{-1}		
Pre water stress	WW[b]	NULL[d]	0.162	0.119	0.178	18.9	19.82	24.75	5.3	6.83	3.11
		SI2	–	0.106	–	–	16	–	–	6.12	–
		SI4	–	0.116	0.215*	–	19.53	25.07	–	5.84	3.14
		SI6	0.147	0.126	0.127	20.21	24.63	20.98	5.89	7.64	3.47
Water stress	WW	NULL	0.124	0.152	0.1	19.01	20.3	22.24	5.5	6.43	3.13
		SI2	–	0.128	–	–	18.13	–	–	7.17	–
		SI4	–	0.169	0.083	–	17.13	20.57	–	6.91	3.3
		SI6	0.152	0.142	0.124	22.01	23.53	24.26	5.9	8.67	3.44
	WD[c]	NULL	0.023	0.075	0.032	10.87	4.85	8.1	2.8	3.6	1.79
		SI2	–	0.119**	–	–	22.53***	–	–	7.76**	–
		SI4	–	0.172**	0.092*	–	22.76***	20.28**	–	6.91**	3.4*
		SI6	0.175***	0.169***	0.129**	21.87**	22.63***	24.87**	6.2**	8.67**	3.38*
Post water stress	WW	NULL	0.172	0.196	0.106**	19.23**	18.05	22.86	6.1**	7.42	3.01*
		SI2	–	0.128	–	–	22.62	–	–	7.84	–
		SI4	–	0.186	0.104	–	21.8	24.26	–	6.72	3.2
		SI6	0.177	0.188	0.201	23.76	23.6	25.74	5.8	7.56	3.42
	WD	NULL	0.078	0.091	0.081	14.22	13.74	14.17	2.3	4.07	2.1
		SI2	–	0.156**	–	–	19.6**	–	–	8.04**	–
		SI4	–	0.203***	0.102*	–	21.96**	21.52**	–	5.83*	3.29*
		SI6	0.127*	0.135**	0.113*	19.87**	20.83**	22.66**	6.1*	9.8***	3.12*

Asterisks indicate significant differences (*$p < 0,05$ **$p < 0,01$ ***$p < 0,001$) between transgenic WD and null WD.
[a] Chlorophyll values are the mean of leave 7.
[b] WW: well watered (plants permanently irrigated).
[c] WD: water deficit (plants water-stressed during the drought period).
[d] Null: Indicates an average of 2011:NSI6; 2012: NSI2, NSI4 and NSI6; 2013: NSI4 and NSI6. Values are the mean (nNULL$_{2011}$ = 30, nSI6$_{2011}$ = 30, nNULL$_{2012}$ = 60, nSI2$_{2012}$ = 20, nSI4$_{2012}$ = 20, nSI6$_{2012}$ = 20, nNULL$_{2013}$ = 34, nSI4$_{2013}$ = 14, nSI6$_{2013}$ = 10).

in events SI4 and SI6, respectively. Water deficit caused a marked decrease in photosynthetic performance of null plants, especially in NSI4 and NSI6, where CO$_2$ assimilation dropped to approximately 50% of the initial value during the water deficit period (Table 3). These plants recovered partially after rewatering, attaining CO$_2$ assimilation rates within 80% of the initial well watered values. In comparison, plants NSI2 showed a stronger decrease of photosynthetic rate during the post-stress period.

All transgenic plants under water deficit stress maintained CO$_2$ assimilation rates similar to those of well watered plants. During the water deficit period, events SI4 and SI6 showed significantly ($p < 0.001$) higher photosynthetic rates than the corresponding null plants. These differences reached approximately twice the value of the null plants. For the event SI2 these differences were observed during the post-water deficit period, due to the low photosynthetic performance of the null plant in this period (Table 3).

In general, stomatal conductance showed the same trend as CO$_2$ assimilation, with all three transgenic events maintaining stomatal conductances under water deficit similar to those of the well watered controls, whereas in the segregating nulls the values decreased significantly under water deficit and did not recover fully one week after resuming normal watering (Table 3).

3.6. Effects of pSARK::IPT on plant senescence during water deficit

Before water deficit, null and transgenic plants had similar chlorophyll content (Table 3). During the water deficit period, the WD plants of the three transgenic lines (SI6, SI2 and SI4) maintained chlorophyll levels similar to those of WW plants. However, chlorophyll content of WD null plants decreased significantly (up to 40%; $p < 0.001$). This decrease was observed in all measured leaves (data not shown), and the observed trend remained constant after rewatering. At this time, transgenic plants subjected to WD
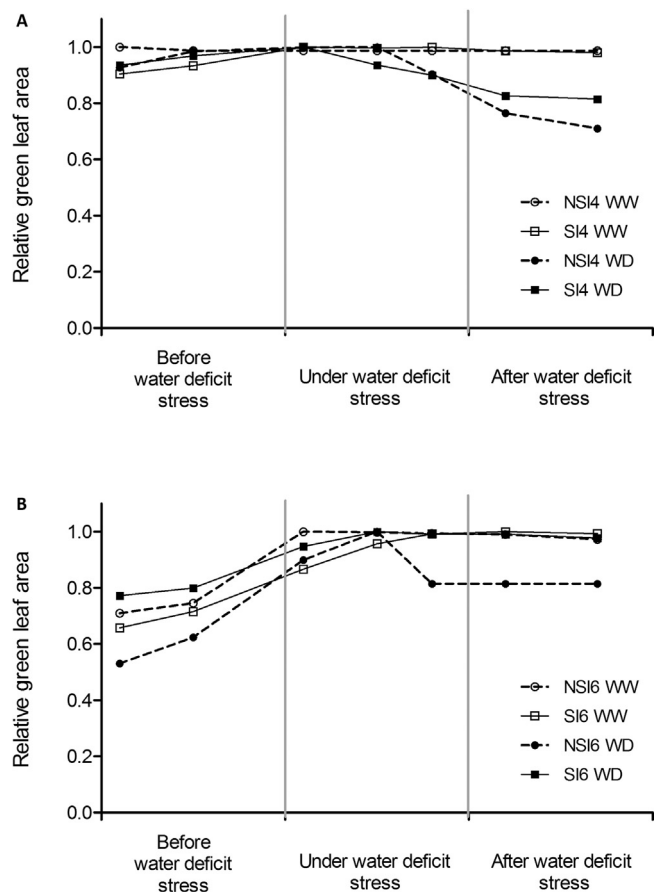
Fig. 4. Relative green leaf area before water deficit (WD), during WD and rewatering periods in (A) SI4 and (B) SI6 $P_{SARK-IPT}$ transgenic plants (2013 experiment). Values are the mean ± SE ($nSI4 = 14$, $nSI6 = 10$).

held chlorophyll values similar to that of their WW counterparts (Table 3).

Leaf expansion of all plants took place until tasseling, when they reached their maximum Total Green Leaf Area (TGLA). Treatments, however, did not affect this process markedly until this stage (Fig. 4). From this stage onwards, significant differences ($p < 0.001$) were detected in TGLA between the null and transgenic plants under drought, which were linked exclusively to leaf senescence. The TGLA decreased severely among all null plants and SI4 plants (up to 25%) under WD, but no difference was detected for this trait between WW and WD SI6 plants (Fig. 4). In fact, the TGLA of SI6 under WD was the same as that of WW SI6 and null WW plants, without effects of water stress in this case (Fig. 4).

At the end of the water deficit period, WD plants of SI4 reached a 15% decrease of their maximum TGLA, while the null plants had a decreased of 20% in this trait. No reduction was detected in maximum TGLA at this time among WW plants.

Finally, it was observed that SI4 plants did not continue decreasing its GLA after the WD period. In contrast, the rate of senescence continued among null plants of SI4 after rewatering, and reached the maximum decline in TGLA (30%) of all evaluated genotypes (Fig. 4).

3.7. Effects of pSARK:IPT on development, growth, source/sink relationships and production traits

Water deficit caused a delayed in most flowering events (Table 4), but this negative trend was more pronounced for female flowering (silking) than for male flowering (anthesis). Conse-

quently, ASI values usually increased among stressed plants, except for transgenics SI16 (Table 4; Fig. 5). The largest difference in ASI caused by WD stress (+12.3 days as compared to the WW condition) corresponded to null plants of SI6 during 2011 (6 days for WW and 18.3 days for WD). The trend was similar for the SI2 genotype, but less pronounced (2012) or even opposite (ASI transgenic > ASI null in 2013) for the SI4 genotype.

Transgenic plants of both SI4 and SI6 tended to be shorter than their null counterparts in potential growing conditions (i.e., WW treatment), and water deficit caused a reduction in plant height for all genotypes (Table 5). However, the effect of water stress on tissue expansion, was always larger among null plants (−10.8% for SI4 and −18.9% for SI6) than among the transgenic plants (−8.2% for SI4 and −6.8% for SI6). Plant and ear growth rates (PGR and EGR) during the treatment period of 2013 decreased due to water deficit effects among plants of the SI4 genotype (Table 5). The decrease in PGR was −52% and −25% for null and transgenic plants, respectively, and it reached similar values for EGR (−56% and −20%, respectively). This trend held among null plants of SI6 (−42% in PGR due to WD stress) but not among their transgenic counterparts (no effect of WD stress on PGR). Interestingly, water deficit caused no negative effect on EGR of the SI6 genotype (Table 5). Mean values of biomass allocation to the ear (i.e., the EGR/PGR ratio) were always larger for transgenic than for null plants of SI4, but not among those of SI6 (Table 5). Nevertheless, due to the described trends in PGR and EGR in response to drought, biomass allocation to the ear (i) did not vary markedly among plants of the SI4 genotype (range between 0.58 and 0.70), and (ii) always increased among WD plants of the SI6 genotype (null and transgenic).

Treatments had a clear effect on final productivity traits (Table 6), represented by total plant biomass and grain yield (PGY). On one hand, transgenesis *per se* usually had no negative effects among plants of the SI4 genotype, for which productivity traits did not differ between null and transformed individuals under WW conditions (Table 6). The exception to this trend was transgenic plants of the SI6 genotype, which exhibited reduced potential productivity (i.e. under WW) as compared to their null counterparts during 2013 (Table 6). On the other hand, water deficit caused a clear decrease in productivity traits of all genotypes, except among transgenic plants of the SI6 (no difference detected between WW and WD). Independently of these considerations, WD effects on total plant biomass were always larger among null (−44% for SI4 and −40% for SI6) than among transgenic plants (−26% for both SI4 and no effect for SI6), and these trends increased for PGY (nulls: −75% for SI4, and −66% for SI6; transgenics: −28% for SI4 and no effect for SI6; averaged across experiments). The decrease in biomass allocation to harvestable organs caused by WD stress was evident as a marked decrease in harvest index (Table 6), which was largest among null genotypes (−63% for SI4 and −49% for SI6) and smallest among the transgenic ones (−14% for SI4 and no effect for SI6).

Most part of the variation in PGY was explained ($r^2 = 0.90$, $n = 24$, $p < 0.01$; across experiments × genotypes × water regimes) by the variation registered in the number of grains set per plant (Fig. 6) and not in individual grain weight (Table 6). Consequently, percent variation in GNP in response to drought followed the trend described for PGY, largest for null genotypes (−71% for SI4 and −56% for SI6) and smallest for their transgenic counterparts (−26% for SI4 and no negative effect for SI6).

Whereas leaf area is crucial for the production of biomass, due to photosynthesis, and because WD was imposed in a period when the number of grains around flowering was established, it was necessary to study the leaf area and its relation to the number of grains. The effect of *IPT* expression and the concomitant CK synthesis on source/sink relationships was determined as the ratio between the leaf green area at the end of the WD period and the number of ker-

Table 4

Treatments effect on flowering characteristics. [1] Anthesis-silking interval (difference between silking and anthesis dates) [2] WW: well watered (plants permanently irrigated). [3]WD: water deficit (plants water-stressed during the drought period). Values are the mean $\pm$ SE (nNSI6$_{2011}$ = 30, nSI6$_{2011}$ = 30, nNSI2$_{2012}$ = 20, nSI2$_{2012}$ = 20, nNSI4$_{2012}$ = 20, nSI4$_{2012}$ = 20, nNSI6$_{2012}$ = 20, nSI6$_{2012}$ = 20, nNSI4$_{2013}$ = 14, nSI4$_{2013}$ = 14, nNSI6$_{2013}$ = 10, nSI6$_{2013}$ = 10). Asterisks indicate significant differences ($*p < 0.05$ $**p < 0.01$ $***p < 0.001$) between transgenic WD and null WD.

| Experiment	Genotype	Water regime	Anthesis date	Silking date	ASI[1] (days)
2011	Null SI6	WW [2]	30-Aug $\pm$ 2.1	5-Sep $\pm$ 1.6	6 $\pm$ 1.8
		WD	1-Sep $\pm$ 2.7	19-Sep $\pm$ 2	18.3 $\pm$ 2.3
	SI6	WW	29-Aug $\pm$ 1.3	7-Sep $\pm$ 1.7	9.8 $\pm$ 1.5
		WD	31-Aug $\pm$ 0.8	9-Sep $\pm$ 3.2	9.5 $\pm$ 2.1***
2012	Null SI2	WW	24-Jul $\pm$ 2.8	1-Aug $\pm$ 3.1	6.4 $\pm$ 1.1
		WD	27-Ju $\pm$ 4.3	12-Aug $\pm$ 2.5	10.5 $\pm$ 1.6
	SI2	WW	24-Jul $\pm$ 5.1	3-Aug $\pm$ 6	7.1 $\pm$ 1.1
		WD	25-Jul $\pm$ 2.6	5-Aug $\pm$ 2.4	7.8 $\pm$ 1.4*
	Null SI4	WW	28-Jul $\pm$ 2.7	3-Aug $\pm$ 2.1	6.3 $\pm$ 2.4
		WD	28-Jul $\pm$ 3.3	6-Aug $\pm$ 4.8	9.4 $\pm$ 4.1
	SI4	WW	29-Jul $\pm$ 0.9	3-Aug $\pm$ 3.5	5.6 $\pm$ 2.3
		WD	28-Ju $\pm$ 1.5	5-Aug $\pm$ 2.5	8.8 $\pm$ 2.7
	Null SI6	WW	24-Jul $\pm$ 3.1	4-Aug $\pm$ 2.2	11.1 $\pm$ 2.6
		WD	24-Jul $\pm$ 1.7	14-Aug $\pm$ 3.3	21.3 $\pm$ 2.5
	SI6	WW	25-Jul $\pm$ 1.2	5-Aug $\pm$ 2.3	11.4 $\pm$ 1.7
		WD	24-Jul $\pm$ 3.2	3-Aug $\pm$ 3.1	10 $\pm$ 3.1***
2013	Null SI4	WW	04-may $\pm$ 1.5 d	10-may $\pm$ 2.5 d	5.6 $\pm$ 2.4
		WD	06-may $\pm$ 3.1 d	11-may $\pm$ 5.0 d	6.0 $\pm$ 2.7
	SI4	WW	06-may $\pm$ 2.7 d	10-may $\pm$ 3.5 d	4.4 $\pm$ 1.7
		WD	06-may $\pm$ 2.6 d	12-may $\pm$ 6.1 d	7.5 $\pm$ 5.3
	Null SI6	WW	04-may $\pm$ 0.5 d	11-may $\pm$ 2.2 d	6.7 $\pm$ 2.2
		WD	08-may $\pm$ 1.9 d	21-may $\pm$ 4.9 d	13.8 $\pm$ 3.9
	SI6	WW	09-may $\pm$ 4.8 d	16-may $\pm$ 4.1 d	7.0 $\pm$ 1.6
		WD	09-may $\pm$ 6.2 d	17-may $\pm$ 6.1 d	5.8 $\pm$ 1.6**
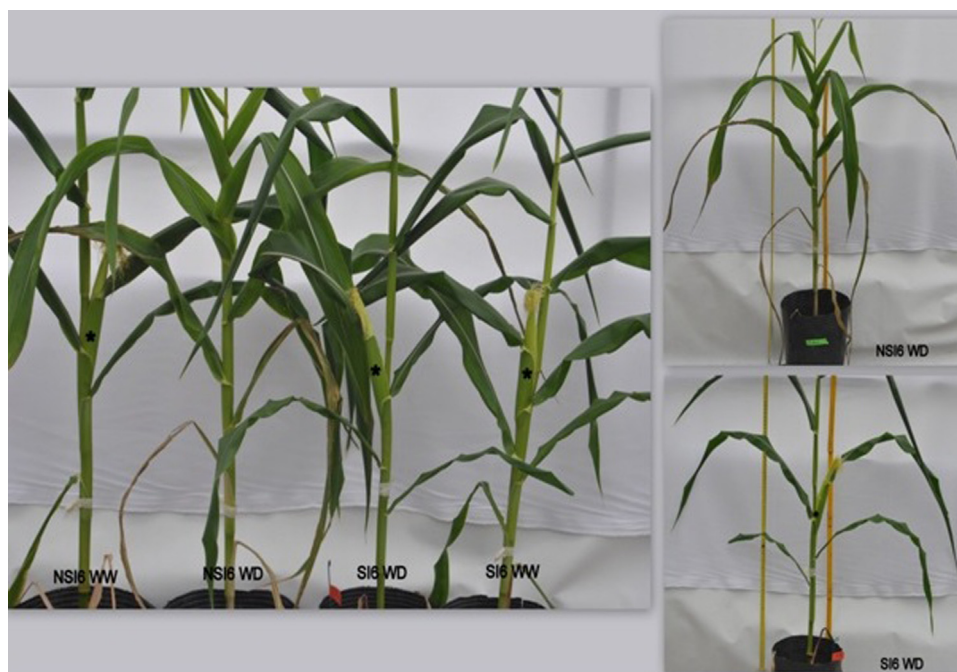
Fig. 5. Water stress effects in NSI6 and SI6 plants (2013 experiment). This photo was taken at the end of the water deficit period. The * indicates the presence of the ear. WW: well watered (plants permanently irrigated). WD: water deficit (plants water-stressed during the drought period). NSI6 WD plants did not shown ears at the end of the WD period.

nels per plant. At the end of the period of WD (R2) transgenic SI4 and SI6 WD plants showed a source/sink ratio similar to the NW plants according to the photosynthesis measurements. On the other hand, significant differences in the source/sink of the NSI4 and NSI6 WD plants were found ($p < 0.001$ and $p < 0.001$ respectively) (Table 6). In both NSI4 and NSI6 null plants under WD, the source/sink ratio increased due to the negative effect of the stress, showing that this negative effect is greater on the number of grains on leaf area.

4. Discussion

The *SARK* promoter is induced in the first stages of senescence and is up-regulated during drought and tissue maturation (Rivero et al., 2007). This approach was successfully demonstrated in both dicots (Rivero et al., 2007; Quin et al., 2011; Kuppu et al., 2013) and monocots (Peleg et al., 2011), and here we demonstrate that the *IPT* expression regulated by the *SARK* promoter could indeed improve water deficit stress tolerance in a C$_4$ plant. Interestingly, transgenic

Table 5

Treatments effect on growth traits during treatment period. [1] Plant growth rate. [2] Ear growth rate. [3] WW: well watered (plants permanently irrigated). [4]WD: water deficit (plants water-stressed during the drought period). Values are the mean $\pm$ SE (nNSI4 = 14, nSI4 = 14, nNSI6 = 10, nSI6 = 10). Asterisks indicate significant differences (*$p < 0,05$ **$p < 0,01$ *** $p < 0,001$) between transgenic WD and null WD.

Experiment	Genotype	Water regime	Plant height	PGR[1]	EGR[2]	EGR/PGR
			(cm)	(g plant^{-1}day^{-1})	(g plant^{-1}day^{-1})	
2013	Null SI4	WW[3]	100 ± 12.6	1.02 ± 0.29	0.65 ± 0.2	0.64
		WD[4]	89 ± 17.5	0.49 ± 0.12	0.28 ± 0.08	0.58
	SI4	WW	91 ± 8.5	1.14 ± 0.26	0.72 ± 0.11	0.65
		WD	83 ± 14.0	0.85 ± 0.23	0.58 ± 0.11	0.71*
	Null SI6	WW	122 ± 12.7	0.92 ± 0.25	0.76 ± 0.14	0.82
		WD	99 ± 7.4	1.58 ± 0.30	0.82 ± 0.17	0.53
	SI6	WW	98 ± 24.2	0.85 ± 0.27	0.53 ± 0.21	0.62
		WD	92 ± 26.4	0.89 ± 0.46	0.66 ± 0.31	0.74*

Table 6

Treatments effect on production traits, the determinants of plant grain yield and final biomass allocation to harvestable grains (harvest index). [1] WW: well watered (plants permanently irrigated). [2]WD: water deficit (plants water-stressed during the drought period). Values are the mean (nNSI6$_{2011}$ = 60, nSI6$_{2011}$ = 60, nNSI2$_{2012}$ = 20, nSI2$_{2012}$ = 20, nNSI4$_{2012}$ = 20, nSI4$_{2012}$ = 20, nNSI6$_{2012}$ = 20, nSI6$_{2012}$ = 20, nNSI4$_{2013}$ = 14, nSI4$_{2013}$ = 14, nNSI6$_{2013}$ = 10, nSI6$_{2013}$ = 10). Asterisks indicate significant differences (*$p < 0,05$ **$p < 0,01$ ***$p < 0,001$) between transgenic events WD and null WD.

Experiment	Genotype	Water regime	Shoot Biomass (g)	Plant Grain Yield (g)	Grain number per plant	Individual grain weight (mg)	Harvest index	Source/Sink relationship
2011	Null SI6	WW	–	28	200.2	188	–	
		WD	–	–	76.4	126	–	–
	SI6	WW	–	27	193.1	172	–	–
		WD	–	35***	205***	152**	–	–
2012	Null SI2	WW	–	15	92.8	169	–	–
		WD	–	4	22.5	133	–	–
	SI2	WW	–	16	100	158	–	–
		WD	–	13***	88***	155*	–	–
	Null SI4	WW	–	20	121	181	–	
		WD	–	4	13	128	–	–
	SI4	WW	–	18	109.3	173	–	–
		WD	–	10*	67.7*	166*	–	–
	Null SI6	WW	–	22	97.3	199	–	–
		WD	–	3	22	139	–	–
	SI6	WW	–	21	112	197	–	–
		WD	–	33**	197**	185**	–	–
2013	Null SI4	WW	46.5	25	150	169	0.54	11.51
		WD	26.2	6	44	144	0.21	24.53
	SI4	WW	47.7	24	148	163	0.49	11.97
		WD	37.4**	17***	110***	139*	0.42***	11.65***
	Null SI6	WW	119.1	71	304	238	0.61	15.77
		WD	71.1	24	134	157	0.31	10.72
	SI6	WW	60	32	166	163	0.45	12.82
		WD	60	33*	198**	186**	0.48**	12.05

plants containing less copies of the SARK:IPT cassette (e.g., line SII6) were less affected by stress than the transgenic lines comprising a larger number of copies (e.g., lines SI12 and SI14). Although the data is insufficient to establish a relationship between SARK:IPT copy number and tolerance, it is possible to speculate that the superior performance of the SI16 lines could be due to higher induction of gene expression during the water-deficit stress episode. This phenomenon was correlated with a relative lower production of ABA versus a higher CK synthesis, similar to what was reported previously in transgenic tobacco (Rivero et al., 2007) and transgenic rice plants (Peleg et al., 2011) expressing SARK:IPT. Whether higher SARK:IPT copy number could contribute to a decrease in expression due to gene silencing requires further investigation.

Cytokinin and chlorophyll contents were less affected by water deficit among transgenic maize genotypes than among their null counterparts, which resulted in a delay in leaf senescence. These results were consistent with cytokinin functions reported in cotton (Liu et al., 2008), arabidopsis (Zhang et al., 2000), alfalfa (Calderini et al., 2007), wheat (Sýkorová et al., 2008), creeping bentgrass (Merewitz et al., 2012) and maize (Robson et al., 2004). We show that the phenotypes of three SARK:IPT maize lines were consistent with the suppression of senescence initiation. While responses related to leaf dehydration can vary among species (Reddy et al., 2004), water deficit stress effects have been well characterized.

These effects include a rapid decrease in stomatal conductance to prevent excessive water loss by transpiration, leading to a decrease in photosynthetic activity by limiting the CO_2 content in the mesophyll (Tardieu and Simonneau, 1998; Lopes et al., 2011). Moreover, at the same time it has been found an increase of ABA and a decrease of cytokinins (Bruce et al., 2002). In our work, the null plants responded to water deficit with a marked decrease in stomatal conductance. On the contrary, the SARK:IPT plants under water deficit kept rates comparable to those of well watered plants. The same trend was observed for photosynthetic activity. This effect on photosynthesis and stomatal conductance in SARK:IPT maize plants is similar to that reported for tobacco (Rivero et al., 2009) and rice (Reguera et al., 2013). Transgenic SARK:IPT maize plants under water deficit stress had stomatal conductance and photosynthetic activity levels similar to those of both transgenic and null well watered plants. This trend matched the low levels of ABA and JA present in SARK:IPT plants under water deficit, which is consistent with the negative relationship between stomatal conductance and the concentration of ABA and JA (Assmann, 1993; Mok and Mok, 2001; Kazan and Manners, 2013). We did not detect stomatal closure, ABA/JA increased or photosynthetic rate reduction among SARK:IPT maize plants, even under water deficit, possibly due to the increase in cytokinin levels in response to reduced water availability.
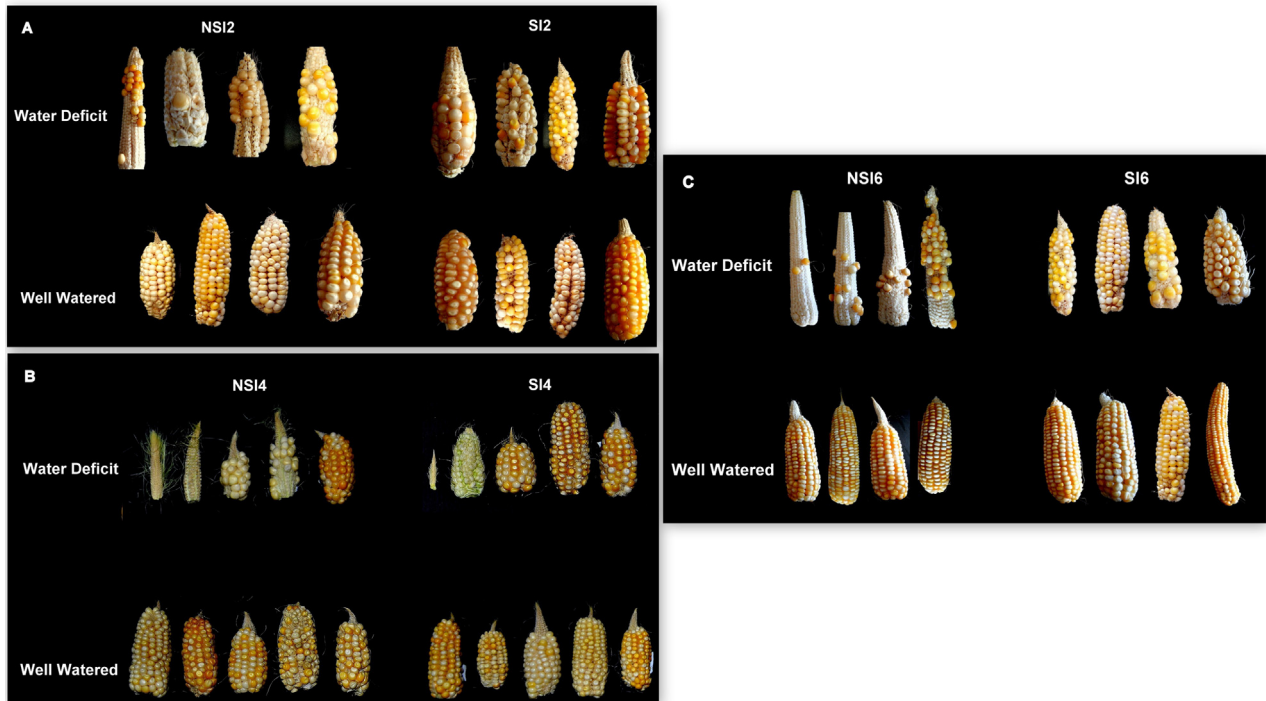
Fig. 6. Water stress effects on yield of null and three $P_{SARK-IPT}$ transgenic plants. WW: well watered (plants permanently irrigated). WD: water deficit (plants water-stressed during the drought period). (A) NSI2 and SI2 plants, (B) NSI4 and SI4 plants and (C) NSI6 and SI6 plants.

Rivero (2007) showed that the senescence-activated cytokinin production caused the inhibition of chlorophyll breakdown in the basal leaves of tobacco, affecting the source/sink relationships. Our work showed that the chlorophyll content and the source/sink relationships of the *SARK:IPT* maize lines under WD remained similar to that of well watered null plants. Moreover, the water deficit treatment affected floral synchrony as well as the potential kernel set and final grain yield of the *SARK:IPT* maize plants, and these trends caused a differential advantage among the different water regime × genotype combinations. These results agree with evidence from Kuppu (2013); Liu et al. (2012); Peleg et al. (2011) and Rivero et al. (2007). Contrary to our findings, results in arabidopsis (Xu et al., 2009) and maize (Robson et al., 2004) suggested that the senescence-activated cytokinin production induced a marked delay in flowering. Delayed anthesis in the maize study of Robson et al. (2004), however, was detected in only one of the three evaluated genotypes. Moreover, authors gave no evidence of negative effects on synchrony between anthesis and silking events (i.e. an increase in ASI), which is the critical response linked to reduced kernel numbers in this species (Hall et al., 1982; Bolaños and Edmeades, 1993). The above-mentioned differences among studies may be due to the promoter used and the time of application of the stress treatment, since water restrictions applied during flowering time have the largest negative effect on grain yield due to reductions in its main determinant, the number of kernels. Other works have previously demonstrated that the enhanced CK synthesis induced changes in source/sink relationships in transgenic SARK:IPT rice plants, promoting sink strengthening under stress conditions (Peleg et al., 2011; Reguera et al., 2013). The increased C-assimilation by the transgenic plants promoted increased C-allocation to the sinks (i.e. grains) resulting in increased yields, and not vegetative growth, under water-deficit.

In our work, expression of *IPT* and the consequent increase in cytokinins content peaked at this critical moment, preserving high photosynthesis levels and consequently high production of assimilates for holding high plant and ear growth rates, with the concomitant benefits on final kernel set (Andrade et al., 1999; Andrade et al., 2002; Echarte and Tollenaar, 2006) and consequently grain yield. In general, differences among genotypes (G) in grain yield performance across environments (E) are driven by a strong GxE effect, which usually includes cross-over responses (Cooper et al., 1996). This effect is evident as a change in ranking among genotypes along a gradient of environments represented by the mean grain yield of each site (Finlay and Wilkinson, 1963). When variation in mean grain yield is linked to the water budget, the cross-over response represents the differences among genotypes in their capacity to produce grain biomass under drought. These differences may be simply associated to cycle duration or may also represent the expression of traits (constitutive or inducible) that confer an advantage under drought. The former is an escape strategy, whereas the second is actual stress tolerance (Connor et al., 2011). Our present data and the previous research on SARK:IPT expression indicate that the response belongs to the second group. However, to define the extent of the GxE interaction, the effects of the transgene must be analyzed in (i) different genetic backgrounds, evaluate whether other genetic interactions modify the described benefits of the transgene (Austin, 1999), and (ii) under different drought scenarios (e.g., terminal drought, alternate drought episodes of short extension, etc.) (Cooper et al., 2005). This will be the focus of future research.

5. Conclusions

We reported the manipulation of cytokinin content by the senescence-regulated expression of the *A. tumefaciens IPT* gene through its control by the SARK promoter in maize, and the resulting effect on water deficit tolerance from metabolic to plant production levels. We demonstrated that the p*SARK*-regulated expression of the *IPT* gene improved the photosynthetic performance and delayed leaf senescence, leading to an improved productivity under water deficit conditions, although. Our results

are promising for the development of transgenic maize able to grow in areas with unfavorable water regimes.

Acknowledgements

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