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# Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress

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## Summary

Drought is the major environmental factor limiting crop productivity worldwide. We hypothesized that it is possible to enhance drought tolerance by delaying stress-induced senescence through the stress-induced synthesis of cytokinins in crop-plants. We generated transgenic rice (*Oryza sativa*) plants expressing an *isopentenyltransferase* (*IPT*) gene driven by $P_{SARK}$, a stress- and maturation-induced promoter. Plants were tested for drought tolerance at two yield-sensitive developmental stages: *pre-* and *post-anthesis*. Under both treatments, the transgenic rice plants exhibited delayed response to stress with significantly higher grain yield (GY) when compared to wild-type plants. Gene expression analysis revealed a significant shift in expression of hormone-associated genes in the transgenic plants. During water-stress (WS), $P_{SARK}$::*IPT* plants displayed increased expression of brassinosteroid-related genes and repression of jasmonate-related genes. Changes in hormone homeostasis were associated with resource(s) mobilization during stress. The transgenic plants displayed differential expression of genes encoding enzymes associated with hormone synthesis and hormone-regulated pathways. These changes and associated hormonal crosstalk resulted in the modification of source/sink relationships and a stronger sink capacity of the $P_{SARK}$::*IPT* plants during WS. As a result, the transgenic plants had higher GY with improved quality (nutrients and starch content).

## Introduction

Water-deficit often combined with high temperature stress is the main abiotic factor limiting crop-plants productivity and food-security worldwide (Boyer, 1982). Moreover, agro-ecological conditions are expected to deteriorate with the projected global climate changes and the rise in food demand for the ever expanding world population (Long and Ort, 2010; Mittler and Blumwald, 2010). Plants subjected to drought respond by a number of physiological mechanisms at the molecular, cellular, tissue, morphological and whole-plant levels (Bray, 1997; Chaves *et al.*, 2003; Witcombe *et al.*, 2008). These responses vary with the species and genotype, the length and severity of stress, and the developmental stage.

Drought is the major environmental constraints to rice productivity in rainfed areas (reviewed by Farooq *et al.*, 2009; Serraj *et al.*, 2009). Although over 50% of the rice-growing area worldwide is rainfed, it produces only one-quarter of total production (Maclean *et al.*, 2002). To meet the ever-growing demand for rice by 2030, a significant increase of at least 35% in yield is needed (Bouman *et al.*, 2007). Thus, developing new rice cultivars with high yield and improved drought tolerance and water-use efficiency is a major challenge.

In cereals, grain yield (GY) is dependent on the plant source/-sink relationship. The top two leaves are the primary source, and the florets are the primary sink for photosynthesis (Hirota *et al.*, 1990; Sicher, 1993). Rice is highly dependent on the source leaves for grains filling as photosynthesis in the developing panicles contributes only little to grain filling. Hormonal transitions, whether ontogenic or environmentally induced, play a major role in source/sink changes, and cytokinins (CK) appear to be a key element affecting these transitions (Roitsch and Ehneß, 2000). Therefore, hormone-induced changes of source/-sink relationships could be an important component for the development of drought-tolerant rice.

Several studies have reported that senescence could be delayed in transgenic plants expressing *isopentenyltransferase* (*IPT*, an enzyme that mediates CK synthesis), using different promoters (Ma, 2008 and references therein). Tobacco plants expressing the *Agrobacterium tumefaciens IPT* gene under the control of a *senescence-associated receptor kinase* (*SARK*; Hajouj *et al.*, 2000), a maturation- and stress-inducible promoter, exhibited enhanced photosynthetic capacity resulting in improved drought tolerance (Rivero *et al.*, 2007, 2009). Here, we investigate the effects of manipulating CK biosynthesis on plant hormone homeostasis during drought in rice. Transgenic plants expressing the $P_{SARK}$::*IPT* were generated for testing the effect of altered hormonal shift on sink/source balance under drought, with emphasis on GY and grain quality.

## Results

### Effects of $P_{SARK}$::*IPT* on yield under water-stress conditions

The *SARK* promoter linked to the *IPT* gene was introduced into rice. Fourteen independent homozygous lines expressing

$P_{SARK}$::*IPT* were generated. Three independent transgenic lines ($T_3$ generation seeds) expressing the $P_{SARK}$::*IPT*, their direct nulls and wild-type (WT) plants were grown under optimal growth conditions in the greenhouse. The transgenic plants did not differ in morphology (i.e. plant height, number of tillers and flowering date; Table S1) from WT and null plants (Figure 1a). The plants were subjected to a severe water-stress (WS) at two critical developmental stages: Pre-anthesis stage (end of booting stage, panicle elongation) and post-anthesis stage (2 weeks after flowering) by withholding watering for a period of 6–10 days (Figure S1). WT and null plants exhibited stress symptoms (i.e. leaf rolling) after 2–3 days of WS, and the $P_{SARK}$::*IPT* plants started to show drought symptoms after 6–10 days (not shown). At this point, all plants were re-watered (Figure 1b) and then grown under optimal watering conditions until harvest. Under well-watered (WW) conditions, the WT and transgenic plants showed similar total biomass and GY (Figure 1c,d). In WT plants subjected to WS, the GY was severely reduced (75.4% and 73.1% reduction for pre-anthesis and post-anthesis WS, respectively) when compared with WW conditions (Figure 1c). The three null lines performed similarly to the WT (data not shown). The $P_{SARK}$::*IPT* plants displayed a significant GY increase with respect to WT plants (144% and 158% for pre-anthesis and post-anthesis WS, respectively) (Figure 1c). Total dry matter (DM) production of the transgenic lines was significantly higher than that of the WT plants under WS (Figure 1d). During the first 5 days of stress, the WT dried significantly faster than the transgenic plants, and no differences were seen afterwards (Figure 1e). Whether the lower water loss rates observed by the transgenic plants were because of faster stomata closing during drought remain unclear.

## Expression pattern of the *IPT* gene in rice

*IPT* expression patterns were measured using qPCR throughout the entire life cycle of the plants grown under WW conditions. While low levels of expression were seen at early developmental stages (vegetative phase), *IPT* expression increased significantly at the reproductive stage during natural senescence (Figure 2a). During the development of WS, *IPT* expression increased and reached a maximum during the third day of stress. At this stage, the $P_{SARK}$::*IPT* plants did not display stress symptoms (Figure 2b). The expression during drought was not only enhanced in older leaves (third and fourth) but also in the younger leaves (second and flag leaf), developing panicles and roots, confirming the drought responsiveness of the $P_{SARK}$ promoter (Figure 2c).

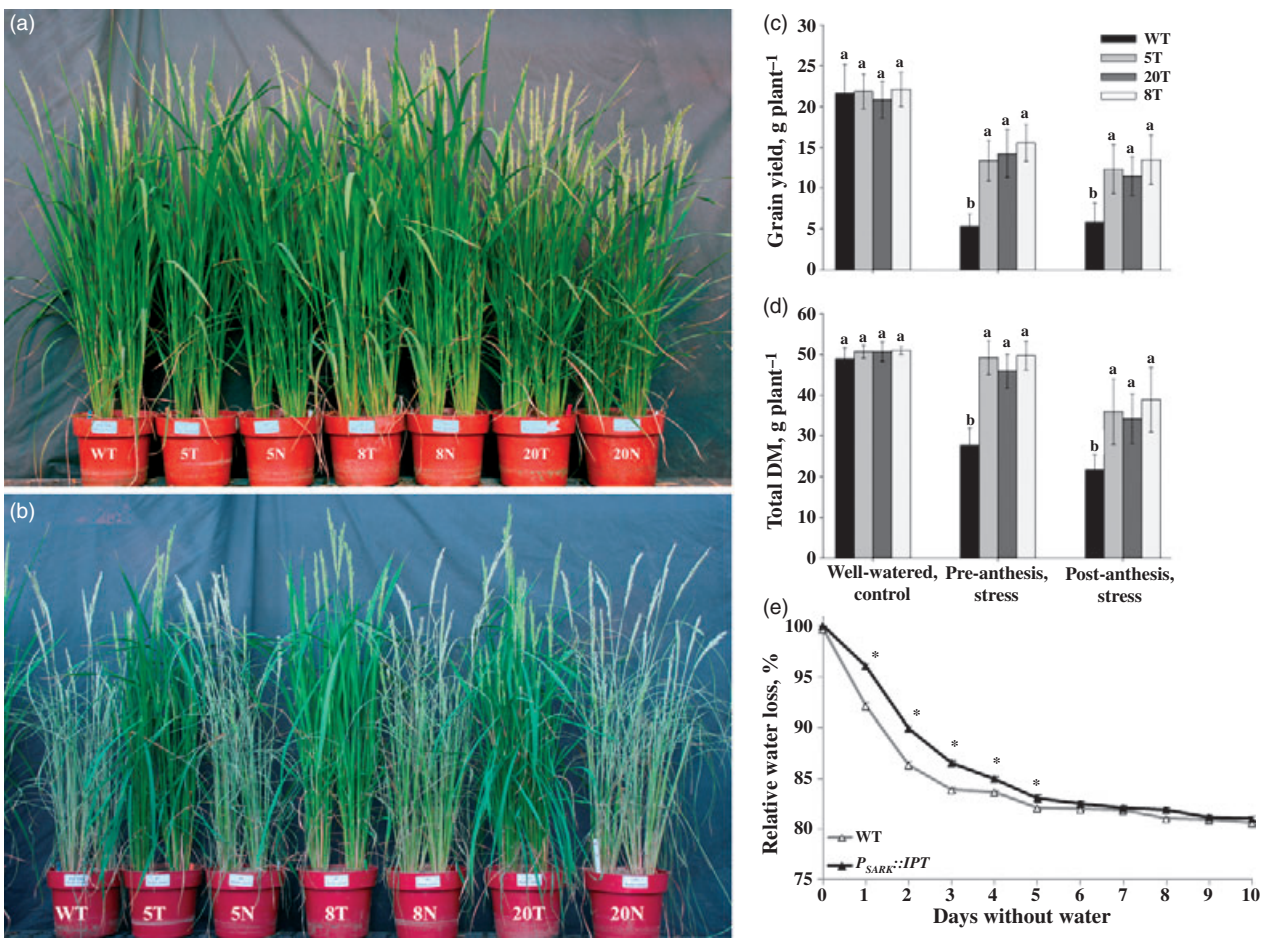


**Figure 1** Effects of water-stress on growth and yield. Wild-type (WT) and transgenic plants expressing $P_{SARK}$::*IPT* (lines 5T, 20T and 8T) and their nulls (5N, 20N and 8N) (a) Plants grown under well-watered conditions; (b) plants subjected to 6 days of water-stress at pre-anthesis stage; (c) grain yield; (d) total dry matter collected at the end of the experiments; and (e) relative water loss rate from pots. Values are the mean ± SD ($n$ = 12). Within each water treatment, different letters indicate significant differences by Tukey LSD test ($P < 0.05$).

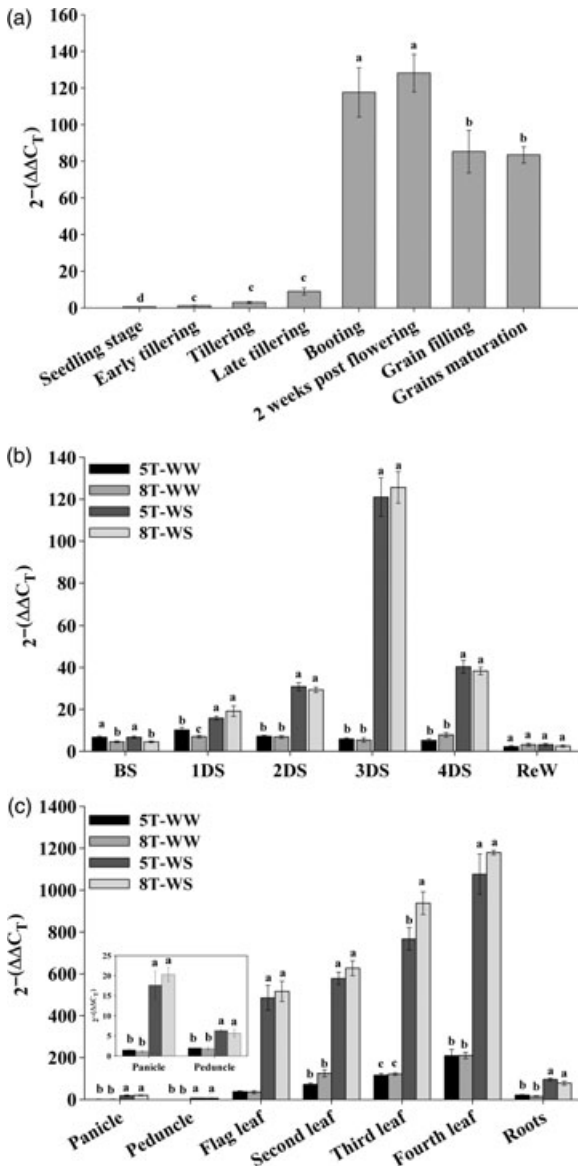


**Figure 2** Relative *isopentenyltransferase* (*IPT*) expression in two independent $P_{SARK}$*::IPT* lines (5T and 8T). (a) plants grown under well-watered conditions; (b) Expression patterns during the development of stress. BS, before stress; (1DS, 2DS, 3DS, 4DS) 1, 2, 3, 4 days of water-stress; RW, after re-watering; WW, well-watered; WS, water-stress. (c) Expression pattern at 3DS in different organs. Values were calculated and normalized using transcription elongation factor as internal control. Values are the mean ± SD ($n$ = 6).

## Changes in the expression of genes encoding hormone-associated pathways

A comparison between WT and $P_{SARK}$*::IPT* plants revealed differential expression of genes encoding enzymes mediating hormone biosynthesis and homeostasis (Figure 3). In general, *IPT* expression in the transgenic plants resulted in the change in expression of genes related to the synthesis and degradation of CK, abscisic acid (ABA), gibberellins (GA), auxin (IAA), salicylic acid (SA), ethylene, jasmonate (JA) and brassinosteroid (BR). Notably, although the transgenic plants grown under WW conditions did not exhibit any phenotypic differences from WT, these plants displayed the differential regulation of a number of hormone-related genes (Figure 3). For example, an IAA-responsive gene (*OsARF7*) was down-regulated, and GA-inactivation genes (*GA2ox 2* and *GA2ox 4*) were up-regulated in $P_{SARK}$*::IPT* plants under WW conditions. Noteworthy, seven of the endogenous rice *IPT* genes (*OsIPT1*, *OsIPT3-8*) did not show expression changes (*OsIPT2* is not present in the Affymetrix GeneChip) (data not shown). A significant up-regulation of genes involved in CK degradation (*CK-O-glucosyltransferase 1-3*) was in agreement with the increased *IPT* expression after 3 days of WS (Figure 3d). Our results are supported by the observed increase in *CK-O-glucosyltransferase* after the exogenous application of trans-zeatin (Hirose *et al.*, 2007), thus suggesting that the up-regulation of these genes could play a role in the homeostatic control of CK activity.

Changes in the expression of genes associated with different hormones were observed in the WT and $P_{SARK}$*::IPT* plants during stress (Figure 3). Nevertheless, because of the significant role(s) during stress adaptation of BR and JA, we will focus on genes associated with the synthesis and homeostasis of these phytohormones. Significant differences between WT and $P_{SARK}$*::IPT* plants were observed in the expression of genes associated with the BR biosynthetic pathways (Figure 3a). Delta14-sterol reductase (*DWF5*) and C-8 sterol isomerase (*HYD1*) are genes encoding proteins involved in sterol synthesis (Fujioka and Yokota, 2003). These genes were highly expressed in the $P_{SARK}$*::IPT* plants under WS. A number of genes involved in BR-regulation and signalling (*BRL3*, *BRI1*, *BRH1*, *BIM1*, *SERK1*, *BSK1*, *BIN4* and *BAK1*; Choe, 2010; Kim and Wang, 2010) were also differently regulated in the $P_{SARK}$*::IPT* plants (Figure 3a). In contrast, genes encoding proteins associated with JA biosynthesis (*OPR2*) and signalling (*MES3* and *JAZ12*) were down-regulated in the transgenic plants in response to WS (Figure 3b). Validation of selected transcripts associated with BR- and JA-related pathways confirmed the trends seen in the array data (Figure 4). Key genes encoding enzymes involved in BR-biosynthesis (*DWF4*, *DWF5* and *HYD1*) and BR-signalling (*BRI1*, *BZR1*, *BAK1*, *SERK1* and *BRH1*) were highly expressed in the transgenic plants under WS. Also, in most cases, the expression of these genes was up-regulated in the transgenic plants under WW conditions (Figures 3a and 4).

Under WS, the expression of genes encoding IAA transporters (*OsPIN3a* and *OsPIN6*) and IAA-responsive genes (*OsIAA5*, *OsIAA21* and *OsARF5*) were down-regulated in the $P_{SARK}$*::IPT* plants (Figure 3e), while the levels of expression of GA-associated genes (*GA receptor*, *OsGAI* and chitin-inducible GA-responsive protein) increased (Figure 3f). In addition, ethylene responsive and signalling genes (Flavonol synthase/flavanone 3 hydroxylase, *ETR2*, *MTHFR2*, *RAP2.4* and *EIN3*) were up-regulated (Figure 3g).

## Detached leaves assays

To test the effects of CK on the delayed senescence of rice leaves and to assess the relationship between increase in CK and transcript level induction of BR synthesis, we compared detached flag leaf sections from WT, $P_{SARK}$*::IPT* plants and WT plants treated with exogenous CK. The first indication for the involvement of CK in senescence came from observations showing that the exogenous application of CK to detached leaves prevented senescence (Richmond and Lang, 1957). Flag leaf sections from the two transgenic lines tested ($P_{SARK}$*::IPT*$_{5T}$ and $P_{SARK}$*::IPT*$_{8T}$) displayed delayed senescence when compared to

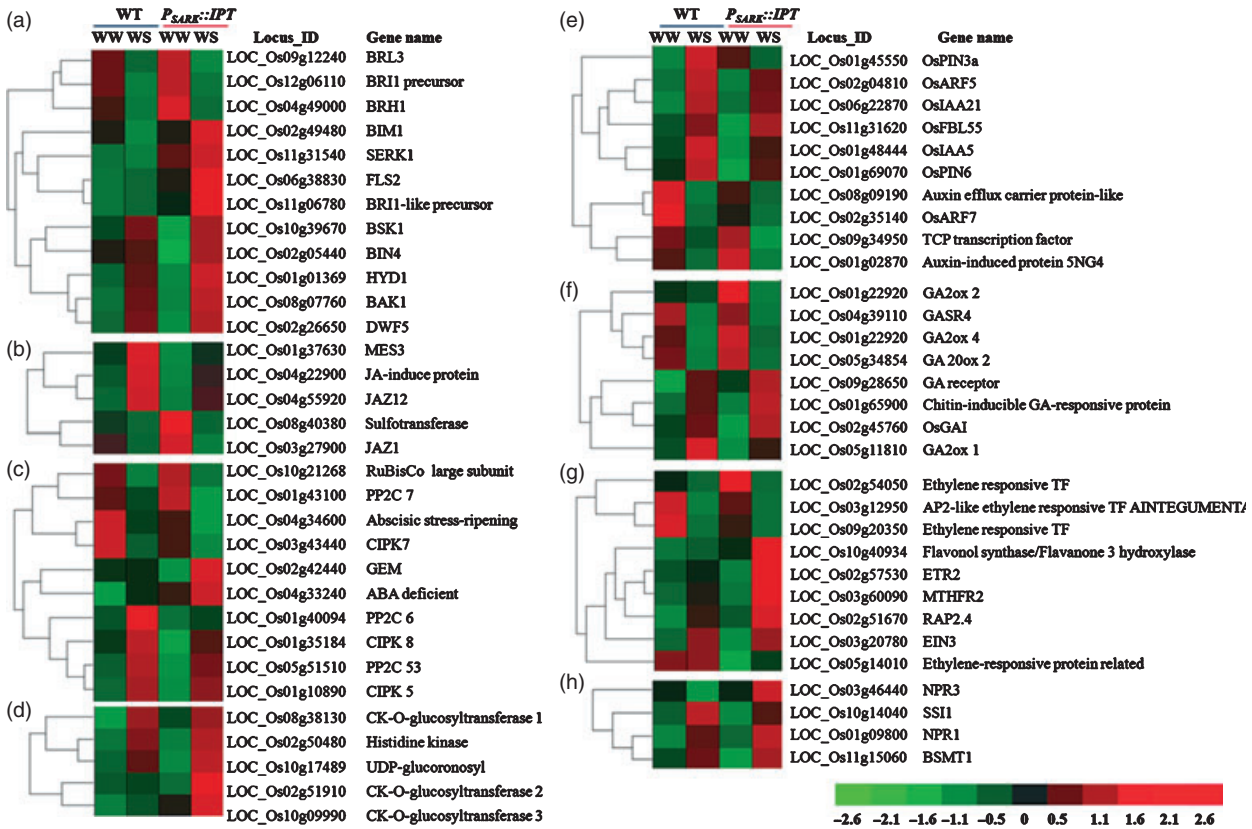


**Figure 3** Changes in hormone-associated genes in wild-type and $P_{SARK}$::*IPT* plants. WW, well-watered, control; WS, 3 days of water-stress at pre-anthesis stage. Red and green represent high and low relative expression when compared to the mean value of expression across all samples, respectively. Scale is $\log_2$ of mean expression value. (a) Brassinosteroid, (b) jasmonate, (c) abscisic acid, (d) cytokinin, (e) auxin, (f) gibberellin, (g) ethylene and (h) salicylic acid. *BRL3*, BRI1-Like 3; *BRH1*, BR-responsive ring-h2; *BIM1*, bHLH protein; *SERK1*, somatic embryogenesis receptor-like; *FLS2*, flagellin-sensitive 2; *BSK1*, BR-signalling kinase 1; *BIN4*, BR-insensitive 4; *HYD1*, C-8 sterol isomerase; *BAK1*, BRI1-associated receptor kinase; *DWF5*, delta14-sterol reductase; *OPR2*, putative 12-oxophytodienoate reductase; *MES3*, methyl esterase 3; *JAZ12*, JA-ZIM-domain protein 12; *JAZ1*, JA-ZIM-domain protein 1; *PP2C* 7, protein phosphatase 2C 7; *CIPK7*, CBL-interacting protein kinase 7; *GEM*, ABA-responsive protein-like; *PP2C* 6, protein phosphatase 2C 6; *CIPK8*, CBL-interacting protein kinase 8; *PP2C* 53, protein phosphatase 2C 53; *CIPK5*, CBL-interacting protein kinase 5; *OsPIN3a*, auxin efflux carrier component; *OsARF5*, auxin-response factor 5; *OsIAA21*, auxin-responsive Aux/IAA gene family member; *OsFBL55*, F-box domain and LRR-containing protein; *OsIAA5*, auxin-responsive Aux/IAA gene family member; *OsPIN6*, auxin efflux carrier component; *OsARF7*, auxin-response factor 7; *GA2ox* 2, GA 2-beta-dioxygenase 2; *GASR4*, GA-regulated GASA/GAST/Snakin family protein precursor; *GA2ox* 4, gibberellin 2-beta-dioxygenase 4; *GA20ox* 2, GA20 oxidase; *OsGAI*, chitin-inducible GA-responsive protein 2; *ETR2*, ethylene receptor 2; *MTHFR2*, methylenetetrahydrofolate reductase 2; *RAP2.4*, ethylene-responsive transcription factor; *EIN3*, ethylene-insensitive 3; *BSMT1*, SAM-dependent carboxyl methyltransferase; *SSI1*, suppressor of salicylic acid insensitivity 1; *NPR1*, Nonexpressors of phaogenesis-related genes1; *NPR3*, NPR1-like protein 3.

WT (Figure 5a) and maintained 69%–75% of the total chlorophyll content after 5 days of incubation in the dark (Figure 5b). Similarly, detached leaves of $P_{SAG12}$::*IPT* and $P_{SAG13}$::*IPT* tomato were still green and exhibited a higher chlorophyll content after 10 days in the dark when compared with the WT (Swartzberg *et al.*, 2006). Sections from the WT leaves treated with exogenous CK did not show chlorophyll degradation after 5 days (Figure 5a,b). Interestingly, the transgenic lines showed a more pronounced reduction in Chl a (35%–28%) than in Chl b (25%–21%), and the WT leaves treated with CK displayed an increase in Chl b (Figure 5b). The expression of transcripts associated with BR-biosynthesis (*DWF4*), regulation (*BZR* and, *BRI1*) and response (*FLS2;* Chinchilla *et al.*, 2009) was up-regulated in transgenic plants expressing $P_{SARK}$::*IPT* and WT plants treated with exogenous CK after 3 days of senescence (Figure 5c). *DWF4*, *BZR1* and *BRI1* were expressed before senescence (day 0) and displayed higher expression during senescence. On the other hand, *FLS2* displayed expression in the transgenic plants and plants treated with exogenous CK during senescence only (Figure 5c).

## Source/sink relationships

Cytokinins are important regulators of plant growth and development (Ma, 2008) and have been shown to control plant source/sink relationships (Roitsch and Ehneß, 2000). The effect of *IPT* expression and the concomitant CK synthesis (Rivero *et al.*, 2007) on sink strength was tested by analysing the contents of mineral-nutrients and the quantification of sucrose and starch in the grain. Electron microscopy sections of flag leaf parenchyma cells showed that under WW conditions, the transgenic plants had a higher number of starch granules than the WT plants, and this difference was not seen under WS (Figure 6a). The starch content of the flag leaves from the transgenic plants was significantly higher than that of the WT plants (58% and 88% for WW and WS treated transgenic plants, respectively). A similar pattern was seen in the mature grains,

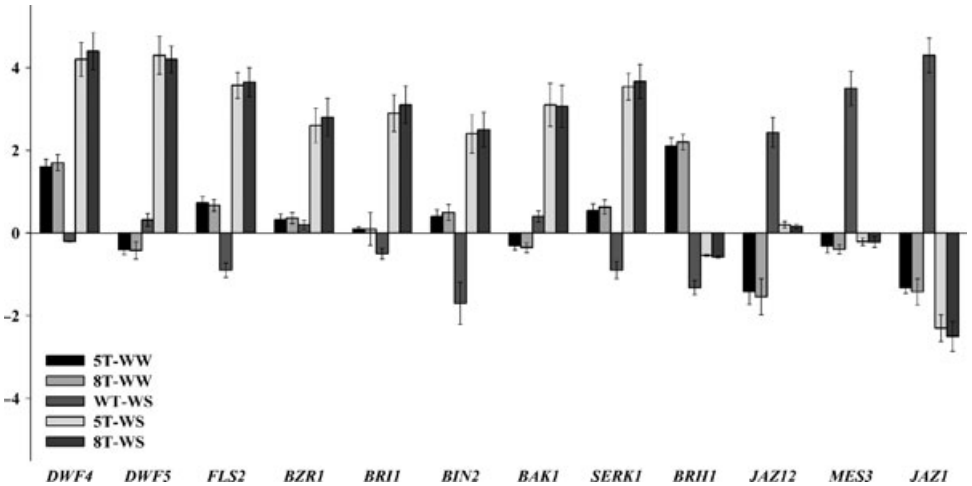


**Figure 4** Validation of subset of BR- and JA-related genes using qPCR assay. The wild-type plants grown under well-watered conditions were used as reference sample ($log_2 = 0$). Values were calculated and normalized using transcription elongation factor as internal control. Values are the mean ± SD ($n = 6$). *DWF4*, Dwarf4; *FLS2*, flagellin-sensitive 2; *BZR1*, brassinazole resistance 1; *BRI1*, BR-insensitive 1; *BIN2*, BR-insensitive 2; *BAK1*, BRI1-associated receptor kinase; *DWF5*, delta14-sterol reductase; *SERK1*, somatic embryogenesis receptor-like; *BRH1*, BR-responsive ring-h2; *JAZ12*, JA-ZIM-domain protein 12; *MES3*, methyl esterase 3; *JAZ1*, JA-ZIM-domain protein 1.

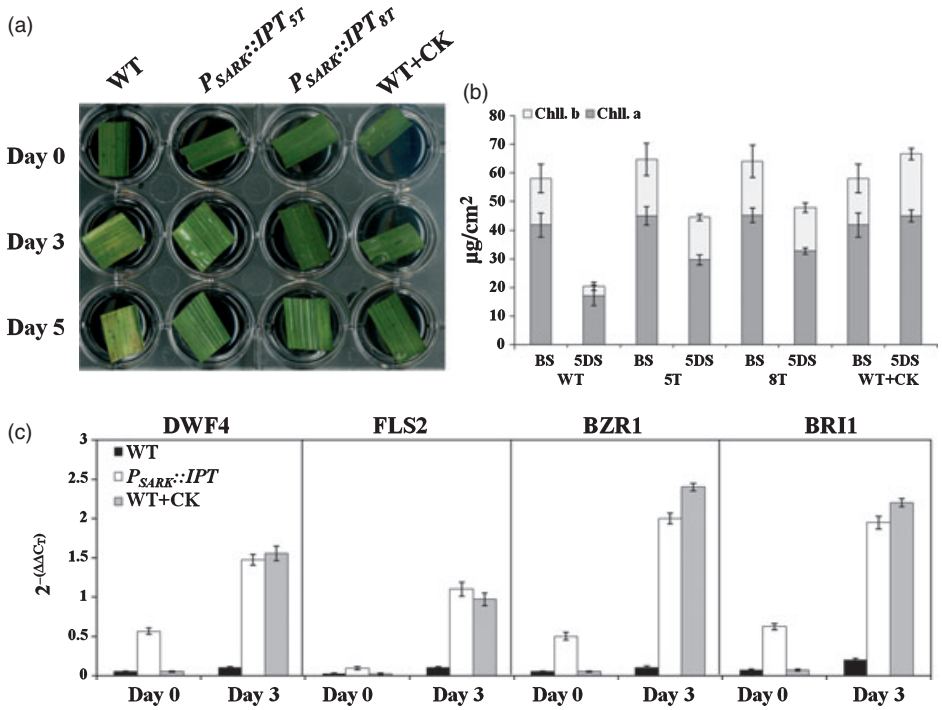


**Figure 5** Detached leaves assay. (a) Flag leaf pieces taken at pre-anthesis stage and kept in $ddH_2O$ for 5 days in dark under 30 °C. (b) Chlorophyll a and b content in wild-type (WT), two transgenic lines (5T and 8T) and WT with exogenous cytokinins (WT + CK) before senescence (day 0, BS) and after senescence (day 5, 5DS). Values are the Mean ± SD ($n = 3$). (c) Relative expression of selected BR-related genes. Values were calculated and normalized using transcription elongation factor as internal control. Values are the mean ± SD ($n = 6$). *DWF4*, Dwarf4; *FLS2*, flagellin-sensitive 2; *BZR1*, brassinazole resistance 1; *BRI1*, BR-insensitive 1.

with significant starch increases of 15% and 30% for grain obtained from transgenic WW and stressed plants, respectively (Figure 6b). Sucrose analysis showed a significantly higher sucrose content in leaves of the transgenic plants grown under both watering regimes (Figure 6b), but no differences in the grain sucrose content. The expression of several genes related to sugar metabolism was altered in the transgenic and WT plants under both water conditions (Figure 6c). Real-time PCR analysis showed that the expression of several genes encoding enzymes belonging to the starch degradation pathway i.e. α-amylase precursor (*α-AMY)*, disproportionating enzyme (*DPE2*), starch debranching enzyme (Isoamylase III, *ISA3*) and starch

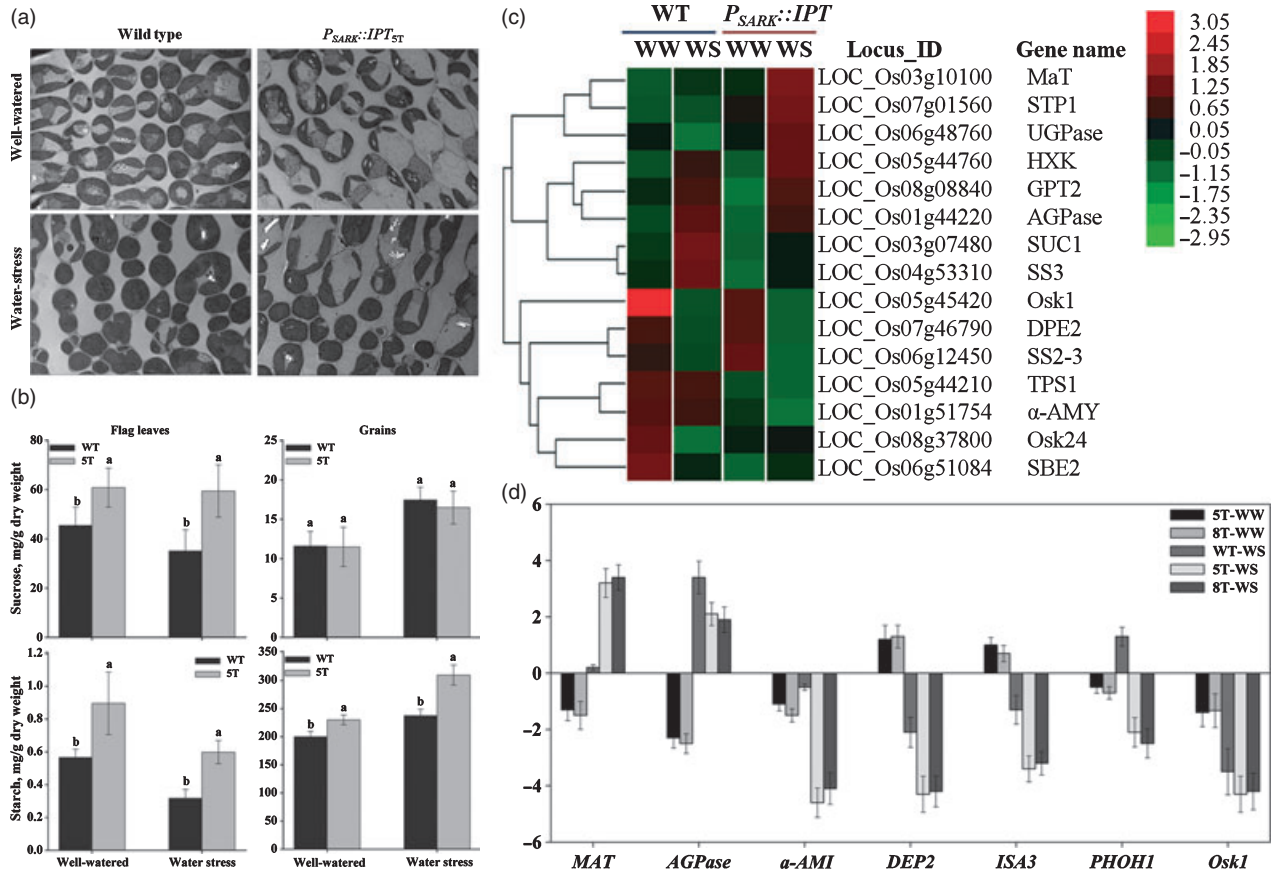


**Figure 6** Sucrose and starch metabolism. (a) Electron microscopy of flag leaves at pre-anthesis stage under water-stress and after re-watering. (b) Quantification of sucrose and starch of wild-type (WT) and transgenic plants at pre-anthesis in flag leaves and after maturation in grains. (c) Heat map of selected sucrose metabolism-related genes derived from hierarchical cluster analysis differently regulated between WT and $P_{SARK}$::*IPT* plants under well-watered (WW) and 3 days of water-stress at pre-anthesis (WS). Red and green represent high and low relative expression when compared to the mean value of expression across all samples, respectively. (d) Validation of a subset of genes using qPCR assay. Values were calculated and normalized using transcription elongation factor as internal control. WT plants grown under well-watered conditions were used as reference sample ($\log_2 = 0$). Values are the mean ± SD ($n = 6$). *MaT*, mannitol transporter; *UGPase*, UTP glucose-1-phosphate urifylyltransferase; *SBE2*, 1,4-alpha-glucan-branching enzyme; *SS3*, starch synthase 3; *SS2.3*, starch synthase 2.3; *GTP2*, antiporter/glucose-6-phosphate transmembrane transporter; *AGPase*, ADP-glucose pyrophosphorylase; *DPE2*, disproportionating enzyme; *SUC1*, sucrose transporter; *TPS1*, trehalose-6-phosphate synthase; *STP*, sugar transporter; Isoamylase III, *ISA3*, starch debranching enzyme; *PHOH*, starch phosphorylase Ha; *α-AMY*, alpha amylase precursor; *Osk1*, *SnRK1a* related protein kinase 1; *Osk24*, *SnRK1b*-related protein kinase 24; *HXK*, hexokinase; *OsMADS57*, MADS-box family gene with MIKCc type-box.

phosphorylase Ha (*PHOH*) was down-regulated in the transgenic plants (Figure 6d). Two *SnRK1*-type of protein kinase genes, *Osk1* (Os05g45420) and *Osk24* (Os08g37800), were found highly up-regulated under WW conditions in WT plants (Figure 6c). The *Arabidopsis* orthologues of these genes are *KIN10* (*SNF1* kinase homologue 10, At3g01090) and *KIN11* (*SNF1* kinase homologue 11, At3g29160). *KIN10* and *KIN11* were reported to regulate starch mobilization during the night, playing an important role during starch starvation in leaves (Baena-Gonzalez *et al.*, 2007). The higher expression of *Osk1* and *Osk24* in WT plants under WW conditions correlated with the lower amount of starch in leaves (Figure 6a,b). These two genes are not only involved in carbohydrate biosynthesis in the source tissues but also in the sink (grain filling). *Osk1* is involved in unloading sucrose via the sucrose synthase pathway and *Osk24* acts in the conversion of sucrose to starch.

More recently, several rice candidate regulators for starch biosynthesis were identified based on co-expression analysis of the rice transcriptome (Fu and Xue, 2010). Expression patterns of 16 candidate transcription factors correlated with known starch synthesis genes that were preferentially expressed in the vegetative tissues (source organ). The expression of one of these candidate transcription factors, *OsMADS57* (a MADS-box gene with a MIKCc type-box, Os02g49840), was found to be up-regulated in $P_{SARK}$::*IPT* plants under both watering conditions (Figure 5c). Notably, the expression of *OsMADS57* increased 37-fold in $P_{SARK}$::*IPT* plants when compared to WT under stress. Further, we mapped the transcript response of *OsMADS57* in the rice microarray database and found that *OsMADS57* was significantly repressed by a drought shock experiment and was strongly induced by exogenous trans-zeatin treatment in the shoots but not in the roots (PLEXdb:Rice).

Under WW conditions, no significant differences were noted in the grain concentration of macro- and micro-nutrients (Table S3). The alteration of source/sink relationships in the $P_{SARK}$::*IPT* plants under WS resulted in increased grain micronutrient (Zn, Fe and Mn) and macronutrient (S, P and Mg) contents. There was a significant increase in Zn (36.6%) and Fe

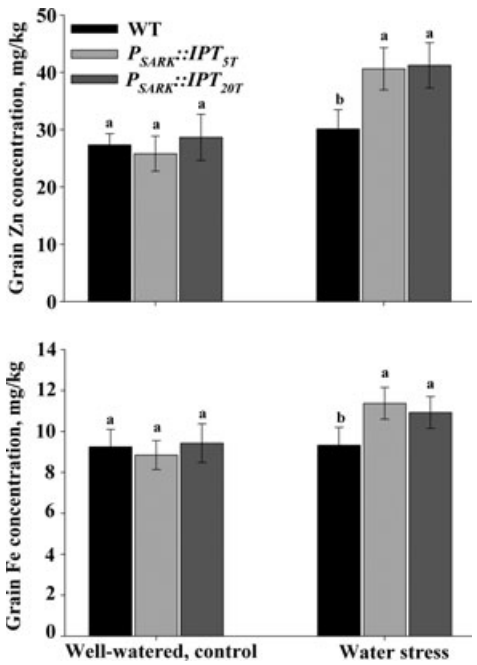


**Figure 7** Grain zinc and iron concentrations of wild-type and two $P_{SARK}$::*IPT* lines (5T and 20T) grown under well-watered, control and water-stress conditions. Values are the mean ± SD ($n$ = 6).

(20.6%) contents in the grain of the transgenic plants subjected to WS (Figure 7). Also, the concentrations of other minerals (Mn, P, S and K) increased in the grain of the stressed transgenic plants (Table S3). The levels of expression of several metal transporters increased in the $P_{SARK}$::*IPT* plants under WS conditions (Figure S2). The expression of *OsZIP1* (Os07g12890) and *OsZIP2* (Os03g29850), belonging to Zrt-, Irt-like proteins (Guerinot, 2000), displayed a 3.5- and 5.5-fold-change increase, respectively. The expression of the rice orthologue (Os02g36940) of *Arabidopsis Plant cadmium resistance 2* (*AtPCR2*) was up-regulated in the $P_{SARK}$::*IPT* plants under water-stress (Figure S2). Recently, *PCR2* was reported to regulate Zn transport from roots to shoot (Song *et al.*, 2010). In addition, several other transporters, *potassium transporter 16* (*OsHAK 16*), *inorganic phosphate transporter 1-1* (*OsPHT1-1*), *inorganic phosphate transporter 1-11* (*OsPHT1-11*) and amino-acid transporter (*LHT1*), were up-regulated in the $P_{SARK}$::*IPT* plants under WS.

## Discussion

The goal of this study was to generate transgenic rice that can survive severe drought episodes with minimal yield losses. Here, we show that the overexpression of *IPT* (inducing the synthesis of CKs), driven by a stress-induced promoter ($P_{SARK}$), affected the expression of genes associated with hormone synthesis and hormone homeostasis, altered the sink-source relationships in the plant and contributed to the enhanced drought tolerance of the transgenic rice plants.

### Effect of $P_{SARK}$::*IPT* on yield

WS at the reproductive stage (flowering and grain development) is the most prevalent problem in most crops under rainfed ecosystems (Blum, 2009). In the current study, we applied a WS at two critical time points of the reproductive stage: flowering stage and during grain filling. Under both pre- and post-anthesis WS, the $P_{SARK}$::*IPT* plants exhibited a delay in stress symptoms (i.e. leaf rolling, senescence and reduction in photosynthesis activity) that contributed to significantly higher biomass and GY production when compared to the WT plants under stress. The higher photosynthetic rates displayed by the transgenic plants under stress further support the yield advantage of the transgenic rice (data not shown). For wild plant species, the senescence of older leaves is a very efficient strategy enabling the survival during WS by reducing the canopy size and minimizing water loss. On the other hand, the yield penalty associated with a reduced canopy size is a disadvantage for crop-plants.

Cytokinins have been associated with the processes leading to leaf senescence (Gan and Amasino, 1997). Transgenic plants expressing *IPT*, under the control of different promoters, displayed delayed senescence (Ma, 2008). In some cases, *IPT* expression also led to delayed flowering (McCabe *et al.*, 2001), nutrient deficiency in the young leaves (Jordi *et al.*, 2000) and reduced seedling establishment in response to WS (Cowan *et al.*, 2005). We have shown recently that the expression of *IPT* under the control of the senescence- and stress-activated $P_{SARK}$ in transgenic tobacco plants resulted in increased drought tolerance, higher photosynthetic capacity and increased biomass (Rivero *et al.*, 2007, 2009).

Here, we showed that $P_{SARK}$, a promoter from a dicot plant, can be used to drive *IPT* expression in the monocot rice (Figure 2). Under WS, the expression of *IPT* in rice plants was significantly higher in the leaves with lower expression in the roots (Figure 2c). Furthermore, detached leaf assays showed a significant delay in leaf senescence of $P_{SARK}$::*IPT* lines when compared to the WT plants (Figure 5a,b). Under stress-induced senescence, the $P_{SARK}$::*IPT* plants were able to maintain relatively higher amounts of chlorophyll b and displayed small reductions in the amount of chlorophyll a. These results support the notion of a CK-dependent protection of photosynthesis during drought stress (Rivero *et al.*, 2009).

### Shift in expression of hormone-associated genes in $P_{SARK}$::*IPT* plants

Phytohormones not only exert intrinsic growth control but also mediate plant adaptations to transiently changing environments (Wolters and Jurgens, 2009). Drought affects the hormone balance in plants, reducing CK synthesis and activating ABA biosynthesis (Davies and Zhang, 1991; Haberer and Kieber, 2002). While CK and ABA roles in plant drought response have been well illustrated (Argueso *et al.*, 2009; Wilkinson and Davies, 2010), the function of other phytohormones, such as GA, IAA, ethylene, BR, JA, SA, nitric oxide and strigolactones, is relatively less characterized. Genomics and physiological data indicated that plant hormones act within a complex network with extensive crosstalk (see Nemhauser *et al.*, 2006). Increase in ABA concentration during drought has been postulated as the main endogenous signal that triggers the plant acclimation response(s) to stress, particularly in the model plant *Arabidopsis thaliana* (Zhu, 2002). The interactions between plant hormones also suggest signalling roles for other phytohormones, and changes in GA, CK, JA and IAA during the response to stress have been shown in crop-plants, such as tomato (Ghanem *et al.*, 2008), wheat (Xie *et al.*, 2003) and rice (Yang *et al.*, 2001; Zhang *et al.*, 2010).

Here, the $P_{SARK}$*::IPT* rice plants displayed the differential expression of genes encoding enzymes associated with hormone synthesis and hormone-regulated pathways. A comparison between the $P_{SARK}$*::IPT* and WT plants revealed the up-regulation of BR-biosynthesis (*DWF5* and *HYD1*) and BR-regulation and signalling genes (*BRL3, BRI1, BRH1, BIM1, SERK1*) in the transgenic plants, either under WW or WS conditions (Figure 3a), suggesting an interaction between CK and BR. This interaction was further supported by measuring the expression of major BR-related genes in $P_{SARK}$*::IPT* lines (higher endogenous CK) and in WT plants treated with exogenous CK. *DWF4,* a gene encoding a sterol C-22 hydroxlase that mediates a key reaction in the BR-biosynthesis pathway, was up-regulated in the $P_{SARK}$*::IPT* plants under WW and WS conditions. Overexpression of *DWF4* in rice resulted in increased grain weight (Wu *et al.*, 2008). Recently, it has been shown that BR-mediated signalling is regulated by ABA through *BIN2* or its upstream components via the *protein phosphatase 2C* (*PP2C*) family of genes (Zhang *et al.*, 2009). In the current study, the relative expression of three *PP2C* genes (*PP2C 7*, *PP2C 6* and *PP2C 53*) increased in WT rice plants under WS (Figure 3c). On the other hand, the expression of *BIN2* was up-regulated in the $P_{SARK}$*::IPT* plants. ABA was shown to inhibit BR effects during the exposure of plants to abiotic stress (Divi *et al.*, 2010). Whether the positive interaction between CK and BR in the $P_{SARK}$*::IPT* plants is a consequence of direct crosstalk between CK, ABA and BR or indirectly mediated by ABA is unclear at this stage.

Jasmonate-related genes (*JAZ12*, *JAZ1*, *OPR2* and *MES3*) were down-regulated in the $P_{SARK}$*::IPT*. JA and its bioactive derivatives are key regulators of plant responses to environmental stresses (for review, see Turner *et al.*, 2002). Transgenic rice expressing $P_{Ubi1}$*::AtJMT* (JA carboxyl methyltransferase) displayed increased JA levels and resulted in a significant GY reduction (because of the lower spikelet number and reduced grain filling rate) (Kim *et al.*, 2009). *OsNPR1 (Nonexpress of phaogenesis-related genes 1)* and *OsNPR3 (NPR1-like protein 3)* were up-regulated in the $P_{SARK}$*::IPT* plants under WS. In *Arabidopsis, NPR1* (also known as *SAI1*) is a key regulator of SA-mediated defence response and is a critical component in the modulation of the crosstalk between SA and JA (Spoel *et al.*, 2003). Recently, it was postulated that ROS-activated *NPR1* monomers bind a BR-activated regulator affecting stress-responsive genes; however, the mechanism(s) remains unclear (Divi *et al.*, 2010). On the other hand, BR was also reported to promote IAA transport (reviewed by Hardtke, 2007).

Auxins play a critical role in plant development. Several studies have shown the crosstalk between CK- and IAA-mediated signalling processes (Moubayidin *et al.*, 2009). For example, CK–IAA antagonistic interactions control root development (Ioio *et al.*, 2008), and a negative effect of CK on IAA signalling and transport has been associated with lateral root formation and leaf position determination (Shimizu-Sato *et al.*, 2009). The antagonistic effects of CK on IAA resulted from the negative control of CK on the PIN-dependent IAA distribution (reviewed by Moubayidin *et al.*, 2009). In *Arabidopsis*, treatment with BL resulted in decreased expression of putative IAA-efflux carrier proteins (*PIN*s gene family), suggesting the control of IAA efflux by BL (Goda *et al.*, 2002). In the current study, the expression of *OsPIN6* and *OsPIN3a* was down-regulated in the $P_{SARK}$*::IPT* (Figure 3e), supporting the notion of a CK–BR and CK–IAA interaction. Further, it has been recently reported that *JAZ1* is the crosstalk point between the JA and IAA pathways and is stimulated by IAA (Grunewald *et al.*, 2009), thus suggesting that the repression of IAA by increased CK leads to the down-regulation of JA signalling and responsive genes.

## Sink-source relationships

Grain yield of cereals is largely determined by source/sink relationships in which florets are the primary photosynthetic sinks while the top two leaves, and the flag leaf in particular, are the primary source (Hirota *et al.*, 1990; Sicher, 1993). In rice, over 80% of the total carbohydrate accumulated in grains is produced by the top two leaves (Gladun and Karpov, 1993). The grain-filling process in rice depends on two main carbon resources: photosynthetic assimilates and carbohydrates stored during pre-anthesis and transported to the grain from vegetative tissues (Yang and Zhang, 2006). The maximum amounts of starch and proteins that accumulate in each grain depend on the number of endosperm cells and the final size of the cells, which is influenced by the rate and duration of grain fill process (Egli, 1998). The reduction in source strength during drought stress affected the plant source/sink relationships, leading to yield reductions. In the $P_{SARK}$*::IPT* plants, the concomitant increase in CKs would enable the plants to maintain source strength during stress and relatively higher yields.

Sugars modulate nearly all fundamental processes in plants including embryogenesis, germination, vegetative growth, development, reproduction, senescence and responses to environmental stimulus (Smeekens, 2000; Halford and Paul, 2003). During the vegetative and early reproductive stages of cereal development, assimilated carbon is temporarily stored as carbohydrates in vegetative sink tissues, such as the stem and leaf sheaths. These carbohydrate reserves are subsequently remobilized for transport to reproductive sink tissues and grain filling in later stages of the plant development. The CK regulation of assimilate partitioning (Brenner and Cheikh, 1995), sink strength (Kuiper, 1993) and source/sink relationships (Roitsch, 1999) is well documented (Herzog, 1982). Furthermore, it has been postulated that CK act in the increase in starch contents under drought-stress (Criado *et al.*, 2009). The delayed senescence exhibited by the $P_{SARK}$*::IPT* plants was found to be associated with higher accumulation of starch in the flag leaves at the pre-anthesis stage under both WW and WS conditions. The amount of starch in leaves at the pre-anthesis stage is a limiting factor for grain carbohydrate content (Perez *et al.*, 1971), and the higher sucrose and starch contents of the flag leaves from the transgenic plants resulted in a significant increase in the grain starch content of the $P_{SARK}$*::IPT* plants. *OsMADS57* was highly expressed in $P_{SARK}$*::IPT* plants under well watered and stressed conditions. This rice transcription factor is co-expressed with starch synthesis genes in vegetative tissues (Fu and Xue, 2010). Genes encoding for starch degrading enzymes were down-regulated in $P_{SARK}$*::IPT* plants. The expression of two *SnRK1*-type of protein kinase genes, *Osk1* and *Osk24,* decreased in the $P_{SARK}$*::IPT* plants and remained unchanged during stress. The *Arabidopsis* orthologues of these genes are *KIN10* and *KIN11 SNF1*, and they encode protein kinases that can initiate multiple transcription cascades in response to sugar depletion under stress, promoting catabolism and suppressing anabolism (Baena-Gonzalez *et al.*, 2007). The decreased expression of *Osk1* and *Osk24* would support the notion of a decrease in starch degradation. The decreased expression of *Osk1* and *Osk24* in the WT plants during stress should be taken with caution because after 3 days of stress, these plants dis-

played severe damage. Transgenic rice overexpressing the ethylene receptor (*ETR2*) was reported to suppress the expression of α-*AMY* (Wuriyanghan *et al.*, 2009). Here, the expression of *ETR2* was up-regulated while the expression of α-*AMY, PHOH* and *DPE2* was down-regulated in the $P_{SARK}$*::IPT* plants under WS, further supporting the involvement of hormonal balance in the regulation of sink-source relationships and improved drought tolerance in rice.

In rice, overexpression of *Iron-regulated Transporter 1* (*OsIRT1*), a ZIP family gene, elevated the iron and zinc content in shoot, roots and mature grains (Lee and An, 2009). On the other hand, CK repressed the expression of a root iron transporter *(AtIRT1)* and a Fe-reductase oxidase (*AtFRO2)* in CK-treated *Arabidopsis* plants grown under low iron supply (Séguéla *et al.*, 2008). Similarly, treatment with other roots growth inhibitors, such as osmotic stress (mannitol or NaCl) or hormones (IAA or ABA), also repressed iron-deficiency response genes (Séguéla *et al.*, 2008). The response of *OsZIP1* and *OsZIP2* in rice seen in this report would support the notion of a differential response of *ZIP* genes that is organ-dependent (roots vs. shoot/leaves) and also depends on plant development (i.e. vegetative vs. reproductive).

In conclusion, we demonstrated that the expression of $P_{SARK}$*::IPT* in rice affected plant hormone homeostasis and altered the source/sink balance during water-deficit. The transgenic plants showed delay in response to drought that resulted in improved tolerance to water-deficit and significantly increased GY. This strategy can be useful approach for tailoring rice plants with improved drought tolerance, particularly during the stress-sensitive reproductive developmental stage.

## Experimental procedures

### Plasmid constructs and plant transformation

The *Agrobacterium tumefaciens ipt* (*isopentenyltransferase*) gene was linked to the 840-bp fragment of the *SARK* promoter and introduced as a *Hin*dIII/*Xba*I fragment into the binary vector *pBI101* as described previously in Rivero *et al.* (2007). Embryogenic calli from mature seeds of rice (*Oryza sativa* L. ssp. *japonica* cv. kitaake) were transformed with $P_{SARK}$*::IPT* in the UC Davis Plant Transformation Facility (http://www.ucdptf.ucdavis.edu/). PCR and segregation analysis were used to identify single insertion events (Table S2). Plants containing a single insertion event were selfed to generate homozygous $T_3$ seeds.

### Drought treatment and physiological characterization

Three homozygous $T_3$ lines (5T, 20T and 8T) expressing $P_{SARK}$*::IPT* alongside with nulls and WT plants were germinated on moist germination paper for 6 days (30 °C in dark). Seedlings were then transplanted into 4.5-L pots, filled with soil (capay series, harvested in California rice field, 38°32′23.93″N,121°48′30.81″W, shredded and steamed for 1.5 h to eradicate soil pathogens), with two plants per pot, and placed in water tubes. Greenhouse conditions were kept 12 h, 30 °C (day)/12 h, 20 °C (night). Plants were fertilized with a solution 50% N:P:K (20:10:20) and 50% ammonium sulphate (total of 0.5 g nitrogen) every 10 days until panicle initiation. Drought treatments were applied at two developmental stages: pre-anthesis (end of booting stage toward panicle emerging) and post-anthesis (2 weeks after flowering, during grain filling stage), by withholding water for 6–10 days followed re-watering. Plants were re-watered when visual stress symptoms (i.e. leaf rolling) appeared in the transgenic plants. To evaluate the rate of water loss during the drought treatment, pots were weighted daily and relative water loss was calculated.

### Electron microscopy

For scanning electron microscopy, the flag leaves of WT and $P_{SARK}$*::IPT* plants under WW and WS were sampled and immediately fixed in an aqueous solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2 h. Samples were post-fixed with 1% $OsO_4$ in the sample buffer during 1 h after the samples were dehydrated. Ultrathin sections (70 nm) of the parenchyma rice cells were obtained using a Reichert Ultracut ultramicrotome stained with uranyl acetate followed by lead citrate. The samples were observed with a Philips CM120 Biotwin lens transmission electorn microscope (FEI, Hillsboro, OR, USA).

### Sucrose and starch quantification

Leaves and grain sucrose and starch were quantified as described (Smith and Zeeman, 2006) with some modifications. Flag leaves were sampled from WW and after 3 days of WS and immediately frozen in liquid-N. Grains were harvested at the end of the experiment. Then 0.25 g of grain powder was incubated in ethanol (80%) for 3 min at 95 °C and centrifuged ≥3000 *g* 10 min, keeping supernatant (soluble carbohydrate fraction). For sucrose quantification, the soluble carbohydrate fraction dissolved in ethanol (80%) was evaporated using a rotary evaporator. The samples were dissolved in $ddH_2O$ and subsequently the sucrose was analysed by high-performance anion-exchange chromatography (HPAEC) on HPLC using a CarboPac PA 1 column (4 × 250 mm diameter) and determined by pulsed amperometric detection (Dionex, Sunnyvale, CA). For starch quantification, the yellowish pellet (insoluble carbohydrate fraction) was incubated allowing ethanol to evaporate and dissolved in $ddH_2O$ 10 min at 95 °C. 0.5 mL of the gelatinized starch granules was transferred to a tube with 0.5 mL 200 mM Na acetate (pH 5.5) and was digested with six units of α-amyloglucosidase and two units of α-amylase for 4 or 24 h at 37 °C, for leaf or grain, respectively. A solution of equivalent volume and composition was incubated without the enzymes and used as control samples. The release of glucose was determined at 340 nm using a Glucose Assay (GAHK20; Sigma-Aldrich, St. Louis, MO) with a spectrophotometer (DU-640; Beckman Coulter, Brea, CA, USA). Starch content was calculated as 90% of glucose content.

### Grain minerals analysis

For the analysis of mineral nutrients, a sub-sample of 10 g of harvested grains was oven dried grained and used for ions analysis. Grain macronutrients (calcium, Ca; magnesium, Mg; potassium, K; phosphorus, P; sulphur, S) and micronutrients (zinc, Zn; iron, Fe; copper, Cu; manganese, Mn) concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES).

### $P_{SARK}$*::IPT* expression analysis

Leaf samples, of the last fully exposed leaf, from the $P_{SARK}$*::IPT* plants were collected throughout the entire life-cycle, to analyse the expression pattern of *IPT* gene. During the drought treatment, flag leaves samples were collected from stresses and WW

*$P_{SARK}$::IPT* plant daily. The expression of *IPT* was also tested in different organs, at 3 days of WS. Total RNA was extracted from plant tissue using RNeasy®Mini kit (Qiagen, Valencia, CA). The quality of RNA was determined using Nanodrop ND-1000. First strand cDNA was synthesized from 1 μg of total RNA with the QuantiTect Reverse Transcription kit (Qiagen). Quantitative PCR was performed on the StepOnePlus™ (Applied Biosystems, Foster City, CA), using SYBR® GREEN. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the endogenous rice transcription elongation factor (*TEF*) gene (Table S2).

### Detached leaf assay

Flag leaves were collected from WT and *$P_{SARK}$::IPT* plants grown under optimal conditions in control greenhouse, at the anthesis stage. Leaf fragments (~3 cm) were placed in tissue culture plates, with $ddH_2O$ or a solution of synthetic analogue of natural cytokinin, benzyladenine (BA $2.2 \times 10^{-5}$ M; Sigma B3408-1G). Six biological replicates and three technical replicates were used for this assay. Leaf segments were kept at 30 °C under dark conditions. Amount of chlorophyll degradation was assessed visually every day, and chlorophyll was extracted after 5 days.

Chlorophyll content analysis was conducted twice: before senescence (day 0) and after senescence (day 5). Three leaf discs (0.6 $cm^2$) were sampled immersed in 2 mL of N,N-dimethylformamide in the dark for 48 h at 4 °C; absorbance of the supernatant at 647 and 664 nm was measured using a spectrophotometer (DU-640; Beckman Coulter, Brea, CA, USA), and chlorophyll a and b concentrations were calculated (Moran, 1982).

### Sample collection and microarray experiments

For microarray analysis, flag leaves were sampled from WT and *$P_{SARK}$::IPT* plants early morning. Three biological replicates were prepared for WW and at 3 days of WS plants. Total RNA was extracted from flag leaves using RNeasy®Mini kit (Qiagen). Residual DNA was removed by performing on-column *DNaseI* digestion. The quality of RNA was determined using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Total RNA (2 μg) of each of these 12 samples was labelled with the GeneChip® 3'IVT Express kit package (Affymetrix, CA) following the manufacturer's instructions (http://www.affymetrix.com). Labelled RNAs were hybridized to the Affymetrix GeneChip®, following the manufacturer's instructions. Washing and staining steps were performed using the GeneChip® Fluidics Station 450. The arrays were finally scanned with the GeneChip® Scanner 3000 7G piloted by the Affymetrix GeneChip® Operating Software. The CELL data files of the 12 Affymetrix GeneChip® results of the current study were deposited in the public microarray database NCBI GEO (https://www.ncbi.nlm.nih.gov/projects/geo), accession GSE23211.

### Processing microarray data

Normalization and statistical analyses were performed using JMP Genomics® (ver.3.2) statistical package (SAS, Cary, NC). Probe intensity signal values were transformed into $\log_2$ scale and background normalization of robust multichip average. A quality control procedure was used to assess the quality of hybridizations between and within arrays. Normalization was performed using the quintile procedure. Analysis of variance was employed to test the effects of two factors: genotype (G) (WT vs. *$P_{SARK}$::IPT*) and treatment (E) (WW vs. WS) and their interactions (G × E). A threshold of significance was $P = 0.05$. The obtained significantly changed transcripts were annotated using HarvEST (http://harvest.ucr.edu).

### Quantitative PCR analysis

RNA was extracted from flag leaves of WT and two transgenic (*$P_{SARK}$::$IPT_{5T}$* and *$P_{SARK}$::$IPT_{8T}$*) plants under WW and WS using RNeasy®Mini kit (Qiagen), with six biological replicates. First strand cDNA was synthesized from 1 μg of total RNA with the QuantiTect Reverse Transcription kit (Qiagen). Primer Express® software (Applied Biosystem, Foster City, CA) was used for primer design. Quantitative PCR was performed on the StepOnePlus™ (Applied Biosystems, Foster City, CA), using SYBR® GREEN. A total reaction volume of 15 μL was used. Reaction included 2 μL template, 0.3 μL of reverse primer, 0.3 μL of forward primer, 7.5 μL SYBR Green Master Mix and 4.9 μL RNA-free water. qPCR assay was performed using the following conditions: 95 °C for 10 min follow by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the endogenous rice *TEF*, whose expression did not changed across different genotype, developmental stage and/or treatment, gene control amplified with the primer sets specified in Table S2.

### Statistical analysis

The JMP ® (ver.7.0) statistical package (SAS Institute) was used for statistical analyses. ANOVA was employed to test the effect of genotype on trait. LSMeans Tukey test was used to compare genotypes means at a probability level of 5%. Levels of significance are represented by asterisks as follows: *, **, *** and n.s. indicate significance at $P \leq 0.05$, 0.01, 0.001 or nonsignificant, respectively.

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## Supporting information

Additional Supporting information may be found in the online version of this article:

**Figure S1** (a) Scheme of the experimental design and time point at which samples were collected for expression analysis (indicated by green ovals). The blue line indicates well-watered conditions, of wild-type and $P_{SARK}$*::IPT* plants. The red line represents both genotypes under water-stress at pre-anthesis stage. (b) The pair-wise differential expression comparisons performed between WT and $P_{SARK}$*::IPT* plants.

**Figure S2** Heat map of selected metal transporters genes derived from hierarchical cluster analysis differently regulated between WT and $P_{SARK}$*::IPT* plants under well-watered (WW) and 3 days of water-stress at pre-anthesis (WS). Red and green represent high and low relative expression when compared to the mean value of expression across all samples, respectively. *Plant cadmium resistance 2* (*PCR2*), *zinc transporter 1* (*OsZIP1*), *zinc transporter 2* (*OsZIP2*), *Inorganic phosphate transporter 1-11* (*OsPHT1-11*), *potassium transporter 16* (*OsHAK 16*), amino-acid transporter (*LHT1*) and *inorganic phosphate transporter 1-1* (*OsPHT1-1*).

**Table S1** Phenological characterization of wild type and three independent transgenic line expressing *IPT* gene.

**Table S2** List of primers used for real time qPCR.

**Table S3** Grain mineral-nutrient concentrations of wild type (WT) and two independent transgenic rice plant expressing $P_{SARK}$*::IPT* grown under well-watered and water stressed conditions.

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